RalGDS is required for tumor formation in a model of skin carcinogenesis

Ana González-García,[1] Catrin A. Pritchard,[2] Hugh F. Paterson,[1] Georgia Mavria,[1] Gordon Stamp,[3] and Christopher J. Marshall[1,*]

[1]Cancer Research UK Centre for Cell and Molecular Biology, Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, United Kingdom
[2]Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, United Kingdom
[3]Department of Histopathology, Imperial College, Du Cane Road, London W12 0HS, United Kingdom
*Correspondence: chris.marshall@icr.ac.uk

Summary

To investigate the role of signaling by the small GTPase Ral, we have generated mice deficient for RalGDS, a guanine nucleotide exchange factor that activates Ral. We show that RalGDS is dispensable for mouse development but plays a substantial role in Ras-induced oncogenesis. Lack of RalGDS results in reduced tumor incidence, size, and progression to malignancy in multistage skin carcinogenesis, and reduced transformation by Ras in tissue culture. RalGDS does not appear to participate in the regulation of cell proliferation, but instead controls survival of transformed cells. Experiments performed in cells isolated from skin tumors suggest that RalGDS mediates cell survival through the activation of the JNK/SAPK pathway. These studies identify RalGDS as a key component in Ras-dependent carcinogenesis in vivo.

Introduction

Through their interaction with different effector proteins, small GTPases of the Ras family control a wide range of cellular functions, including proliferation, differentiation, survival, and cell movement. Of particular interest in oncogenic signaling by small GTPases has been the mechanism used by oncogenic Ras to transform cells. The identification of multiple proteins that preferentially interact with active Ras raises the potential for multiple effector pathways, and has led to a number of studies attempting to elucidate the critical oncogenic signaling pathways downstream of Ras (for recent review, see Repasky et al., 2004).

The activity of small GTPases is modulated by the opposing effects of the guanine nucleotide exchange factors (GEFs), which promote the formation of the active GTP bound state in response to extracellular signals, and the GTPase accelerator proteins (GAPs), which terminate GTPase signaling by facilitating GTP hydrolysis to GDP. Interestingly, several GEFs appear to be effector proteins of other small GTPases, thereby providing a link between the activation of one small GTPase and another. The RalGEF family of proteins control the activity of RalA and RalB, and at least three members, RalGDS, Rgl, and Rgl2/Rlf, have a Ras binding domain and therefore may be Ras effectors (D'Adamo et al., 1997; de Bruyn et al., 2000; Shao and Andres, 2000; Wolthuis and Bos, 1999). Ral proteins can also be activated by Ras-independent pathways, via interaction with other small GTPases or in a calcium dependent manner (Hofer et al., 1998; Linnemann et al., 2002; Rosario et al., 2001; Wolthuis et al., 1998). RalGEF proteins are widely expressed in mouse tissues, but to date, it is not clear whether different members of the family regulate different cellular functions, or if they act in a tissue or growth factor-specific manner. Insight into the role of the Ral pathway in Ras-mediated oncogenic transformation came with the observation that dominant negative Ral mutants can block Ras-mediated transformation of fibroblasts in culture (Urano et al., 1996). Furthermore, the use of Ras effector loop mutants capable of activating RalGEF proteins without affecting the activity of Raf or PI3K supports a role for the Ral pathway in Ras-dependent transformation (Rodriguez-Viciana et al., 1997; White et al., 1995). Finally, activated versions of RalGEFs can synergize with ERK and PI3K signaling in transformation assays of cells in culture, and can contribute to experimental metastasis (Ward et al., 2001). These results suggest that activation of the Ral pathway alone has a weak oncogenic effect, but can complement the roles of other

SIGNIFICANCE

Ras oncogenes are mutated in around 15% of human tumors, so it is important to delineate the oncogenic signaling pathways activated by this GTPase. Ras has multiple effectors, including Raf protein kinases, phosphoinositide-3 kinases, and guanine nucleotide exchange factors for the small GTPases Ral and Rac. While most studies have focused on the role of Raf and PI3-kinase signaling, there is increasing interest in studying the Ral pathway in oncogenesis. Although previous evidence using overexpression and dominant negative approaches in tissue culture has suggested that Ral is involved in Ras-mediated transformation, the results shown here using a mouse model of carcinogenesis provide genetic evidence for the involvement of Ral signaling, and suggest that regulation of cell survival is the mechanism.

Ras effectors in promoting cell transformation in tissue culture; however, it has recently been argued that the role of the Ral pathway in Ras-dependent transformation could be more critical in human cells compared to rodent fibroblasts (Hamad et al., 2002; Rangarajan et al., 2004). Part of the role of Ral in oncogenesis may be to stimulate transcription from the cyclin D1 promoter, the c-*fos* serum response element, and the TATA binding protein promoter (Henry et al., 2000; Johnson et al., 2000; Murai et al., 1997). Additionally, Ral has been implicated in the regulation of the AFX and c-*jun* transcription factors, which are phosphorylated in response to Ral signaling and might regulate cell proliferation (de Ruiter et al., 2000; Kops et al., 1999). Aside from its role in cell transformation, the presence of Ral GTPases in both the plasma membrane and transport vesicles (Bielinski et al., 1993; Feig et al., 1996) suggests that this pathway is involved in membrane trafficking. In fact, GTP-bound Ral interacts with Sec5 (Brymora et al., 2001; Moskalenko et al., 2002), a component of the exocyst complex implicated in the delivery of secretory vesicles to specific sites in the plasma membrane (Hsu et al., 1999). The importance of Ral in intracellular trafficking is also reflected in its ability to regulate endocytosis of EGF and insulin receptors (Nakashima et al., 1999). Finally, Ral plays a role in the control of phospholipase D activation, suggesting the involvement of this GTPase in the regulation of membrane lipids (Jiang et al., 1995). However, despite the recent advances in our understanding of the Ral pathway, the physiological consequences of Ral activation have yet to be resolved.

