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Binding of Ras to Phosphoinositide 3-Kinase p110α Is Required for Ras-Driven Tumorigenesis in Mice

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SUMMARY

Ras proteins signal through direct interaction with a number of effector enzymes, including type I phosphoinositide (PI) 3-kinases. Although the ability of Ras to control PI 3-kinase has been well established in manipulated cell culture models, evidence for a role of the interaction of endogenous Ras with PI 3-kinase in normal and malignant cell growth in vivo has been lacking. Here we generate mice with mutations in the *Pi3kca* gene encoding the catalytic p110α isoform that block its interaction with Ras. Cells from these mice show proliferative defects and selective disruption of signaling from growth factors to PI 3-kinase. The mice display defective development of the lymphatic vasculature, resulting in perinatal appearance of chylous ascites. Most importantly, they are highly resistant to endogenous Ras oncogene-induced tumorigenesis. The interaction of Ras with p110α is thus required in vivo for certain normal growth factor signaling and for Ras-driven tumor formation.

INTRODUCTION

Activating point mutations in the genes encoding the Ras subfamily of small GTP-binding proteins contribute to the formation of a large proportion of human tumors. Analysis of over 40,000 human tumor samples indicates an activating mutation rate of 22%, 8.2%, and 3.7% for *KRAS*, *NRAS*, and *HRAS*, respectively (Catalogue Of Somatic Mutations In Cancer, http://www.sanger.ac.uk/genetics/CGP/cosmic/). The signaling pathways by which activated Ras protein controls cell growth and contributes to malignant transformation are therefore of considerable continuing interest. Several direct Ras effector enzyme families have been characterized, the best studied of which are Raf kinases, type I phosphoinositide (PI) 3-kinases, Ral-guanine nucleotide exchange factors (Ral-GEFs), the Rac exchange factor Tiam1, and phospholipase C ε (Downward, 2003). Of these, the genes encoding both B-Raf and the p110α PI 3-kinase catalytic subunit have been found to be frequently activated by somatic mutation in human cancer (Weir et al., 2004), with overall mutation frequencies around 10%–15% for each. PI 3-kinase activity is further implicated in carcinogenesis by the frequent inactivation of the tumor suppressor gene PTEN, which encodes the phosphatase that reverses the incorporation of phosphate at the 3′ position of the inositol ring (Cully et al., 2006). However, genetic analyses of Ras signaling in development of worms and flies has provided only very limited evidence for a significant role for Ras effector pathways other than Raf (Prober and Edgar, 2002).

In recent years, intense efforts have been made to determine the relative importance of the different effector pathways in the process of Ras-induced tumor formation. For the most part, this has involved the exogenous expression in cultured cells of activated forms of the effector proteins or of partial loss-of-function mutant Ras proteins that have selective ability to interact with one effector compared to another (Repasky et al., 2004). These experiments have revealed varying effector dependencies of Ras-induced transformation in different cell types and species. However, the physiological significance of these studies may be limited by the difficulty of using exogenously expressed, artificially activated constructs in cultured cells to mimic the amplitude and duration of Ras effector activation in naturally occurring human tumors.

Another line of investigation has been to study chemical carcinogenesis using dimethylbenzanthracene (DMBA), which mutationally activates the endogenous Hras gene, and phorbol ester to induce skin tumors in mice (Quintanilla et al., 1986). When applied to mice in which genes for key Ras effectors have been deleted, it has been found

that the presence of each of Tiam1, PLCε, and Ral-GDS is required for skin tumor formation (Bai et al., 2004; Gonzalez-Garcia et al., 2005; Malliri et al., 2002). This implicates these effectors in Ras-induced tumorigenesis although does not formally prove that Ras acts through them, or whether the tumor-suppressive effects of deletion of these effectors are specific to Ras, rather than other oncogene, induced carcinogenesis. Unfortunately it has not been possible to undertake a similar analysis for the other major Ras effectors due to the early lethality of mice deleted for the Raf-1, B-Raf, p110α, and p110β genes (Vanhaesebroeck et al., 2005; Wellbrock et al., 2004).

While the importance of endogenous PI 3-kinase and other non-Raf effectors in Ras-driven tumorigenesis remains the subject of debate, it is even less clear how important these pathways are for normal growth factor signaling in a physiological context. Overexpression of a dominant-negative Asn17 Ras mutant partly inhibits induction of PIP_3 by epidermal and nerve growth factor in cultured PC12 cells (Rodriguez-Viciana et al., 1994) and by fibroblast growth factor in SKF5 cells (van Weering et al., 1998). However, the specificity of this inhibitor of Ras activity is uncertain. Little has been done to address this issue in a whole animal context.

In this report we set out to study the importance of the interaction of endogenous PI 3-kinase p110α with endogenous Ras in development and tumorigenesis in mice. Animals were made with point mutations knocked into the *Pi3kca* gene that prevented interaction with activated Ras. These mice show defects in lymphatic development and are highly resistant to endogenous Ras oncogene-induced tumorigenesis. The interaction of Ras with p110α thus represents an important physiological component of both normal and malignant Ras signaling.

RESULTS

Generation of Mice Bearing Mutations in PI 3-Kinase p110α that Block Interaction with Ras

In order to investigate the role played by the interaction of PI 3-kinase p110α with Ras in mammalian development and tumorigenesis, we set out to generate mice in which the interaction was disrupted by the introduction of point mutations into the endogenous p110α gene. A previous study of the structure of active Ras bound to a different type I PI 3-kinase isoform, p110γ, had identified four critical amino acids that are absolutely required for this interaction (Pacold et al., 2000). Although the degree of homology between different type I PI 3-kinase isoforms is not extremely high in the Ras-binding domain (RBD) (Vanhaesebroeck and Waterfield, 1999), two of the four amino acids characterized as critical to the interaction of Ras with p110γ were conserved in human, cow, mouse, and fly p110α (threonine 208 and lysine 227 in mammalian pl10α, Figure 1A).

To determine whether mutation of these residues would be sufficient to fully disrupt the interaction of Ras with p110α, we changed threonine 208 to aspartic acid and lysine 227 to alanine, either separately or together. We avoided reversing the charge of these sites as previous studies had shown that mutation of lysine 227 to glutamic acid had a significant stimulatory effect on the basal catalytic activity of p110α, although substitution with neutral alanine did not (Rodriguez-Viciana et al., 1996). We investigated whether GST-fusion proteins of the RBD of the mutant forms of p110α were capable of binding to Ras. As shown in Figure 1B, wild-type p110α very efficiently interacted with both H- and K-Ras, but both the single and double mutations on p110α abrogated its ability to bind. Similar results were obtained in the reverse experiment, where full-length wild-type and mutant forms of p110α were mixed with GTP-bound GST-V12 H-Ras fusion protein (Figure 1C).

