# The role of senescence and prosurvival signaling in controlling the oncogenic activity of FGFR2 mutants associated with cancer and birth defects

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**Mutations in fibroblast growth factor receptors (FGFRs) cause human birth defect syndromes and are associated with a variety of cancers. Although forced expression of mutant activated FGFRs has been shown to oncogenically transform some immortal cell types, their activity in primary cells remains unclear. Here, we show that birth defect and cancer-associated FGFR2 mutants promote DNA-damage signaling and p53-dependent senescence in primary mouse and human cells. Senescence promoted by FGFR mutants was associated with downregulation of c-Myc and forced expression of c-Myc facilitated senescence escape. Whereas c-Myc expression facilitated senescence bypass, mutant FGFR2 signaling suppressed c-Myc-dependent apoptosis and led to oncogenic transformation. Cells transformed by coexpression of a constitutively activated FGFR2 mutant plus c-Myc appeared to be become highly addicted to FGFR-dependent prosurvival activities, as small molecule inhibition of FGFR signaling resulted in robust p53-dependent apoptosis. Our data suggest that senescence-promoting activities of mutant FGFRs may normally limit their oncogenic potential and may be relevant to their ability to disrupt morphogenesis and cause birth defects. Our results also raise the possibility that cancers originating through a combination of constitutive FGFR activation and deregulated Myc expression may be particularly sensitive to small molecule inhibitors of FGF receptors.**

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## INTRODUCTION

Fibroblast growth factor signaling plays crucial roles during embryonic development by regulating cell proliferation, differentiation, survival, cell–cell communication and cell fate specification. The biological effects of FGF ligands are mediated by binding to FGF receptors, which induce receptor dimerization, autophosphorylation and the assembly of signaling complexes. Fibroblast growth factor receptor (FGFR) activation initiates several different phosphorylation cascades whose combined actions dictate their effects on cell behavior. Signaling pathways controlled by FGFs include the phospholipase C-gamma (PLC-$\gamma$), phosphatidylinositol-3 kinase (PI3K) and Ras/mitogen-activated protein kinase (MAPK) pathways (1). The strength, anatomical location and duration of FGF signaling are of critical importance during embryonic

development and mutations in FGFRs that either cause constitutive FGFR activation, altered receptor dimerization or influence ligand-binding specificity or affinity, can lead to a variety of birth defects (2,3).

In addition to causing developmental defects, activating or gain-of-function mutations in *FGFRs* are found sporadically in a number of different types of cancer. For example, mutations in *FGFR2* are found in endometrial and gastric tumors (4–7), mutations in *FGFR3* are found in urothelial carcinomas (8) and multiple myeloma (9,10), and mutations in *FGFR1* have been found in glioblastoma (11). Mutations in *FGFR3* are also found in benign epidermal nevi and seborrheic keratoses (12–14) and single nucleotide polymorphisms in the *FGFR2* gene that lead to increased *FGFR2* transcription are associated with increased risk of breast cancer (15–17). In addition to

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*FGFR* mutations, *FGFR* gene amplification, particularly of *FGFR2* (18), is associated with several different cancers.

Consistent with direct participation in tumorigenesis, a number of studies have shown that expression of cancer-associated *FGFR3* and *FGFR2* mutations can promote oncogenic transformation of immortal mouse cell lines (7,19–22). However, their effects in both immortal and primary cells appear to be highly cell type specific and proliferation of some cell types is strongly inhibited by FGFR signaling (reviewed in 1,23). Although the mechanisms responsible for the different responses that cells have to mutant FGFRs remain unclear, it has been postulated to be related to their ability to engage signaling pathways that can both promote proliferation and suppress proliferation, as well as by triggering negative feedback mechanisms (1).