Results and discussion

To investigate the role of Ral in oncogenic transformation and to study Ral guanine nucleotide exchange factors critical for this cellular response, we have generated mice deficient in RalGDS, one of the RalGEFs that regulate Ral activity. A targeting vector was designed in which loxP sites flanked exons 9 to 15 of RalGDS (Figure 1A). These exons comprise part of the catalytic domain of RalGDS and residues involved in the binding of the exchange factor to Ras. Embryonic stem cells in which the homologous recombination event had occurred were transiently transfected with Cre recombinase to excise the floxed sequences, resulting in a RalGDS^{n} allele that lacks exons 9 to 15 (Figures 1A and 1B). Cells carrying this allele were used for the generation of chimeric mice that transmitted the disrupted allele through the germline (Figure 1C). Intercrossing of RalGDS$^{+/-}$ mice yielded the expected Mendelian ratios, indicating that disruption of RalGDS does not result in embryonic lethality. Moreover, male and female RalGDS$^{-/-}$ mice are fertile, and no major defects have been observed in any of the organs analyzed, suggesting that RalGDS is dispensable during mouse development. Experiments in both *Drosophila* and *Xenopus* have shown that disruption of the Ral signaling pathway through expression of Ral mutants results in developmental defects, probably due to a role of Ral in regulating the actin cytoskeleton (Lebreton et al., 2003; Sawamoto et al., 1999). Although the lack of developmental defects in the RalGDS mutant mice can be due to the differences between mice, *Drosophila*, and *Xenopus*, it is more likely that it reflects different consequences of affecting a single Ral exchange factor rather than blocking the action of all Ral exchange factors through expression of dominant negative Ral. Other members of the RalGEF family may compensate for the absence of RalGDS during mouse development, since Northern blot analysis has shown an overlapping pattern of expression for several members of the RalGEF family (Albright et al., 1993; Shao and Andres, 2000; Wolthuis et al., 1996).

Northern blot analysis of mouse embryonic fibroblasts (MEFs) isolated from RalGDS$^{-/-}$ mice shows the presence of a smaller RalGDS transcript derived from the targeted allele (Figure 1D). Lack of appropriate antibodies prevents us from determining whether this transcript results in the expression of a truncated protein. However, transfection of a cDNA plasmid encoding the predicted protein resulting from the gene targeting event shows that the truncated RalGDS protein does not bind to Ras and is also unable to activate Ral (Supplemental Data and Figure 1E). Therefore, if this protein is expressed in the RalGDS$^{-/-}$ mice, it would not respond to Ras-mediated signaling. In agreement with these data, we found that growth factor-mediated activation of Ral was impaired in MEFs isolated from RalGDS$^{-/-}$ embryos compared to cells isolated from wild-type littermates (Figure 1F). Similarly, we observed that 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment induced Ral activation in the skin of wild-type animals, but this activation was reduced in RalGDS$^{-/-}$ mice (Figure 1G).

To assess the role of RalGDS in Ras-dependent tumor formation, we used a well-established chemical carcinogenesis protocol where tumors are initiated in epidermal keratinocytes by topical application of 7,12-dimethylbenz[*a*]anthracene (DMBA) and subsequently promoted by TPA treatment. This procedure results in the development of benign papillomas with a high incidence of H-Ras mutations (Quintanilla et al., 1986). Some of these tumors progress to squamous cell carcinomas, which can undergo epithelial-mesenchymal transition to spindle cell carcinomas. In RalGDS$^{+/+}$ mice, tumors appear within 7 weeks of DMBA application, with 50% of the animals being affected by week 8–9. However, in the RalGDS$^{-/-}$ group, we observed a delay of four weeks in the onset of papillomas (Figure 2A). The resistance to tumor development in RalGDS$^{-/-}$ mice was also reflected in the number of papillomas per animal, which was reduced by 4-fold compared to wild-type littermates (Figures 2B and 2D). Finally, RalGDS deficiency also affected tumor growth, with a significant reduction in the size of the papillomas that developed in RalGDS$^{-/-}$ mice. This impairment in tumor growth was most evident 15 and 18 weeks after tumor initiation (Figure 2C). To examine whether loss of functional RalGDS would affect transformation by oncogenic Ras in tissue culture, MEFs isolated from RalGDS$^{+/+}$ and RalGDS$^{-/-}$ embryos were first immortalized by infection with a retrovirus encoding SV40 large T oncoprotein, and then pools of immortalized cells were infected with a retrovirus expressing H-RasV12 oncoprotein. Figure 2E shows that oncogenic Ras produced 50% fewer foci in RalGDS$^{-/-}$ cells compared with wild-type fibroblasts. Reinstatement of RalGDS expression in the immortalized RalGDS$^{-/-}$ cells by infection with a retrovirus encoding RalGDS restored the induction of transformed foci by H-RasV12 to that obtained by infection of wild-type cells. Altogether, these data indicate an important role for RalGDS in Ras-dependent cell transformation and extend previous observations in the requirement of Ral signaling in Ras-driven oncogenesis to an in vivo setting.