Based on the ability of the mutations T208D and K227A to block the interaction of p110α with Ras, we set out to introduce these two changes into the endogenous gene, Pik3ca, encoding p110α in mice. Homologous recombination was used in ES cells to replace exon 3 with a version containing the double mutation. These cells were used to generate mice from which the neo selection cassette was subsequently removed by breeding onto a strain expressing Cre in the germline (see schematic in Figure 1D). Crosses of heterozygous mutant Pik3ca mice generated live-born mice with wild-type, heterozygous mutant, and homozygous mutant genotypes as determined by PCR analysis (Figure 1D inset).

Disruption of the Interaction of p110α with Ras Attenuates Growth Factor Signaling to the PI 3-Kinase Pathway and Cell Proliferation

Mouse embryonic fibroblasts (MEFs) were made from embryonic day (E) 13.5 embryos from wild-type, heterozygous mutant, and homozygous mutant genotypes. These cells were used to determine the importance or otherwise of the direct interaction of endogenous p110α with endogenous Ras for the ability of various growth factors to activate downstream signaling pathways, in particular ones where Ras could be directly involved, such as Raf/MEK/ERK and PI 3-kinase/Akt. Stimulation of wild-type MEFs with EGF, FGF2, or PDGF led to the activation of both the ERK and Akt pathways as determined by the appearance of phosphorylated ERK and phosphorylated Akt, as expected. However, in cells homozygous for the mutation in the RBD of p110α, we found that Akt activation in response to EGF was significantly reduced and FGF2 signaling to Akt was completely abrogated (Figure 2A). In contrast, PDGF signaling was unaffected in mutant cells as well as ERK signaling in response to EGF and PDGF.

Analyzing a dose-response curve for suboptimal concentrations of PDGF revealed no difference in the level of Akt or ERK activation between mutant and wild-type cells but deficient signaling to Akt, but not ERK, throughout the whole range of EGF concentrations (Figure 2B). Since PI 3-kinase has been shown to be important in insulin signaling and glucose homeostatsis (Foukas et al.,

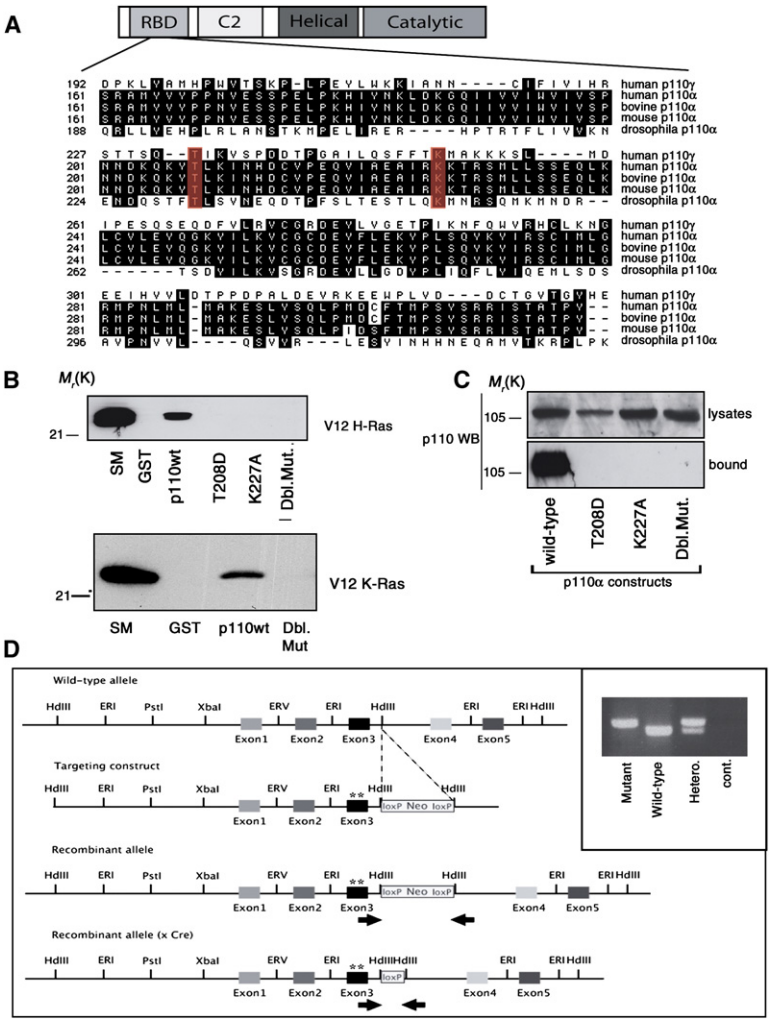

Figure 1. Generation of Mice Bearing Mutations in PI 3-Kinase p110α that Block Interaction with Ras

(A) Schematic of the domain structure of p110α and alignment of p110 isoforms from several species. The red blocks indicate mutations made to disrupt the interaction of p110α with Ras. The alignment was carried out using the clustal algorithm of Lasergene Navigator (DNASTAR).

(B) Wild-type and mutant forms of the GST-tagged Ras-binding domain (RBD) of p110α were expressed in COS7 cells, along with the activated V12 mutant form of H-Ras or K-Ras. The cells were lysed and GST-p110 RBD was recovered on glutathione agarose. Bound V12 Ras was detected with pan-Ras antibody.

(C) Untagged p110α and p85α were transiently overexpressed in COS7 cells. Cell extracts were made and added to GTP-loaded V12 H-Ras GST fusion protein bound to glutathione agarose. The Ras beads were washed and bound p110α detected by western blotting with p110α antibodies.

(D) Schematic representation of the strategy for introduction of the T208D and K227A mutations into exon 3 of pik3ca, the gene encoding p110α in mice. Arrows indicate the location of genotyping primers. Inset: genotyping PCR of samples after Cre-mediated excision of the neomycin selection cassette. The upper band represents the mutant allele (containing a residual 30 base pairs of the loxP site). The lower band represents the wild-type allele.

2006), we also looked at the insulin responsiveness of wild-type and mutant MEFs but found little difference (data not shown). To confirm that the differences seen in Akt activation reflected alterations in PIP_3 levels in the cell, immunocytochemistry with a PIP_3-specific monoclonal antibody was used. As seen in Figure 2C, in response to EGF, mutant MEFs showed highly reduced levels of PIP_3 compared to wild-type MEFs, while PDGF stimulation was similar in the two genotypes.