Although FGF receptors can activate different signal transduction pathways, the Ras/Raf/MAPK pathway appears to be of critical importance in mediating the effects of activated FGF receptors. For example, it was recently shown that small molecule-mediated suppression of Ras/MAPK signaling rescued craniosynostosis in a mouse model of Apert syndrome (24), a disease caused by mutations in FGFR2 that result in broadened ligand-binding specificity and increased ligand affinity (25–27). Since the same mutations that cause Apert syndrome are also found in some cancers (4–7), it seems likely that increased Ras/MAPK signaling plays an important role in their oncogenic activities. However, although activating mutations in *RAS* family members and other components of the *RAS/MAPK* pathway are frequent events in cancer, their expression in at least some primary cell types induces senescence (28–30). Similarly, the PI3K pathway, a major pathway activated by FGFRs, has been demonstrated to promote senescence when hyperactivated (31). While senescence appears to be triggered by multiple mechanisms, recent evidence indicates that senescence induced by mutant Ras and several other oncogenic proteins is associated with the development of DNA strand breaks that may arise due to defects in DNA replication (32,33). The induction of senescence and/or apoptosis by oncogenic proteins is associated with chronic activation of DNA-damage signaling that normally functions to arrest cell proliferation and promote repair. This cellular response to oncogenic proteins appears to provide a significant barrier to further progression towards a malignant state (reviewed in 34–36).

Here we investigated the potential role that senescence might play in regulating the oncogenic activities of mutant forms of FGFR2 that are associated with human birth defects and cancer. We show that gain-of-function and constitutively active FGFR2 mutants promote DNA-damage signaling and p53-dependent senescence. Induction of senescence was linked to downregulation of c-Myc and forced expression of c-Myc facilitated senescence escape and cooperated with mutant activated FGFR2 in oncogenic transformation. The mechanism of oncogenic cooperation between mutant FGFR2 and c-Myc involved both c-Myc-facilitated senescence escape and protection against c-Myc-dependent apoptosis by FGFR2. Moreover, cells transformed by the combination of constitutively active FGFR2 and c-Myc become highly addicted to the prosurvival signaling of the mutant receptor, as these cells could be effectively eliminated by treatment with small mol-

ecule FGFR inhibitors. These studies provide insight into mechanisms underlying the differential effects of mutant FGFRs on cell proliferation and help define the properties of mutant FGFRs that make them highly oncogenic, but also susceptible to drug-induced cell death.

## RESULTS

### Birth defect and cancer-associated FGFR2 mutants induce senescence in primary cells

FGFR2$^{S252W}$, a mutation that affects ligand binding and causes Apert syndrome, and FGFR2$^{K659E}$, a predicted constitutively active receptor mutated in the tyrosine kinase region, have been identified in cancers (5–7). The FGFR2$^{K659E}$ mutation is equivalent to FGFR3$^{K650E}$, which is also found some cancers and causes the neonatal lethality syndrome Thanatophoric Dysplasia Type II (3). Because FGFRs signal through the PI3K and MAPK pathways, which both promote senescence when hyperactive, we examined whether FGFR2$^{S252W}$ and FGFR2$^{K659E}$ promoted senescence in primary mouse embryonic fibroblasts (MEFs). Wild-type FGFR2, FGFR2$^{S252W}$ and FGFR2$^{K659E}$ were introduced into primary MEFs at passage 2–3 using retroviral expression vectors, briefly drug selected, then stained for senescence-associated (sa) β-galactosidase activity. Cells expressing Ras$^{G12V}$ were used as positive controls in these experiments. In contrast to vector alone or wtFGFR2, expression of the FGFR2 mutants, like Ras$^{G12V}$, led to a significant increase in the number of cells that stained positive for sa-β-galactosidase (Fig. 1A and B). sa-β-Galactosidase staining corresponded to the appearance of many enlarged and multinucleated cells exhibiting a flattened morphology, and suppressed cell proliferation (Fig. 1A and C). Similarly, expression of FGFR2$^{S252W}$ and FGFR2$^{K659E}$ in human IMR90 lung fibroblasts, like Ras$^{G12V}$, led to an increase in the number of sa-β-galactosidase-positive cells (Fig. 1E and F). A constitutively active FGFR3 mutant (FGFR3$^{K650E}$) behaved essentially identical to FGFR2$^{K659E}$ in these assays (data not shown). The induction of senescence observed was associated with only a moderate increase in levels of the FGFR2 mutants compared with endogenous FGFR2 in MEFs and IMR90 fibroblasts (Fig. 1D and G). These results suggest that although birth defect and cancer-associated FGFR mutants generally promote proliferation and oncogenesis in immortal cells, they promote senescence in primary cells.