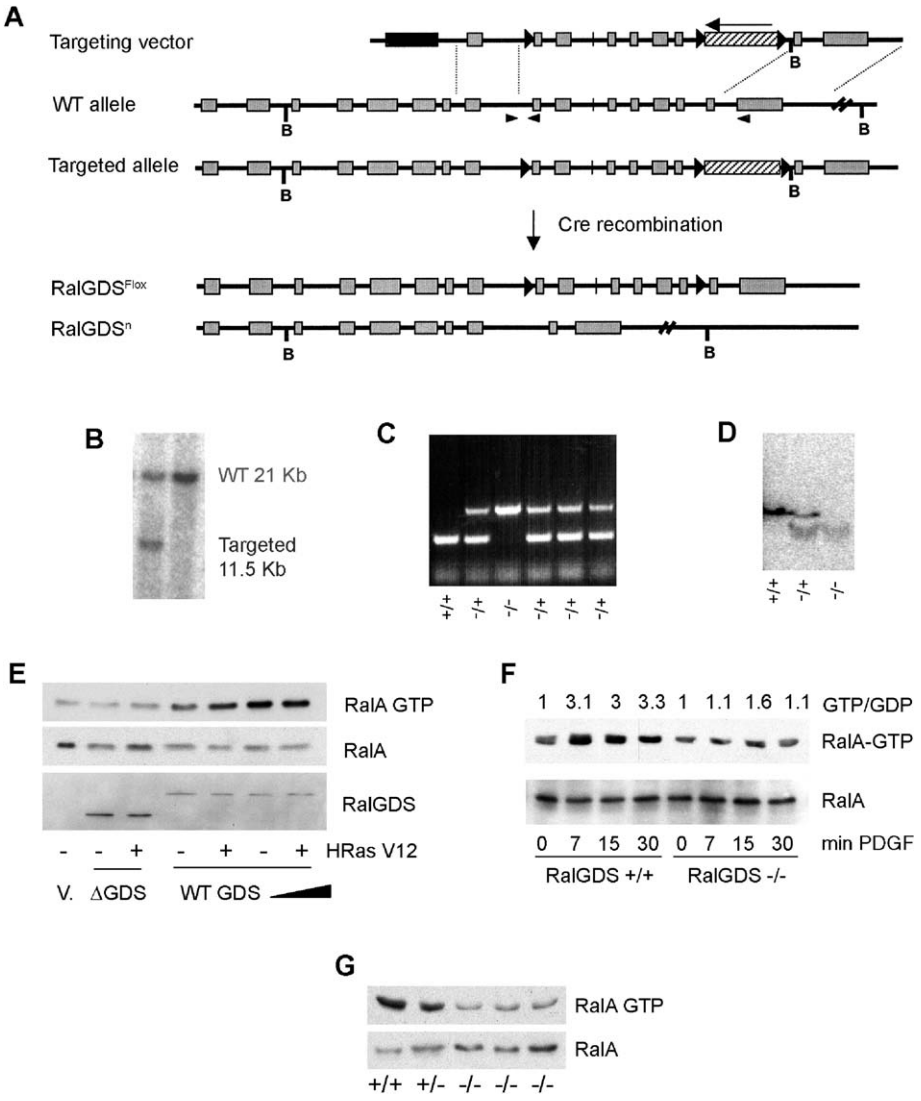

Figure 1. Generation of mutant mice

A: Diagram of the targeting strategy. Schematic representation of the RalGDS allele, targeting construct, and the expected alleles after recombination. Arrows indicate the positions of the PCR primers used to genotype mice. Gray boxes represent exons, black triangles represent loxP sites, the black box represents a diphtheria toxin cassette, and the patterned boxes represent the neomycin cassette.

B: Southern blot of BamHI-digested DNA showing homologous recombination in the RalGDS locus. An XmaI-HincII cDNA probe comprising exons 4 and 5 was used.

C: PCR of tail biopsies from litters obtained from RalGDS$^{+/-}$ matings.

D: Northern blot of RNA isolated from mouse embryonic fibroblast of the indicated genotypes.

E: Activity of truncated RalGDS. Pulldown analysis of RalA activity in lysates from 293 cells transfected with prk5-myc-RalGDS (30 or 60 ng) and prk5-myc-ΔRalGDS (700 ng) in the presence or absence of H-RasV12. It was necessary to transfect different amounts of myc-RalGDS and ΔRalGDS plasmids to ensure similar expression levels of the proteins.

F: Pulldown analysis of RalA activity in protein extracts from RalGDS$^{+/+}$ or RalGDS$^{-/-}$ MEFs after PDGF stimulation. To measure Ral activation, Ral bands were quantified using ImageQuant software. The GTP/GDP ratios relative to nontreated cells are shown.