To establish whether these differences arose due to altered regulatory signaling to the mutant p110α or fundamental defects in its enzymatic activity, we characterized the protein in further detail. As shown in Figure S1A available with this article online, the in vitro lipid kinase activity of immunoprecipitated mutant p110α is indistinguishable from that of the wild-type p110α. Also the amount of p85 coimmunoprecipating with mutant p110α was similar to that with wild-type p110α, thereby suggesting that the binding affinity of p110α for p85 is not affected by the RBD mutations (Figure S1B). In addition, the binding of p85 to tyrosine-phosphorylated proteins following growth factor stimulation also remains unaltered in cells bearing the p110α RBD mutations (Figure S1C). Furthermore, the level of expression of p110α in MEFs was the same for each genotype, as were the expression levels of p110β and p85 (Figure S1D). p110δ and p110γ expression was not detectable by immunblot in these cells (data not shown).

It thus appears likely that the mutations in the RBD do not interfere with the basic enzymatic activity of p110α, its coupling to the p85 regulatory subunit, or expression of PI 3-kinase components, suggesting that the biological effects of these mutations are indeed reflecting altered coupling to Ras. To further confirm this, dominant-negative mutant Ras (N17) was cotransfected with epitope-tagged Akt into wild-type MEFs, which were subsequently stimulated with growth factors. Tagged Akt was immunoprecipitated and its kinase activity assayed in vitro. As shown in Figure S1E, the ability of EGF and FGF2 to activate Akt was completely abolished by dominant-negative Ras in this assay, while that of PDGF was much less affected. Similar observations have been previously

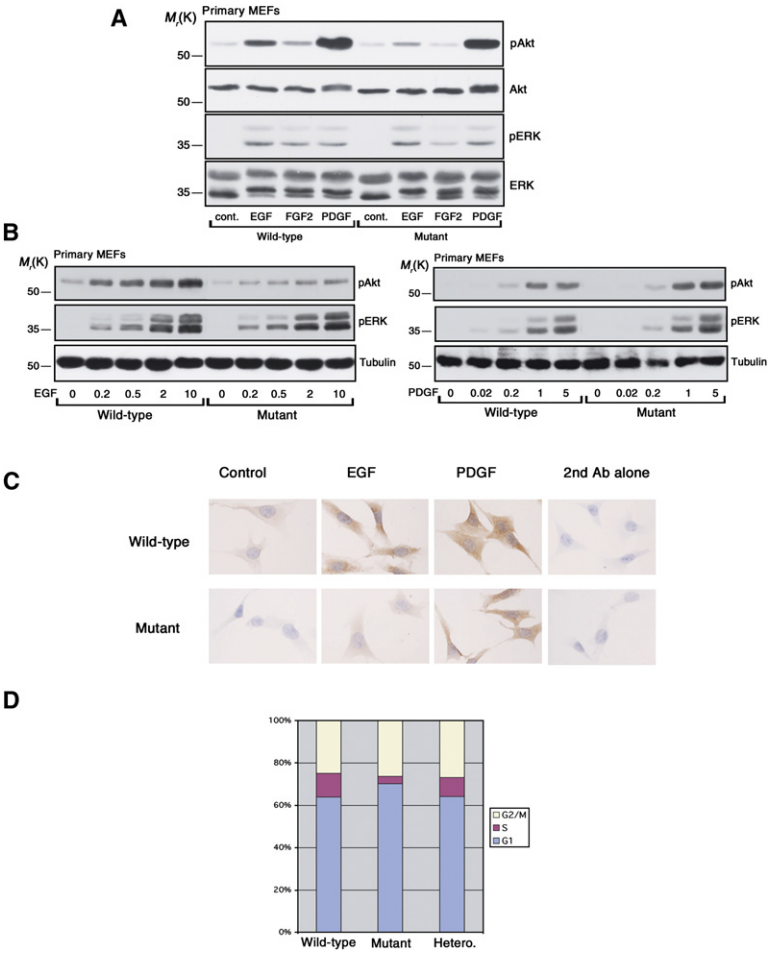

Figure 2. Disruption of the Interaction of p110α with Ras Attenuates Growth Factor Signaling to the PI 3-Kinase Pathway and Cell Proliferation

(A) Serum-starved wild-type and p110α mutant MEFs were stimulated with EGF (50 ng/ml), FGF2 (20 ng/ml), or PDGF (20 ng/ml) for 10 min prior to lysis of cells and analysis of the phosphorylation state of ERK and Akt by Western blotting.

(B) Serum-starved wild-type and p110α mutant MEFs were stimulated with EGF or PDGF at the indicated concentrations (ng/ml) for 10 min prior to lysis of cells and analysis of the phosphorylation state of ERK and Akt by Western blotting.

(C) Serum-starved wild-type and p110α mutant MEFs were stimulated with EGF (50 ng/ml), FGF2 (20 ng/ml), or PDGF (20 ng/ml) for 10 min prior to immunocytochemistry staining for PIP_3.

(D) To quantify effects of p110α mutation on cell-cycle progression, wild-type, mutant, and heterozygous MEFs were pulse-labeled with BrdU in serum-containing medium. Cells were analyzed by FACS for staining with antibody to BrdU and for total DNA content with propidium iodide. The proportions of cells in G1, S, and G2/M phases of the cell cycle are shown graphically.

made in other cell systems (Rodriguez-Viciana et al., 1994; van Weering et al., 1998).

The decreased responsiveness of the PI 3-kinase pathway, a key regulator of cellular proliferation, survival, and metabolism, to certain growth factors raised the issue of whether there was a defect in the growth of cells in which the Ras-p110α interaction was disrupted. To quantify the proportion of actively dividing cells in the different populations, we pulse-labeled MEFs growing in serum containing medium with BrdU. The proportion of homozygous mutant cells in S phase was considerably reduced compared to wild-type and heterozygous cells, and a larger proportion of the mutant cells had accumulated in the G1 and G2 phases of the cell cycle (Figure 2D). There was little difference, however, in the number of apoptotic cells or in cell size in the different MEF populations (data not shown).

Activation of the PI 3-Kinase Pathway by Oncogenic Mutant Ras Requires Direct p110α-Ras Interaction

The ability of expression of oncogenic mutant Ras to induce activation of PI 3-kinase is well established (Rodriguez-Viciana et al., 1994). However, the possibility existed that this effect was indirect, driven by interaction of Ras with effectors other than PI 3-kinase, possibly through the stimulation of transcriptional changes leading to autocrine growth factor production. To address whether the direct interaction of Ras with p110α is needed in order for oncogenic mutant Ras to stimulate the PI 3-kinase pathway, we made use of a posttranslationally inducible activated form of Ras, ER:V12 H-Ras (Dajee et al., 2002). In this construct, the hormone-binding domain of the estrogen receptor is fused to the amino terminus of an activated mutant of H-Ras. On addition of the ligand 4-hyroxytamoxifen (4HT), steric hindrance of Ras is relieved leading to its ability to interact with effectors. In serum-starved wild-type immortalized MEFs stably expressing ER:V12 H-Ras, addition of 4HT rapidly leads to activation of both ERK and Akt, with phosphorylation of these targets visible within 10 min (Figure 3). In contrast, MEFs expressing the RBD mutant p110α fail to activate Akt significantly even after 24 hr induction with 4HT, although ERK activation is normal. These data therefore confirm that direct interaction of Ras with PI 3-kinase is crucial for Akt activation.