### Activation of DNA-damage signaling by FGFR2 mutants

We next investigated whether senescence caused by mutant FGFR2 proteins is related to DNA-damage signaling and activation of DNA-damage checkpoints that promote cell cycle arrest. Primary MEFs and IMR90 fibroblasts subjected to retroviral expression of wild-type FGFR2, FGFR2$^{S252W}$, FGFR2$^{K659E}$ or Ras$^{G12V}$ at passage 3 were examined for several proteins that participate in the DNA-damage response at passage 5. When compared with control cells infected with empty vector or wild-type FGFR2, expression of either FGFR2$^{S252W}$, FGFR2$^{K659E}$ or Ras$^{G12V}$ resulted in an increased percentage of cells exhibiting the characteristic speckled pattern of γH2AX (Ser139-phosphorylated H2AX) (Fig. 2A
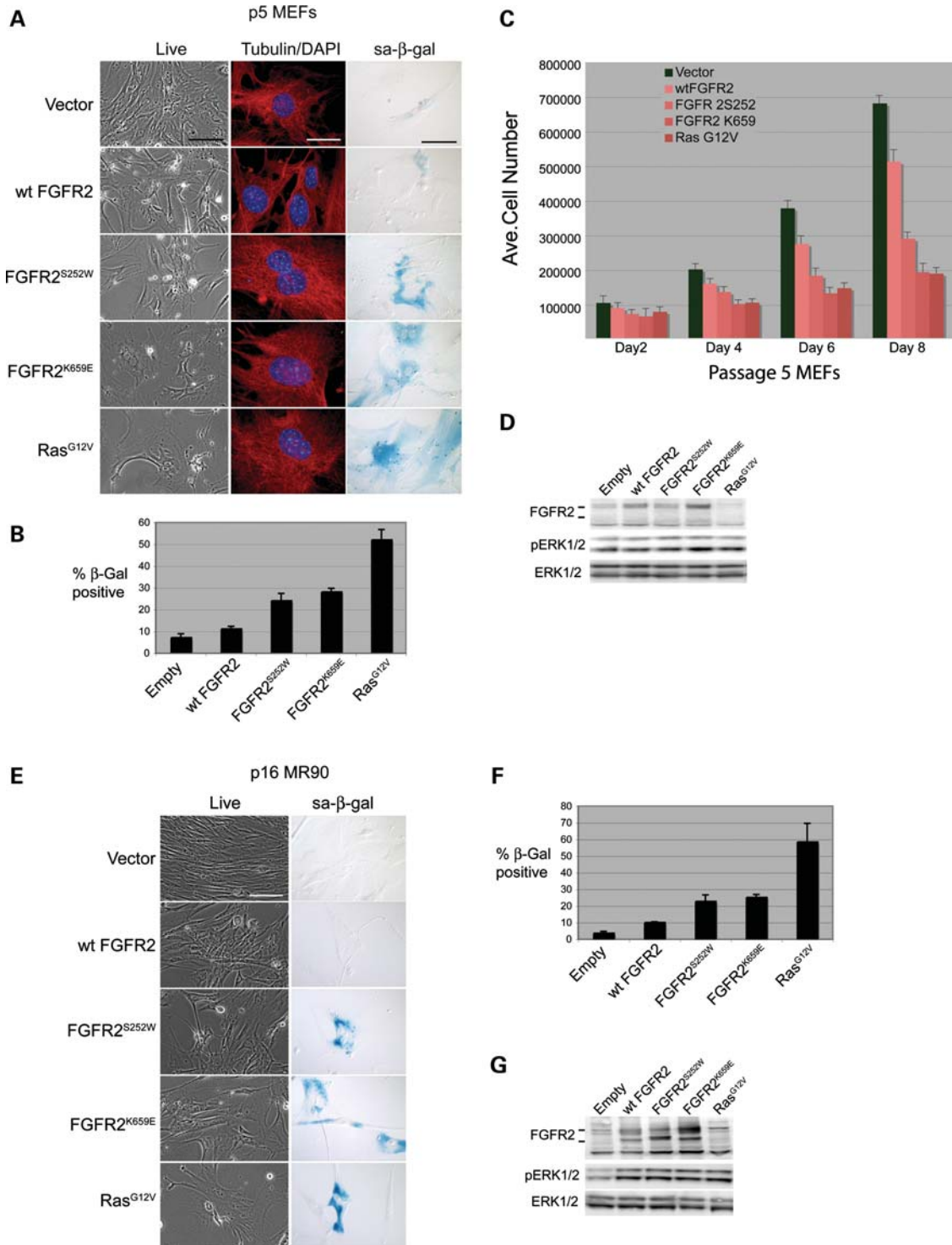
**Figure 1.** FGFR2 mutants promote senescence in primary cells. (**A**) Morphology (live cells and tubulin/DAPI stained cells) and sa-β-galactosidase staining of primary MEFs. (**B**) The mean percentage of β-galactosidase-positive cells and standard deviation based on three independent experiments. (**C**) Mean number of cell counts and standard deviation from passage 5 MEFs plated at 50 000 cells per dish (day 0) and counted every 2 days. (**D**) Western blots showing levels of FGFR2, phospho-ERK1/2 (T202/Y204) and total ERK in MEFs. (**E**) Live and sa-β-galactosidase staining of primary human IMR90 fibroblasts expressing the indicated proteins. (**F**) Mean percentage of sa-β-galactosidase-positive IMR90 cells and standard deviation based on triplicate samples from three independent experiments. (**G**) Western blot of IMR90 fibroblasts as in (D). Scale bars: live cells and sa-β-gal—50 μM, tubulin/DAPI—10 μM.