G: RalA activity in skin treated once with PMA; extracts were made from skin isolated from three separate RalGDS$^{-/-}$ mice—one RalGDS$^{+/-}$ and one RalGDS$^{+/+}$.

As activated versions of RalGDS have been previously shown to be involved in experimental metastasis, we investigated the role of RalGDS in tumor progression. Tumors dissected from the different groups of mice were subjected to histopathological analysis. Papillomas isolated from RalGDS$^{+/+}$ and RalGDS$^{-/-}$ mice were histologically similar. However, we observed significant differences in the frequency of malignant conversion between RalGDS$^{+/+}$ and RalGDS$^{-/-}$ tumors. Progression from papillomas to malignant carcinomas in RalGDS$^{+/+}$ mice started within 16 weeks after DMBA initiation, and ultimately, 36% of the tumors progressed to malignancy. In contrast, no malignant tumors were detected before week 28 in the RalGDS$^{-/-}$ group, and only 9% of the epidermal lesions had a malignant phenotype (Table 1). These data suggest that RalGDS is not only required for Ras-dependent tumor formation, but also influences tumor progression.

To determine the reason for a reduced tumor growth in RalGDS$^{-/-}$ mice, we have analyzed the consequences of PMA and DMBA treatment in the skin of wild-type and RalGDS-deficient mice. DMBA treatment induces formation of adducts in skin DNA that lead to transforming mutations. We tested papillomas isolated from RalGDS$^{-/-}$ mice and control littermates for the presence of Ras mutations. A to T transversions in Ras codon 61 are the most frequent mutations found in two-stage skin carcinogenesis induced papillomas (Quintanilla et al., 1986). This mutation was found in RalGDS$^{-/-}$ papillomas to the same extent as in RalGDS$^{+/+}$ tumors (data not shown), suggesting that the mechanism of mutagenesis after DMBA treatment is not altered in the absence of RalGDS.

This result does not explain the reduced incidence of tumors in RalGDS$^{-/-}$ mice; therefore, we measured the levels of cell proliferation and apoptosis in papillomas isolated from the different groups of mice. Animals were injected with 5-bromo-2-deoxyuridine (BrdU) and the level of proliferating cells within the tumors quantified. As shown in Figures 3A and 3B, the number of proliferating cells is similar for papillomas isolated from the three different genotypes. However, when the levels of cell death in papillomas isolated from RalGDS$^{+/+}$ and

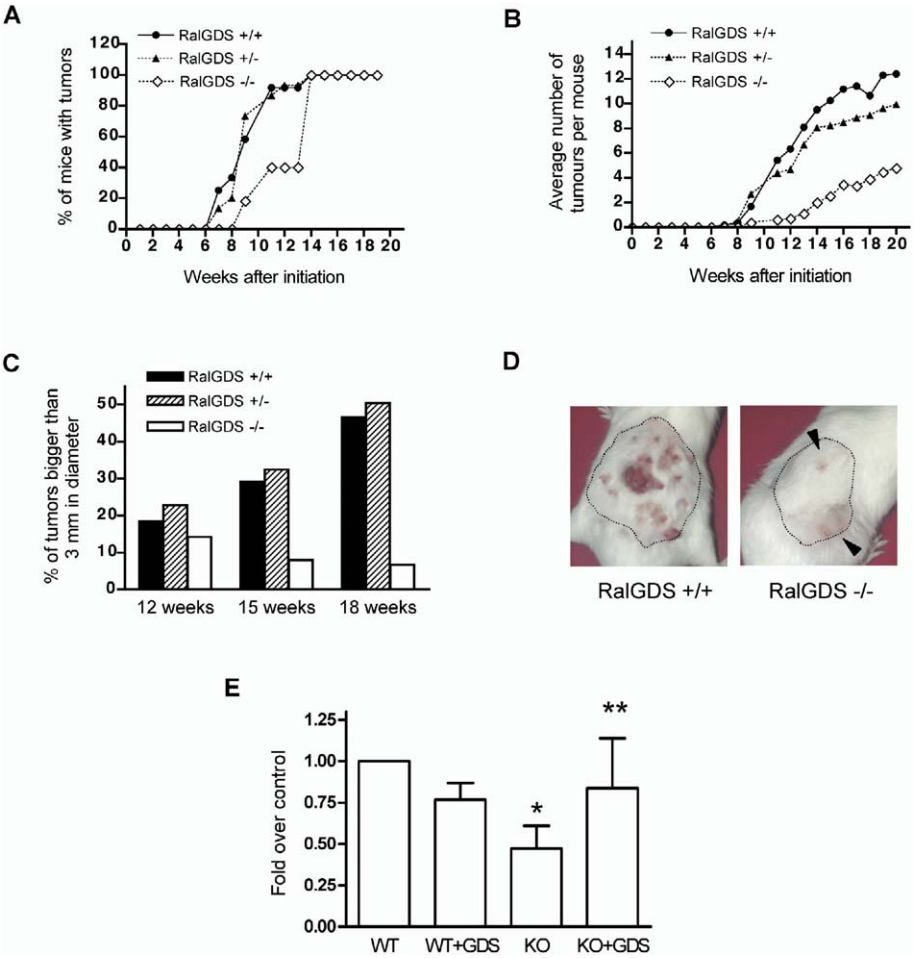

Figure 2. Skin carcinogenesis in wild-type and RalGDS mutant mice

A: Incidence of tumors in wild-type and RalGDS mutant mice.