Mice Deficient in the Interaction of p110α with Ras Have Defective Lymphatic Development

When animals heterozygous for the p110α mutations were interbred, we observed that the number of homozygous

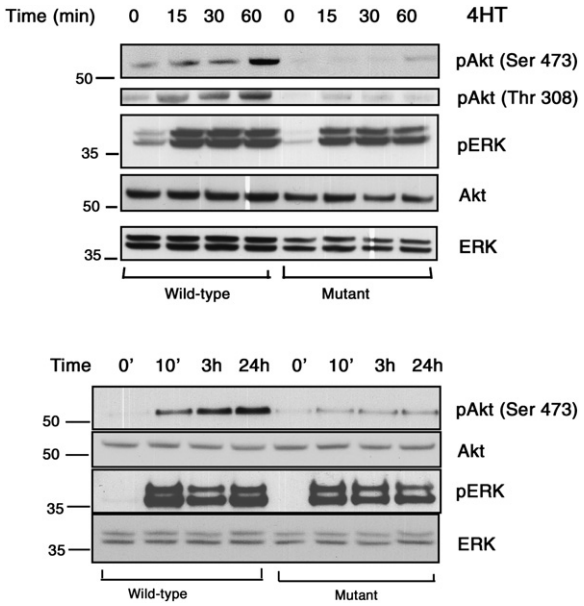

Figure 3. Activation of the PI 3-Kinase Pathway by Oncogenic Mutant Ras Requires Direct p110α-Ras Interaction

Wild-type and mutant MEFs were immortalized with SV40 T antigen and then infected with a retroviral construct, ER:V12 H-Ras, expressing inducibly activatable Ras. Stable drug-selected populations of cells were assessed for the ability of 4-hyroxytamoxifen (4HT) to induce phosphorylation of Akt and ERK. Cells were serum starved for 16 hr followed by induction with 100 nM 4HT for the indicated times. Cells were then harvested and probed with the indicated antibodies by Western blotting.

mutant animals obtained on genotyping at two weeks of age (2%) was significantly below that expected (Table 1). Analysis of E18.5 embryos of similar litters demonstrated a ratio of homozygous mutant animals that was close to Mendelian (19%), while the ratio for one-day-old pups was 9%, suggesting that some mutant animals were being lost around the time of birth. The number of pups counted shortly after birth was higher than the number that remained after 14 days because a number of apparently sick animals had to be culled in the first few days after birth, in accordance with local animal welfare regulations. Closer examination of pups just after birth revealed that most mutant animals exhibited a milky appearance of the peritoneum (Figure 4A). This is a sign of chylous ascites, a condition caused by the leakage of lymphatic fluid into the peritoneal cavity, where it can cause an inflammatory response that can lead to death. In newborn animals, this is usually associated with developmental malformation of the intestinal lymphatic system (Press et al., 1982).

To determine the cause of the suspected chylous ascites, sections of bowel from newborn pups were examined for any abnormality in their tissue architecture. The jejunum demonstrated an edemal accumulation of fluid in the submucosal layer of the intestinal wall (Figure 4B). Because this region of the submucosa contains the lymphatic network collecting chyle from the intestinal lacteals, we suspected that the p110α mutant mice had an abnormally developed lymphatic system in this section of the intestine. Closer examination of the lymphatic vasculature system in the mutant mice revealed deficient branching and development of the network. Staining of E18.5 embryos for the receptor tyrosine kinase VEGFR-3, the receptor for VEGF-C and VEGF-D, which is expressed in the lymphatic endothelium (Dumont et al., 1998, Kaipainen et al., 1995), showed that although major lymph vessels in the thoracic cavity, such as the thoracic duct, were present in the mutants, the finer vessels of the lymphatic network, such as the intercostal vessels, were completely absent (Figure 4C). Similar analysis of the skin and diaphragm of E18.5 embryos showed highly reduced numbers of vessels and a much-reduced level of network branching (Figure 4D). Staining for another marker of the lymphatic network, the homeodomain transcription factor PROX-1, confirmed the lack of a fully developed lymphatic network in the mesenterium of p110α mutant mice (Figure 4E).

A relatively small proportion of the p110α mutant mice survived into adulthood. These mice appeared to be healthy, and any signs of chylous ascites disappeared. Analysis of the lymphatic vasculature in these surviving adult mice revealed it to be normal (data not shown), suggesting either that the lymphatic system recovers from its earlier retarded development or that the severity of the developmental defect is variable and in a minority of mutant mice the lymphatics develop normally. However, the mutant mice that survived to adulthood exhibited a reduced body weight that remained below wild-type levels at all stages of growth (Figure S2).

Table 1. Disruption of the Interaction of p110α with Ras Leads to Reduced Numbers of Surviving Mice

Parental cross	#1: het × het	#2: het × het	#3: het × het
Litter age at genotyping	14 days after birth	1 day after birth	Embryonic day 18.5
Wild-type	124	30	23
Heterozygous mutant	283	77	33
Homozygous mutant	8	10	13
% homozygous mutant obtained	2%	9%	19%

Table showing numbers of animals produced from heterozygote/heterozygote crosses for each genotype when analyzed at different ages. Each group represents an independent set of animals.

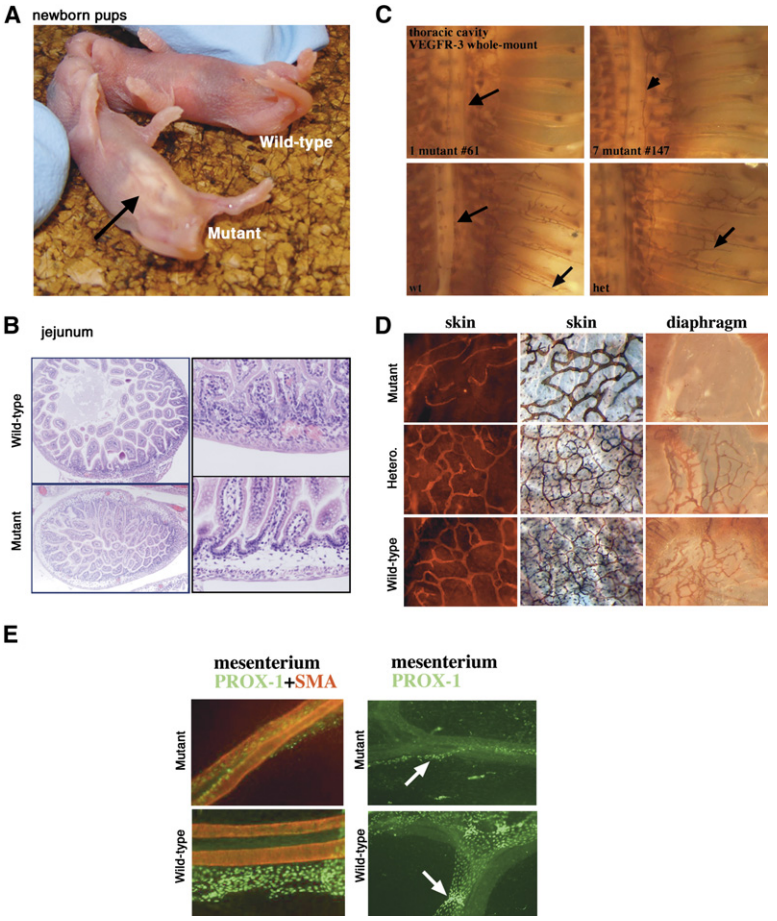

Figure 4. Mice Deficient in the Interaction of p110α with Ras Have Defective Lymphatic Development