and B), an alternative histone that associates with Ataxia telangiectasia mutated (ATM) and accumulates at DNA-damage foci (35). IMR90 fibroblasts similarly showed increased

γH2AX staining and γH2AX staining co-localized with serine 1981-phosphorylated ATM (Fig. 2C–E). Very similar results were achieved when MEFs and IMR90 fibroblasts
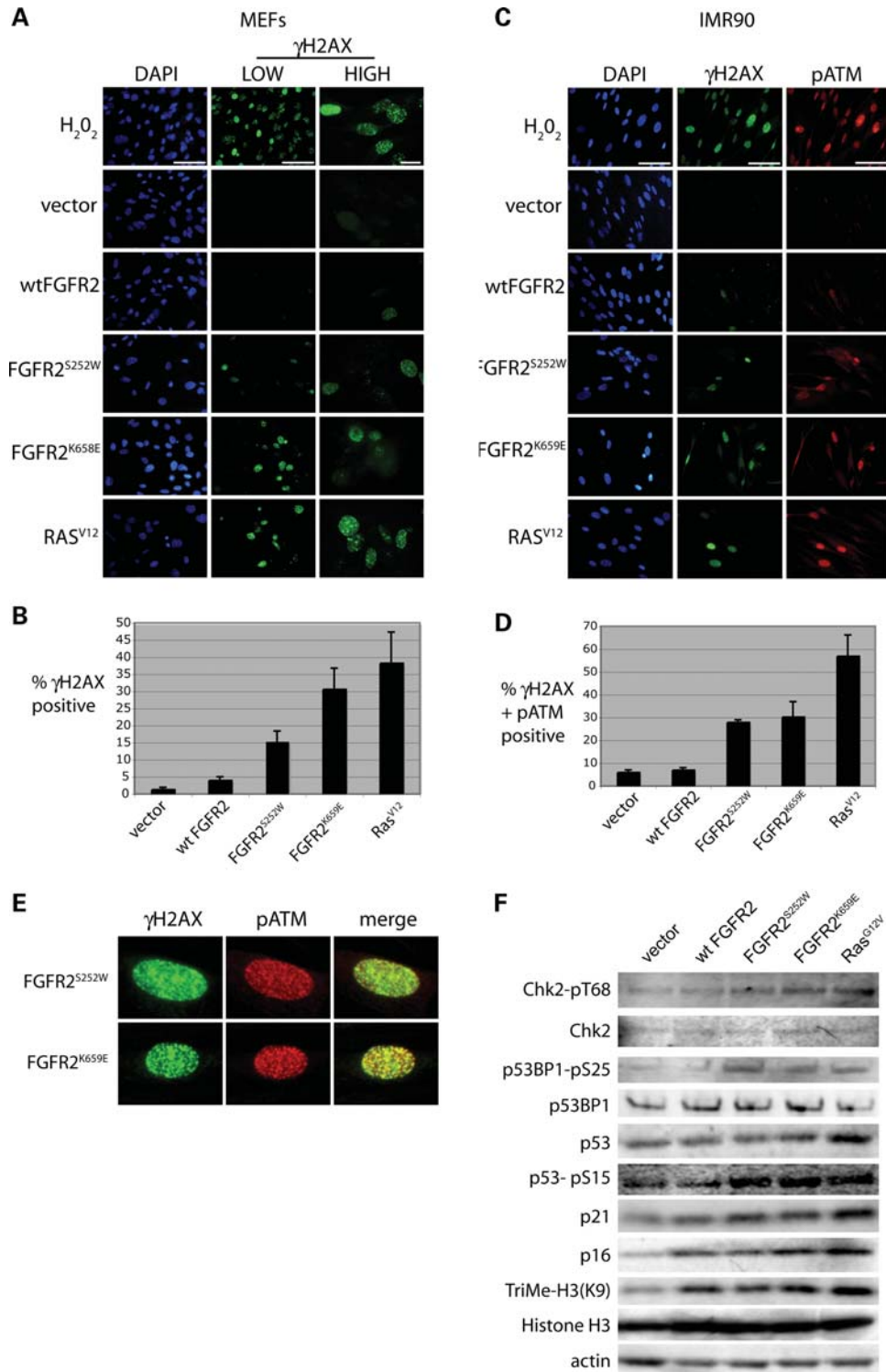
**Figure 2.** Induction of DNA-damage response by FGFR2 mutants. (**A**) γH2AX immunoflourescense in primary MEFs expressing the indicated proteins. Low and higher magnification views are provided to show both the general increase in the number of cells staining for γH2AX, and the speckled pattern of γH2AX staining in positive cells. (**B**) Mean percentage of γH2AX-positive MEFs with standard deviation from three independent experiments. (**C**) γH2AX and p(ser1981)-ATM staining in IMR90 cells expressing the same set of proteins as in (A). (**D**) Mean percentage of γH2AX- and pATM-positive (cells showing co-localization) IMR90 fibroblasts with standard deviations calculated from three independent experiments. Hydrogen peroxide treated cells were used as a positive control, and DAPI staining was used to visualize nuclei in these