B: Average number of tumors per mice. The difference in tumor number between RalGDS$^{+/+}$ and RalGDS$^{-/-}$ groups is statistically significant ($p < 0.001$, Bonferroni's multiple comparison test).

C: Rate of tumor growth. The difference in growth rate between RalGDS$^{+/+}$ and RalGDS$^{-/-}$ groups is statistically significant ($p < 0.0001$, χ^2 test).

D: Appearance of RalGDS mice and wild-type littermates 19 weeks after DMBA initiation.

E: Transformation of immortalized MEFs in culture. SV40 large T immortalized MEFs from RalGDS$^{+/+}$ and RalGDS$^{-/-}$ littermates were infected with a retrovirus expressing RalGDS or empty vector, and pools of drug-resistant clones isolated and then infected with a retrovirus expressing oncogenic H-RasV12. Transformed foci were counted 10–14 days after infection. The number of foci in RalGDS$^{+/+}$ cells infected with H-RasV12 was set to unity and the number of foci in other conditions compared to this (mean ± SD of 4 experiments is shown). The differences in the foci number between KO and WT (*, $p < 0.001$) and KO+GDS and KO (**, $p < 0.05$) are statistically significant by the Student's t test analysis.

RalGDS$^{-/-}$ mice were examined by TUNEL, a significant difference between animal groups was observed. There was a 4-fold increase in the number of apoptotic cells per millimeter of basement membrane in RalGDS$^{-/-}$ papillomas compared to tumors isolated from the RalGDS$^{+/+}$ mice, while the apoptotic index was similar in papillomas from heterozygous and wild-type animals (Figures 3C and 3D). Consistent with the observation that there was a higher rate of apoptosis in RalGDS$^{-/-}$ tumors, we found that a restoration of RalGDS in a tumor cultured from a RalGDS$^{-/-}$ mouse resulted in a decreased response to the apoptotic stimuli of UV and H_2O_2 (Figure 3E). Interestingly, in the light of the known requirement for TNF-α in this model of skin carcinogenesis (Moore et al., 1999), the absence of RalGDS did not affect TNF-α-induced apoptosis, suggesting that RalGDS does not function to protect tumor cells against apoptosis induced by this cytokine (data not shown).

Ral signaling has been shown to be a regulator of the JNK/SAPK signaling pathway (de Ruiter et al., 2000). The JNK/SAPK pathway has been shown to be implicated in the regulation of apoptotic cell death, and may have either pro- or anti-apoptopic effects, depending on the system being analyzed (reviewed in Lin, 2003). Therefore, we investigated whether dif-

Table 1. Histological analysis of skin tumors from RalGDS$^{+/+}$, RalGDS$^{+/-}$, and RalGDS$^{-/-}$ mice subjected to DMBA/TPA treatment

	RalGDS$^{+/+}$		RalGDS$^{+/-}$		RalGDS$^{-/-}$	
	n	%	n	%	n	%
Benign lesions	38	64%	21	58%	32	91%
Malignant lesions	21	36%*	15	42%	3	9%*
Carcinomas in situ	(8)	(38%)	(12)	(80%)	(0)	(0%)
Squamous cell carcinomas	(13)	(62%)	(3)	(20%)	(3)	(100%)

Tumors were isolated from 15 RalGDS$^{+/+}$, 11 RalGDS$^{+/-}$, and 10 RalGDS$^{-/-}$ animals.
*The differences in the rate of malignant conversion between RalGDS$^{+/+}$ and RalGDS$^{-/-}$ are statistically significant ($p < 0.005$; χ^2 test).