(A) p110α mutant mice exhibit chylous ascites: picture of mutant as compared to wild-type new-born mouse pups demonstrating the accumulation of chyle in the abdominal cavity.

(B) Histological analysis of the intestine of newborn mice reveals an abnormality in the submucosal layer of the jejunum in p110α mutants.

(C) p110α mutant mice exhibit deficient development and branching of the lymphatic system. Whole-mount examination with VEGFR3 staining of mutant E18 embryos demonstrates the presence of the thoracic duct (arrowhead) but shows the lack of an extensive branching network in the intercostal region of the thorax (arrows).

(D) Similar examination of the diaphragm and the skin of p110α mutant E18 embryos shows complete absence of the lymphatic system in the diaphragm and deficient branching of the lymphatic vessels in the skin.

(E) Staining of the mesenteric lymphatic network with PROX-1 antibody indicates reduced numbers of lymphatic endothelial cells in mutant animals.

Disruption of the Interaction of p110α with Ras Inhibits In Vitro Transformation of Fibroblasts

To assess the requirement of the interaction of 110α with Ras for the ability of Ras and other oncogenes to transform murine fibroblasts in vitro, MEFs from wild-type, heterozygous, and homozygous mutant p110α animals were immortalized by expression of SV40 large T antigen, then infected with retroviral vectors expressing activated mutant H-Ras, activated mutant EGF receptor L858R, or polyoma virus middle T oncogene. These cell populations were then tested for their degree of transformation by assessing their ability to form colonies in soft agar (Figure 5A). As quantified in Figure 5B, wild-type MEFs expressing activated H-Ras grew independently of anchorage whereas homozygous mutant MEFs completely failed to show transformation by H-Ras. The heterozygous cells showed somewhat reduced transformation efficiency by H-Ras. All three genotype MEFs were found to be expressing H-Ras at similar levels as determined by immunoblotting (data not shown). The ability of activated EGF receptor to transform MEFs was also compromised by homozygous mutation of the p110α RBD, but polyoma middle T transformed all three genotypes equally well. Since anchorage-independent growth is a hallmark of oncogenic transformation in vitro, these data support a direct cell-autonomous role for the Ras-PI 3-kinase interaction in Ras-mediated tumorigenesis and also in transformation by some other oncogenes known to act upstream of Ras, such as EGF receptor. In addition to effects on transformation by H-Ras, p110α RBD mutation also compromised the ability of K-Ras to transform MEFs (Figure S3).

Disruption of the Interaction of p110α with Ras Blocks Carcinogenesis Driven by K-Ras

In order to investigate whether the ability of endogenous p110α to interact with endogenous Ras plays a role in carcinogenesis caused by oncogenic mutation of the K-Ras gene in vivo, the p110α mutant mice were bred with K-Ras LA2 mice. In this strain, an activating mutation has been introduced into one allele of the K-Ras gene, but expression is silenced by a disrupting sequence. This is lost by homologous recombination at very low frequency, resulting in the expression of oncogenic K-Ras protein at endogenous levels in a small number of cells in each tissue. The mice develop lung adenocarcinomas at high frequency, with most of the mice dying due to tumor burden by 200 days of age (Johnson et al., 2001).

Mice that were homozygous for the *Pi3kca* mutation and carried one copy of the K-Ras LA2 transgene showed similar survival rates in the first few weeks of life to

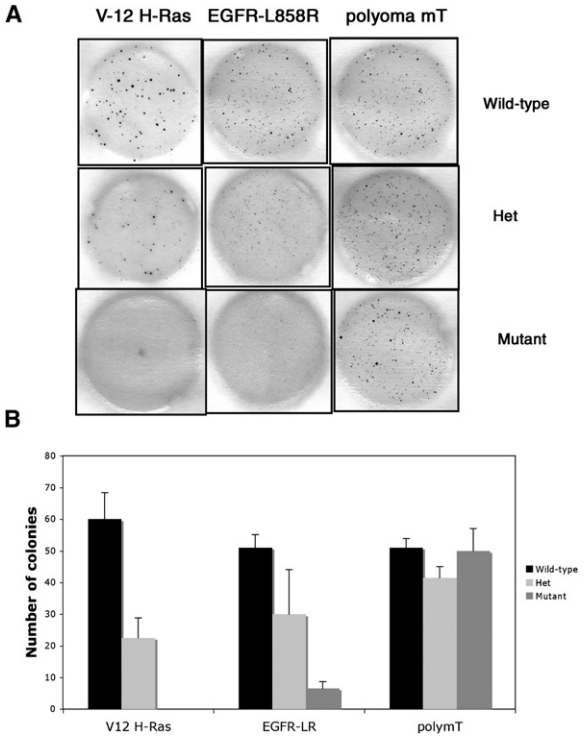

Figure 5. Disruption of the Interaction of p110α with Ras Inhibits In Vitro Transformation of Fibroblasts

MEFs from wild-type, heterozygous, and homozygous mutant animals were immortalized by expression of SV40 large T antigen. Immortalized cells were then infected with activated mutant H-Ras V12, activated mutant EGF receptor L858R, or polyoma middle T. Selected stable populations of cells were tested for their ability to grow in an anchorage-independent manner in soft agar.

(A) Representative images of macroscopic colonies formed after 3 weeks are shown.

(B) Quantification of the numbers of colonies formed. Error bars shown are standard errors of the mean for triplicate samples.

equivalent mice lacking K-Ras mutation. These mice were sacrificed at 4 months and the numbers of tumor nodules visible on the lung surface were counted. The number of tumors occurring in the *Pi3kca* mutant mice was massively reduced compared to those with wild-type PI 3-kinase. While the K-Ras LA2 mice typically had forty or so macroscopic tumors on the surface of the lung, the *Pi3kca* mutant mice had between zero and two visible tumors, and these were much smaller (Figure 6A). One *Pi3kca* mutant K-Ras LA2 mouse was aged to 6 months and found to be still entirely free of macroscopically detectable lung tumors (data not shown).