experiments. (**E**) γH2AX and pATM staining showing coincident staining in IMR90 fibroblasts expressing FGFR2^S252W or FGFR2^K569E as indicated. (**F**) Immunoblot analysis of the indicated proteins. Residues recognized by phospho-specific antiserums is indicated. Actin was used as a general loading control. Scale bars: all are 50 μm, except 'high' (magnification) which is 10 μm.
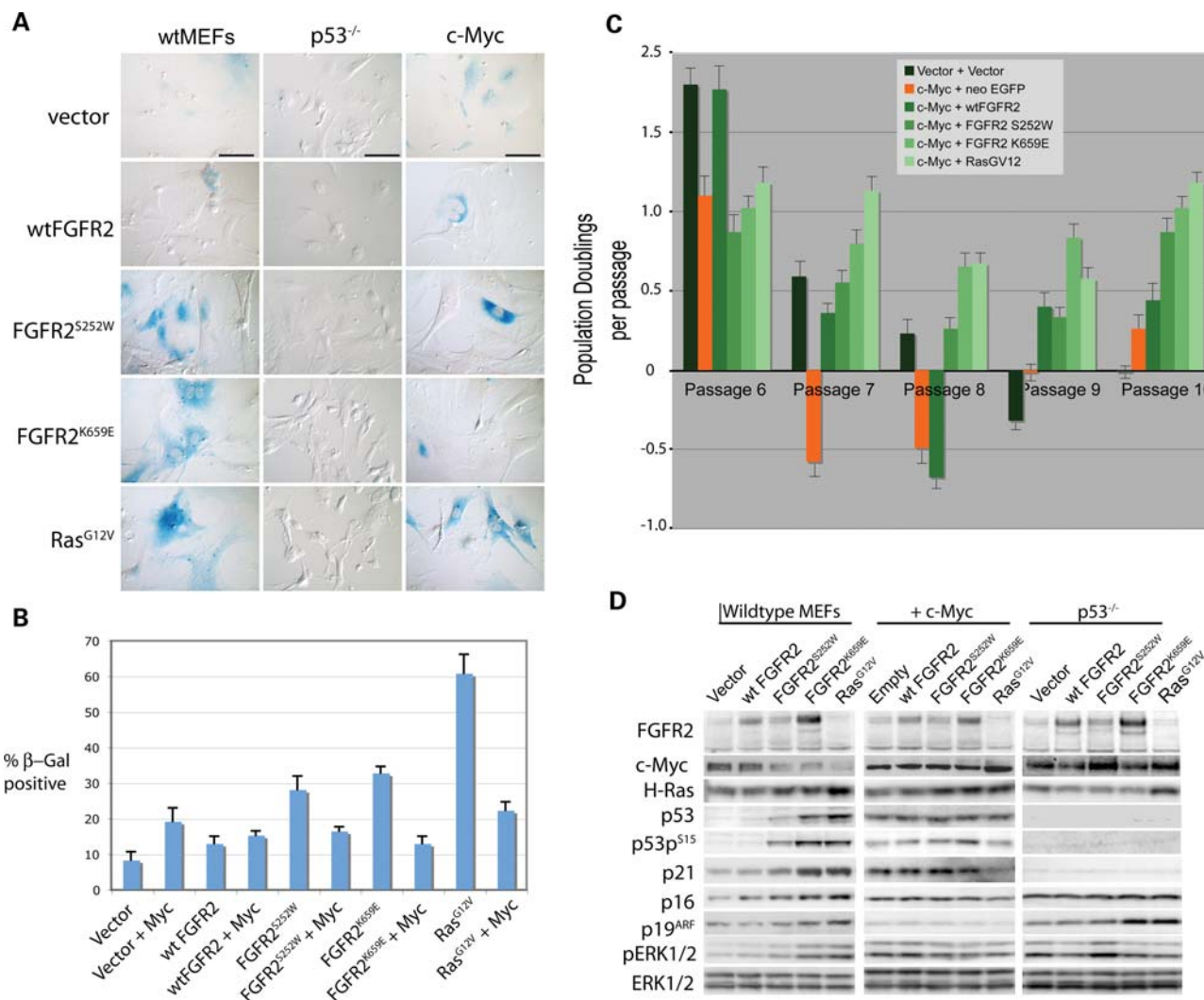
**Figure 3.** Suppression of mutant FGFR2-induced senescence by coexpression of c-Myc or loss of p53. (**A**) sa-β-Galactosidase staining of cells infected with retroviruses expressing the indicated proteins in wild-type MEFs, p53-null MEFs or MEFs expressing c-Myc. (**B**) Mean percentage and standard deviation of sa-β-galactosidase-positive cells for infected wild-type MEFs and MEFs expressing exogenous c-Myc. (**C**) The average number of population doublings calculated during successive cell passages for the various cell strains expressing c-Myc. Data are from two independent experiments performed in triplicate and standard deviations are shown. (**D**) Immunoblot analysis of DNA damage and senescence markers in primary wild-type MEFs and p53-null MEFs expressing the indicated proteins. Scale bars: 50 μм.