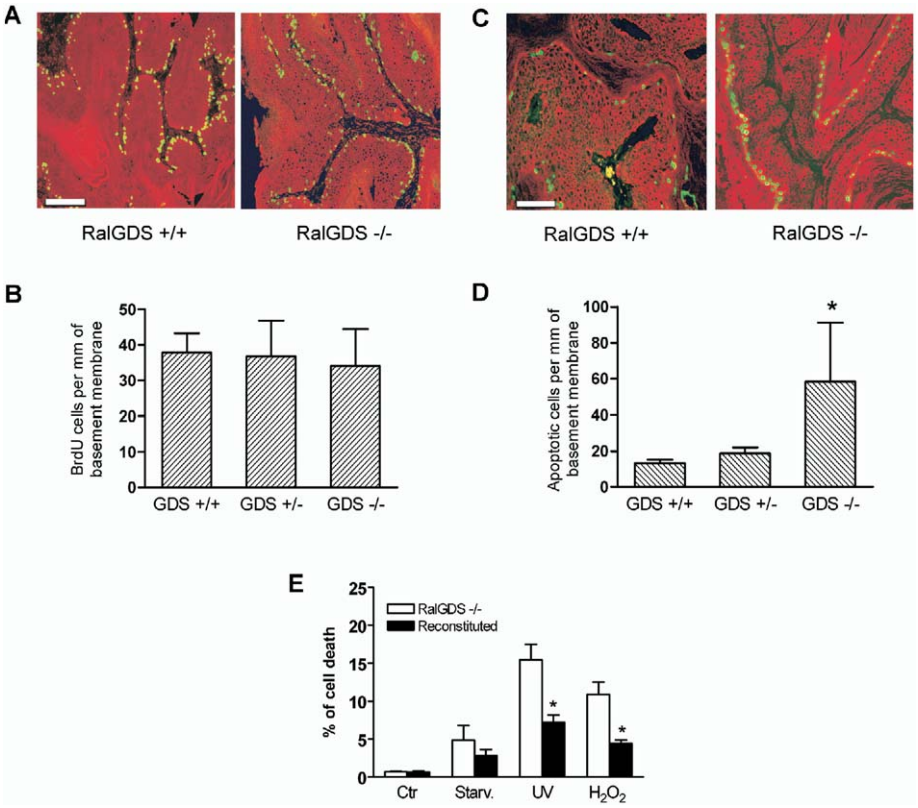

Figure 3. Proliferation and apoptosis in skin tumors

A: BrdU staining (green) of large papillomas (>3 mm in diameter) isolated from the indicated mice. Keratin 14 is shown in red as a marker for the epithelial compartment of the tumor. Scale bars represent 200 μm.

B: Average number of proliferating cells per mm of basement membrane (mean ± SD is shown). Ten papillomas from each genotype were examined.

C: TUNEL staining (green) of large papillomas (>3 mm in diameter) isolated from the indicated mice. Keratin 14 is shown in red as a marker for the epithelial compartment of the tumor. Scale bars represent 100 μm.

D: Average number of apoptotic cells per mm of basement membrane (mean ± SD is shown). Ten tumors of each genotype were examined. * indicates $p < 0.0005$ by the Student's t test analysis.

E: Apoptosis in RalGDS$^{-/-}$ tumor cells. Cells obtained from an explanted tumor isolated from a RalGDS$^{-/-}$ mouse and reconstituted with RalGDS where treated for 7 hr with the stimuli shown and the percentage of apoptosis determined by counting pyknotic nuclei (mean ± SD of four experiments is shown). * indicates $p < 0.01$ by the Student's t test analysis.

ferences in the activation of the JNK/SAPK pathway might be responsible for the enhanced susceptibility to some apoptopic stimuli in RalGDS$^{-/-}$ cells. As shown in Figure 4A, UV light, but not TNF-α, induces JNK activation in RalGDS$^{-/-}$ cells, but the activation of this pathway is more sustained when the expression of RalGDS is restored, showing that prolonged activation of JNK/SAPK signaling requires RalGDS function. These results corroborate previous data indicating that the Ral pathway regulates the activity of JNK/SAPK (de Ruiter et al., 2000). To determine whether JNK activation is responsible for the increased survival in RalGDS reconstituted cells, we blocked JNK activation with SP600125, a reversible JNK inhibitor (Bennett et al., 2001). Following UV light irradiation, JNK inhibition resulted in an increase in apoptotic cell death to similar levels

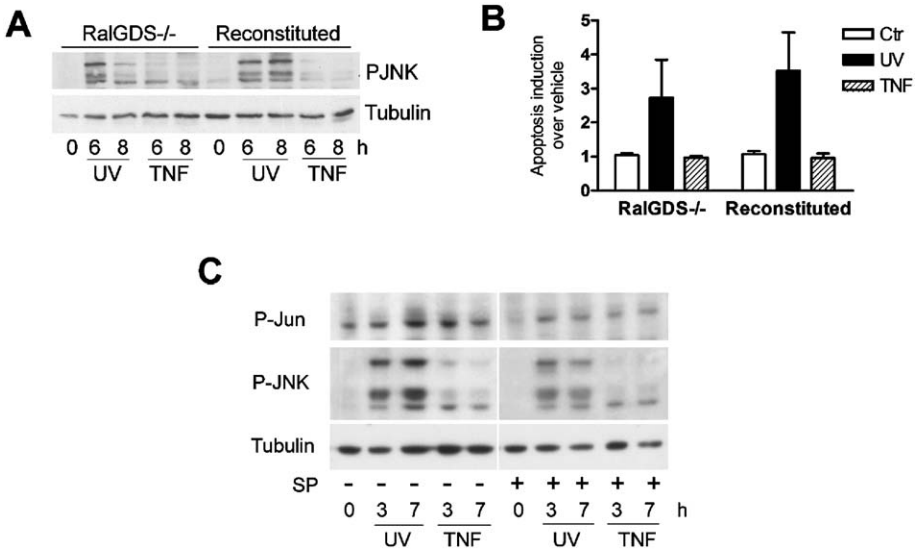

Figure 4. RalGDS mediates cell survival through the activation of the JNK pathway

A: Activation of JNK in RalGDS$^{-/-}$ and RalGDS reconstituted tumor cells upon UV light irradiation. JNK activation was determined in a Western blot using phosphospecific antibodies.

B: UV light and TNF-α-induced apoptosis after treatment with the JNK inhibitor SP600125. The inhibitor was added to the cells 90 min before UV or TNF-α treatment. For each treatment, the percentage of cell death in the presence of vehicle was set to unity and the percentage of cell death after treatment with inhibitor compared to this (mean ± SD of 4 experiments is shown).