Histological analysis of the *Pi3kca* mutant mouse lung tissue revealed that they did contain some foci of abnormal cells that might represent premalignant lesions. However, these were very small compared to tumors found in K-Ras LA2 animals on a wild-type background (Figure 6B). In addition, we used immunohistochemistry with phosphospecific antibodies to study the activation state of Akt in the tumors and premalignant lesions in the K-Ras LA2 animals on the *Pi3kca* mutant and wild-type backgrounds. As shown in Figure S4A, there is evidence that Akt is activated in the Ras-induced tumors found in the wild-type mice but not in the lesions found in the PI 3-kinase mutant background.

Furthermore, the levels of cell proliferation, apoptosis, and senescence were studied in the tumors and premalignant lesions of these animals. As shown in Figure S4B, TUNEL assays revealed low levels of apoptosis in Ras-induced tumors found on a wild-type background, but there were signs of significant levels of apoptosis occurring in the small lesions found on the PI 3-kinase mutant background. The proliferative rates in the lesions and tumors in the two backgrounds were similar, as measured by phospho-histone H3 staining for mitotic cells (Figure S4C), so it is likely that the difference in the sizes of the tumors in the wild-type and the PI 3-kinase mutant backgrounds is at least in part due to elevated rates of cell death in the absence of direct Ras binding to p110α.

Another way of looking at the efficiency of Ras-induced tumor formation is to use the two-stage skin carcinogenesis protocol using initiation with the mutagen DMBA (7,12-dimethylbenzanthracene), which causes activating mutations in the murine H-ras gene, and promotion with the phorbol ester TPA (12-O-tetradecanoylphorbol-13-acetate). As shown in Figure S5, PI 3-kinase mutant mice were much more resistant than wild-type mice to the formation of skin tumors by this means. This experiment was performed on a mouse background, C57BL/6, that is relatively resistant to papilloma formation, so overall tumor numbers formed are low, even for the wild-type mice.

DISCUSSION

A Physiological Role for Ras in the Regulation of PI 3-Kinase by Growth Factors

The creation of mice lacking the ability of their p110α PI 3-kinase catalytic subunit to interact with activated Ras provides an opportunity to definitively address the significance of this interaction in growth factor signaling both in vivo and in vitro. In cultured mouse embryo fibroblasts, loss of p110α binding to Ras strongly reduces PI 3-kinase activation by EGF and FGF-2, but not by PDGF. This differential requirement for Ras may reflect the fact that the activated receptor for PDGF directly binds the p85 regulatory subunit of PI 3-kinase at the plasma membrane (Kazlauskas and Cooper, 1989), whereas the others do not. EGF receptor is thought to direct PI 3-kinase activation more indirectly, either via Gab1 and Grb2 or via ErbB3 (Mattoon et al., 2004; Soltoff et al., 1994). In the case of FGF-2, its receptor phosphorylates the docking protein FRS2, which in turn binds Grb2 and Gab1 (Ong et al., 2001). Where PI 3-kinase is recruited to receptor complexes indirectly, it is possible that smaller numbers of p110 molecules are activated, perhaps making a costimulatory role for Ras binding more essential. On the other

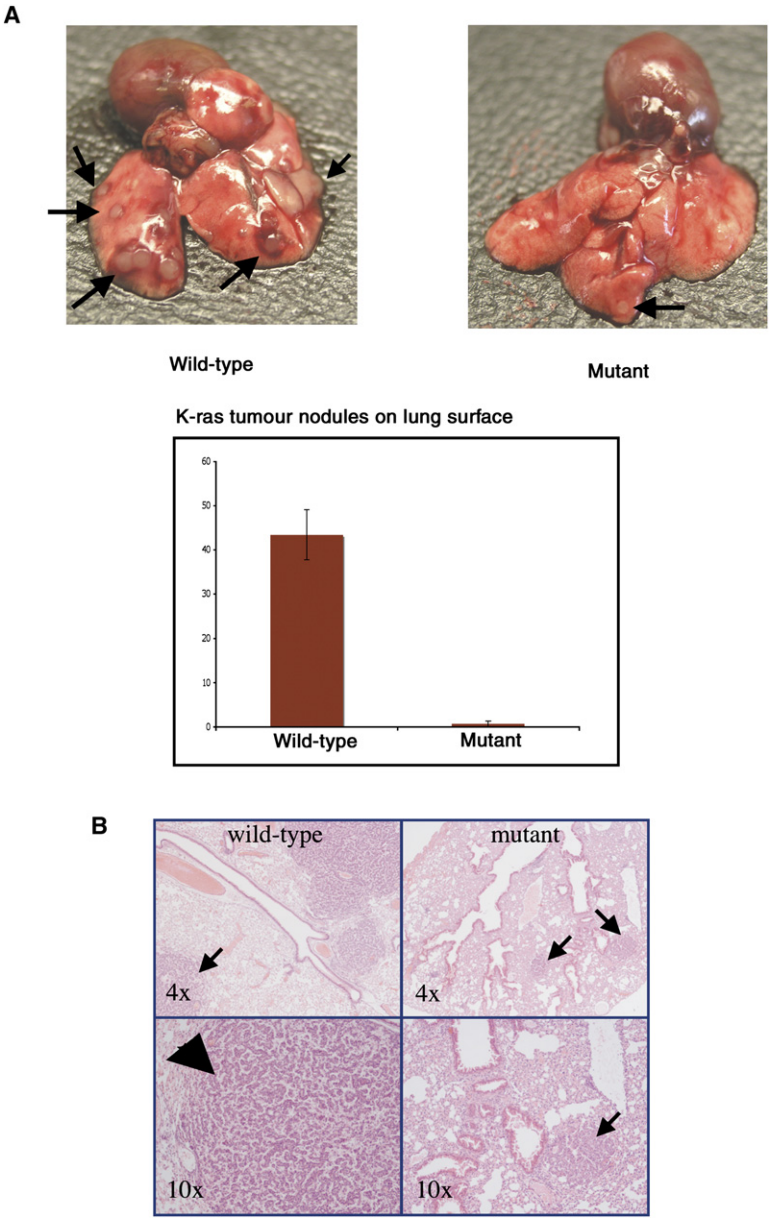

Figure 6. Disruption of the Interaction of p110α with Ras Blocks Carcinogenesis Driven by K-Ras

(A) Tumour nodules on the surface of the lungs in K-Ras LA2 mice on wild-type and *Pi3kca* mutant backgrounds. Note the appearance of just one tumor nodule (~1 mm diameter) on the mutant lung and several macroscopic nodules of different sizes on the wild-type lung. The lower panel shows quantitation of the tumors found in three *Pi3kca* mutant and six wild-type K-Ras LA2 mice at 120 days. Error bars shown are standard errors of the mean for triplicate samples.