C: Jun phosphorylation after treatment with the JNK inhibitor SP600125. RalGDS reconstituted cells were treated as in **B** and Jun phosphorylation determined using phosphospecific antibodies.

in both RalGDS$^{-/-}$ and reconstituted cells, showing that in this system, JNK/SAPK signaling provides a survival signal. Consistent with the lack of JNK/SAPK activation by TNF-α, SP600125 had no effect on cell death induced by this cytokine (Figure 4B). These results show that RalGDS signaling to JNK/SAPK activation mediates protection against some apoptotic stimuli, but not others, and argue that the increased apoptosis that we see in tumors of RalGDS$^{-/-}$ mice may be due to a defect in JNK/SAPK activation. In a number of other tumor systems, JNK/SAPK signaling has been shown to provide a survival signal (Hess et al., 2002), and of particular interest in this context, the JNK/SAPK cascade has been shown to give a survival signal in mouse skin carcinogenesis protocols (Chen et al., 2001). Further experiments will be needed to determine the mechanism of JNK/SAPK survival signaling in tumor cells. It has been shown that cyclin D1 is a c-*jun* target gene, and expression of this cyclin is also Ral-dependent (Albanese et al., 1995; Henry et al., 2000). Deletion of cyclin D1 in mice results in inhibition of Ras-dependent tumor formation (Robles et al., 1998), a similar phenotype to that observed in the RalGDS$^{-/-}$ mice. Although our results cannot exclude the involvement of cyclin D1 in the reduced tumorigenesis of RalGDS$^{-/-}$ mice, the normal levels of cell proliferation in tumors isolated from these animals (Figures 3A and 3B) suggest this is not the case.

Taken together, our results show that RalGDS is required for Ras-induced tumor formation, and suggest that it regulates tumor growth by providing a survival signal to tumor cells. Although tissue culture experiments have suggested that activation of RalGEF proteins and the Ral pathway are required for proliferation in a variety of cell types (Goi et al., 1999; Rosario et al., 2001; Wolthuis et al., 1997), we cannot detect any impairment in cell proliferation in RalGDS-deficient papillomas (Figures 3A and 3B). This might be due to different cellular requirements in tissue culture and in an in vivo situation. Our data, however, support a role for RalGDS in tumor cell survival. A role for the Ral pathway in cell survival has been previously suggested for transformed cells (Chien and White, 2003).

The identification of numerous binding partners for activated Ras and consequently the possibility of multiple signaling pathways downstream of Ras raises the question of which pathways are critical in oncogenesis (see Repasky et al., 2004). While there is a large body of evidence supporting a key role for Raf/MAPK pathway in oncogenic Ras signaling, for example the restriction of Ras and BRAF mutations to the same tumor types and the mutual exclusivity of Ras and BRAF mutations (Davies et al. 2002), questions have been raised about the significance of Ras signaling through the Raf/MAPK pathway during carcinogenesis, because some cells containing oncogenic Ras alleles have reduced activation of ERK MAP kinases (Tuveson et al., 2004). Such observations, together with the potential for multiple Ras signaling pathways, suggest that other Ras-dependent pathways could be critical in cell transformation. Our data show that Ras signaling through RalGDS is required for oncogenic transformation. Similarly, genetic ablation of other potential Ras effectors, Tiam1, an exchange factor for the Rho-family small GTPase Rac, (Malliri et al., 2002), and recently Phospholipase-C epsilon (Bai et al., 2004), has been shown to reduce tumor formation in the DMBA/TPA skin carcinogenesis model. Therefore, it appears that at least four Ras signaling pathways, Raf, RalGDS, Tiam1, and phospholipase-C epsilon, are involved in oncogenic Ras signaling. It is possible that different Ras effectors cooperate in the oncogenic process by regulating different aspects of tumor biology. Interestingly, genetic ablation of either Tiam1 (Malliri et al. 2002), phospholipase-C epsilon (Bai et al., 2004), or RalGDS results in viable mice, whereas ablation of Raf-1 or BRAF leads to death of embryos (Wojnowski et al., 1997; Wojnowski et al., 1998); these observations raise the possibility that Tiam1, phospholipase-C epsilon, or RalGDS may be better therapeutic targets in cancer, because their inhibition might be less toxic than the inhibition of Raf signaling.

The data we present here suggests that RalGDS is an important effector of Ras-mediated oncogenesis in a mouse model system. Recent work from Hamad et al. (2002) and Rangarajan et al. (2004) suggests that the RalGDS pathway may be more critical to transformation by oncogenic Ras in human cells than mouse; however, their studies were all carried out in tissue culture systems and may therefore have missed a role for Ral signaling in rodent tumor induction in vivo. While our data provide evidence for RalGDS involvement in Ras-dependent tumor formation, further studies will be needed to evaluate the requirements for RalGDS in other oncogenic pathways not dependent on Ras activation; however, preliminary studies in p53 deficient mice indicate that ablation of RalGDS does not affect the incidence of tumors (data not shown).

Experimental procedures

Generation of RalGDS mutant mice

Mouse RalGDS genomic clones were obtained by screening a 129/SvJ BAC library (Incyte). The RalGDS targeting vector was constructed using a DNA fragment extending from intron 7 to the 3′ untranslated region, which was then cloned into the pKO scrambler 901 vector (Stratagene). A neomycin cassette flanked by two loxP sites was cloned upstream of exon 16, introducing a new BamHI site for genotyping purposes. A third loxP site was located in intron 8. The targeting vector also contains a diphtheria toxin selection cassette (Figure 1A). This construct was electroporated into RW4 ES cells (Incyte) and homologous recombination was checked by Southern blot analysis after BamHI digestion using a cDNA probe containing exons 4 and 5 of RalGDS. Positive clones were then transiently transfected with PcrePac vector to eliminate the neomycin cassette. As a result of Cre-mediated recombination, two different targeted alleles were obtained (Figure 1A): a RalGDS floxed allele in which loxP sites flank exons 9 to 15 and a RalGDS null allele (RalGDS^{n}) that lacks the exons 9–15. Cells carrying the RalGDS^{n} allele were injected into MF-1 blastocysts, and germline-transmitting chimeric mice were obtained. Animals with a mixed MF-1/129SvJ background were used throughout the experiments.

Ral pulldown experiments

For pulldown assays in MEFs, cells were starved for 16 hr in the absence of serum and then stimulated for the indicated time points using 25 ng/ml PDGF (Sigma). Cells were washed once in PBS and lysed in PBS containing 1% Triton X-100, 40 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium fluoride, 10 mM pyrophosphate, and 1 mM sodium vanadate. Lysates containing 200 μg of protein were incubated for 1 hr at 4°C with glutathione-Sepharose beads that had been precoupled to recombinant glutathione *S*-transferase (GST)-RalBP1 RalBD as described previously (Wolthuis et al., 1998). The beads were then washed once with lysis buffer and twice with 25 mM Tris (pH 7.2), boiled, and the samples analyzed by Western blotting with an anti-Ral A antibody (Pharmingen).

For pulldown assays in skin extracts, mice were shaved and treated with 150 μl 10^{-4} M 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma). 24 hr later, skin was dissected and snap frozen. Tissue was then powdered in liquid nitrogen using a pestle and mortar, lysed in pulldown buffer, and lysates processed as above.

Multistep skin carcinogenesis

The backs of age-matched groups of mice (15 animals of each genotype), consisting of littermates generated from intercrosses of RalGDS$^{+/-}$ mice on a mixed MF-1/129SVj background, were shaved, and the next day animals were topically dosed with 150 μl of 125 μg/ml 7,12-Dimethylbenzanthracene (DMBA, Sigma). Starting one week later, mice were treated twice weekly for 20 weeks with 150 μl 10^{-4} M 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma). Tumor incidence and multiplicity in the different animal groups was recorded weekly. Mice were killed if distressed, if any individual tumor became ulcerated or reached a diameter of 1.5 cm, or at the termination of the experiment (50 weeks after initiation). The experiment was performed twice with similar results.

Focus formation assay

Passage 2 MEFs were immortalized by infection with a retrovirus encoding SV40 large T antigen, and pools of drug-resistant clones containing at least 70–80 independent infection events were isolated. These cell pools were then infected either with an empty vector or with a RalGDS containing retrovirus and drug-resistant pools selected as described above. Cells were then transformed by infection with a retroviral vector encoding H-RasV12 and the number of foci determined 10–14 days after infection.

Histology and immunohistochemistry

Tumors were dissected and fixed in 10% buffered formalin or embedded in OCT compound (Sakura) and frozen as required. The tumors were then histologically classified by hematoxylin and eosin staining of paraffin sections. The degree of hyperplasia and cytological atypia were assessed as mild, moderate, and severe, according to established guidelines (Arbeit et al., 1994; Hennings et al., 1993; van Hogerlinden et al., 1999). Tumors were classified as carcinomas when there was clear evidence of stromal invasion, with reduced nuclear polarization and disordered keratinization, associated with stromal fibroblastic reaction (desmoplasia) and/or evidence of infiltration into the panniculus carnosus muscle layer in the subcutaneous tissue.

For proliferation analysis, mice received an intraperitoneal BrdU injection (1 mg in 200 μl PBS) 1 hr before sacrifice. Detection of BrdU incorporation was performed in frozen sections using an in vivo proliferation detection kit (Roche) according to manufacturer's instructions. Apoptotic cells were detected in deparaffinized sections by TUNEL using an in situ cell death detection kit (Promega). The number of TUNEL- or BrdU-positive cells from several independent fields was evaluated per mm of basement membrane by confocal microscopy. The antibody for detection of keratin 14 was obtained from Babco.

Apoptosis induction

A cell line was obtained by explanting a skin tumor isolated from a p53$^{+/-}$ RalGDS$^{-/-}$ mouse. These cells were infected either with an empty retrovirus or with one encoding RalGDS, and pools of drug-resistant clones containing at least 50 independent infection events were isolated. For apoptosis induction, cells were starved in the absence of serum for 24 hr and then treated with 30 nM hydrogen peroxide (H_2O_2), 30 nM TNF-α in the presence of 5 μM cyclohexamide, or exposed to ultraviolet irradiation (40 mJ/cm^2). Cells were fixed 7 hr later with 4% paraformaldehyde and stained with DAPI. The percentage of apoptotic cells was determined by counting the number of pyknotic nuclei in 10 consecutive fields. For the analysis of JNK effect on apoptotic cell death, 40 μM SP600125 was added to the cells 90 min prior to apoptotic cell death stimuli.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/3/219/DC1/.

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