(B) H&E staining of oncogenic K-Ras mouse lungs. The arrows indicate small adenomatous hyperproliferations seen in the lungs of *Pi3kca* mutant mice. The large arrowhead indicates a typical adenocarcinoma in a mouse with wild-type *Pi3kca*.

hand, other considerations may also be involved, as insulin signaling to PI 3-kinase, which involves IRS family adaptors, does not appear to be majorly dependent on Ras interaction with p110α. Another possible explanation might be the extent to which particular growth factor receptors use different isoforms of p110. Deletion of p110α in mouse embryo fibroblasts results in more complete inhibition of EGF than PDGF signaling to Akt (Zhao et al., 2006). It has been suggested that this might reflect an ability of PDGF, but not EGF, receptor to regulate p110β, which is also expressed in these cells.

Very recently, mice have been made where a similar set of mutations has been introduced into a different isoform of PI 3-kinase, p110γ, whose expression is largely restricted to hematopoietic cells. This resulted in loss of accumulation of PIP_3 in neutrophils in response to chemoattractants (Suire et al., 2006). In addition, flies with a similar mutation introduced into Dp110 show greatly reduced egg-laying ability and are small in size (Orme et al., 2006). This suggests that blocking Ras interaction with p110 can attenuate PI 3-kinase regulation in other systems as well.

Interaction with Ras has been shown to allosterically activate PI 3-kinase via a change in the structure of the catalytic pocket (Pacold et al., 2000), in a manner that is synergistic with binding of p85 to pYXXM peptides (Rodriguez-Viciana et al., 1996). By itself, the interaction of Ras with p110 is insufficient to drive membrane translocation of PI 3-kinase, a necessity for its activation (Suire et al., 2002), suggesting that the ability of oncogenic

mutant Ras by itself to drive PI 3-kinase activation may be dependent on low-level signaling input from receptor tyrosine kinases, especially EGF receptor family members that respond to autocrine ligands produced in response to activation of the Raf/ERK branch of Ras downstream pathways (Schulze et al., 2001).

The phenotype of mice homozygous for the p110α RBD mutation suggests that in vivo there is also a requirement for the Ras/PI 3-kinase interaction for some growth factors to signal correctly. Most obviously, there is a partial failure and delay of development of the lymphatic system, resulting in the accumulation of chylous ascites in newborn pups. This developmental phenotype is similar to that of VEGF-C$^{+/-}$ mice (Karkkainen et al., 2004) and also to some extent angiopoietin 2 knockout mice (Gale et al., 2002). The sprouting of the first lymphatic vessels from embryonic veins appears to be highly dependent on VEGF-C signaling, with homozygous deletion of VEGF-C resulting in embryonic edema from E12.5, complete lack of lymphatic vasculature, and death in utero. The similarity of the phenotype of the VEGF-C heterozygotes and p110α RBD mutant homozygotes could suggest that failure of Ras to engage p110α directly might result in vivo in a roughly 50% reduction in the ability of VEGF-C to signal to a critical downstream effector system, such as PI 3-kinase/Akt. It has been demonstrated that VEGF-C promotes survival and proliferation of lymphatic endothelial cells in vitro and induces Akt and ERK activation (Makinen et al., 2001b). VEGF-C signals through two receptor tyrosine kinases, VEGFR2 and VEGFR3, of which VEGFR3 is critical for its role in control of lymphatic development (Joukov et al., 1996; Makinen et al., 2001a, 2001b). VEGFR3 makes a good candidate for a receptor that might require Ras to signal to PI 3-kinase as, like EGFR and FGFRs, it lacks good p85-binding motifs and presumably must engage the pathway indirectly. However, it is also possible that signaling through other lymphangiogenic growth factors and their receptors, such as angiopoeitin 2 and Tie2, could also be defective in the p110α RBD mutant mice.

It is striking that one of several defects suffered by mice deleted for the Pik3r1 gene, which encodes the PI 3-kinase regulatory subunits p85α, p55α, and p50α, is the formation of chylous ascites (Fruman et al., 2000). These mice have reduced levels of p110α protein and lipid kinase activity but show much more widespread problems, including extensive liver necrosis and hypoglycaemia, indicating the relatively selective nature of the impact on PI 3-kinase signaling of disrupting the Ras/PI 3-kinase link. However, in the animals reported here, in addition to the effects on lymphatic development, it is possible that other growth factor signaling pathways may be defective that are not essential under the husbandry conditions employed. Moreover, the decreased size of these animals may reflect defects in systemic growth regulation, although at present it cannot be ruled out that it is an indirect manifestation of the perinatal lymphatic development defect.

The Importance of Ras Interaction with PI 3-Kinase in Tumorigenesis

While the results discussed above clearly show that Ras interaction with PI 3-kinase is needed for some normal growth factor signaling, the ability of activated mutant Ras to cause transformation in vitro and tumor formation in vivo also requires PI 3-kinase p110α to have a functional RBD. The striking effect of homozygous mutation of p110α RBD on the ability of a sporadically activated single D12 K-Ras allele expressed at endogenous levels to cause lung tumor formation suggests that, at least in this setting, oncogenic Ras is highly dependent on its ability to stimulate PI 3-kinase directly in order to drive tumorigenesis in vivo.

Hundreds of lung adenocarcinomas form in K-Ras LA2 mice by 4 months of age, resulting in death by 6 months (Johnson et al., 2001). The rate of lung tumor formation is cut by more than 95% by the introduction of point mutations in p110α blocking its interaction with Ras. Normally, the adenocarcinomas in K-Ras LA2 mice show evidence of Akt activation, suggesting that Ras is inducing this effector pathway in the tumors. Very few tumors form in the PI 3-kinase mutant animals, although they do show significant areas similar to atypical adenomatous hyperplasia (AAH), thought to be a premalignant condition that may progress to adenocarcinoma (Nakahara et al., 2001). The AAH-like lesions in the PI 3-kinase mutant mice do not exhibit Akt activation. It appears likely that these hyperplastic lesions fail to develop into tumors due to defective Akt activation, resulting in increased rates of apoptosis. In this manner the lung epithelial cells differ from the embryonic fibroblasts derived from these mice, where the major effect of the mutation of PI 3-kinase appeared to be on cell proliferation rather than survival. We have not been able to observe obvious signs of senescence in the AAH lesions using β-galactosidase staining, although this has been observed in a different K-Ras-driven mouse lung cancer model (Collado et al., 2005).

The simplest explanation for the failure of tumors to develop in the PI 3-kinase mutant mice is that lung epithelial cells expressing activated Ras fail to activate Akt, which is directly required for their proliferation and survival. Clones of activated Ras-expressing epithelial cells thus fail to grow beyond a limited size. However, it is also possible that the defect is not entirely cell autonomous: the PI 3-kinase mutant epithelial cells may be deficient in their interaction with surrounding stroma. They could be unable to induce angiogenesis, although the lesions may well be too small to require new vasculature. It is also conceivable that they have an altered interaction with the host immune system. In each case these possible problems could be caused either by defects in the activated Ras-expressing epithelial cells themselves or in the surrounding host tissues, which also carry the PI 3-kinase mutation, even though they lack expression of activated Ras. The possibility of defects in tumor neovascularization might be raised by the deficiencies in the related lymphangiogenesis system in the PI 3-kinase mutants, although there is no

indication that there is any developmental problem with normal blood vessel formation in these mice. Defects in lymphangiogenesis itself are very unlikely to account for failure of primary tumors to develop, although the lymphatics are important for metastatic spread of established tumors (Saharinen et al., 2004). In any case, the lymphatic system is apparently normal in the lungs of the PI 3-kinase mutant mice once they reach adulthood.

Study of the ability of exogenously introduced Ras to transform immortalized fibroblasts from normal and PI 3-kinase mutant mice in vitro suggests that there is at least a major cell-autonomous component to the impact of PI 3-kinase RBD mutation on Ras-induced tumorigenesis. This in vitro system also allows rapid study of the specificity of the effect of this mutation on different oncogenic signaling pathways. Like activated Ras, oncogenic mutant EGF receptor requires Ras interaction with p110α to induce colony formation in soft agar, but the ability of polyoma virus middle T antigen to transform fibroblasts is not affected. Polyoma middle T activates PI 3-kinase strongly via direct binding to p85 via sites phosphorylated by c-Src (Whitman et al., 1985), presumably without need for synergistic input from Ras. We are also in the process of addressing the specificity of the inhibitory effect of the p110α RBD mutation on tumorigenesis in vivo for oncogenes other than Ras.

The results presented here prove that the direct interaction of Ras with the catalytic subunit of type I PI 3-kinase is important in both malignant and normal developmental growth signaling. The strong reduction in Ras-induced lung tumor formation when this binding is prevented suggests that specific targeting of the interaction of Ras with PI 3-kinase may have therapeutic value in the treatment of tumors, such as lung, colon, and pancreatic carcinoma, with high incidences of Ras mutation. In addition, since adult mice lacking Ras binding to PI 3-kinase are apparently healthy, drugs targeting this link might be expected to be well tolerated.

EXPERIMENTAL PROCEDURES

Reagents

The murine p110α genomic clones (gm35 and gm45) were a kind gift from Dr. Tom Roberts (Dana Farber Cancer Institute). Bovine p110α, V12 H-Ras, V12 K-Ras, and Myc expression constructs were described previously (Rodriguez-Viciana et al., 1997). EGF, PDGF-AB, and FGF2 were obtained from R&D systems. BrdU was purchased from Sigma. The oncogenic K-Ras mouse strain (*Kras*-LA2) was from the MMHCC mouse repository (NCI-Frederick). The ER:V12 H-Ras construct (Dajee et al., 2002) was a kind gift from Dr. Paul Khavari (Stanford University).

Antibodies

The pan-ERK, phospho-ERK (E10) monoclonal, and phospho-Akt (Ser473) antibodies were all purchased from Cell Signaling Technology. The Pan-Ras (Ab-4) antibody was obtained from Calbiochem, the p85 (06-195) antibody from Upstate, and the p110β (Sc-602) antibody from Santa Cruz. The antibody recognizing bovine p110α was from Transduction Laboratories. The monoclonal hybridoma tubulin (TAT-1), Akt, and GST antibodies were generated in-house. The VEGFR-3 antibodies were from R&D Systems, the Cy3-conjugated SMA antibody was from Sigma, and the PROX-1 antibody was a kind gift from Dr. Tatiana Petrova. The mouse-specific p110α antibody was a kind gift from Antonio Biliancio and Bart Vanhaesebroeck (Ludwig Institute for Cancer Research, University College London). HA antibody monoclonal antibody 12CA5 was from Abgent, and in vitro kinase assays on HA-Akt immunoprecipitates were performed as in Alessi et al. (1996).

Proliferation Assays

Cells were pulsed with BrdU for 1 hr, prior to trypsinization and fixation in ethanol. FACS analysis was performed on a FACScalibur (Becton Dickson).

PIP_3 Immunocytochemistry

Wild-type and mutant MEFs cultured on coverslips were serum-starved overnight and stimulated with EGF (50 ng/ml), FGF2 (20 ng/ml), or PDGF (20 ng/ml) for 10 min. Reactions were stopped by washing the cells with cold TBS. Cells on glass coverslips were fixed with 4% formaldehyde and then permeabilized with 0.5% Triton X-100 in TBS. Immunocytochemistry was performed using a Histostain-Plus Kit (Invitrogen), according to the manufacturer's protocol. PIP_3 levels were detected with mouse anti-PIP_3 monoclonal antibody (Z-G345; Echelon) diluted at 1:50 overnight at 4°C.

Whole-Mount Immunostaining

Tissues were fixed in 4% paraformaldehyde and used for whole-mount immunofluorescence or immunoperoxidase staining. For immunofluorescence, tissues were stained with goat anti-mouse VEGFR-3, Cy3-conjugated anti-SMA, or rabbit anti-mouse PROX-1 antibodies. Secondary antibodies were Alexa-conjugated (Molecular Probes). For immunoperoxidase staining, tissues were incubated with biotinylated goat anti-mouse VEGFR-3 antibody followed by Vectastain Elite ABC reagent (Vector laboratories). Peroxidase activity was detected with 3,3′-diaminobenzidine (Sigma).

Cell Culture and Soft Agar Assays

MEFs of the three genetic backgrounds were isolated following standard protocol and maintained in DMEM supplemented with 10% fetal bovine serum. Phoenix cells were used to generate ecotropic viruses. MEFs were infected with filtered (pore size 0.45 μm) viral supernatant, supplemented with 8 μg/ml polybrene. Primary MEFs were immortalized by infecting with SV40 T antigen. Selected pools of immortalized cells were infected with H-rasV12 pBABE puro or empty pBABE puro as control. Twenty-four hours after infection, cells were selected with 1 μg/ml puromycin for at least 7 days. To monitor the capacity of MEFs to grow in semi-solid medium in vitro, cells were transferred to 2 ml complete DMEM containing 0.35% low-melting agarose. 1 × 103 cells were seeded in duplicate into 60 mm dishes containing a 2 ml layer of solidified 0.6% agar in complete medium. Colonies were stained 2 weeks later with Giemsa stain.

Supplemental Data

Supplemental Data include Experimental Procedures and five figures and can be found with this article online at http://www.cell.com/cgi/content/full/129/5/957/DC1/.

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