were stained with antiserum directed against phosphorylated (Ser25) 53BP1 (Supplementary Material, Fig. S2), a p53 interacting protein activated in response to DNA damage (35). Further, immunoblot analysis showed increased DNA damage-associated phosphorylation of Chk2, p53BP1 and p53 in IMR90 fibroblasts expressing mutant FGF receptors or Ras (Fig. 2D). In addition, levels of p53, p21 and p16 were increased in these cells (Fig. 2D, also see Fig. 3D), a result consistent with activation of the DNA-damage checkpoint and suppressed proliferation and/or senescence. Finally, levels of trimethylated (K9) Histone H3, a marker of silenced (hetero) chromatin, were increased by the expression of mutant FGF receptors and Ras.

The constitutively active FGFR2$^{K659E}$ mutant was generally more effective at inducing DNA-damage foci and senescence-associated markers than FGFR2$^{S252W}$, but not as effective as oncogenic Ras$^{G12V}$ (Fig. 2A–D), a result that

generally correlated with their abilities to promote senescence (Fig. 1).

## Senescence induced by mutant FGFRs can be bypassed by increased Myc or loss of p53

The well-documented role of p53 in senescence (34) and the apparent activation of p53 and p53 targets associated with senescence by FGFR2 mutants in IMR90 fibroblasts (Fig. 2F) and in MEFs (Fig. 3D), suggested that p53 might be playing a critical role in the senescence observed. To examine the role of p53, wild-type FGFR2, FGFR2$^{S252W}$, FGFR2$^{K659E}$ and Ras$^{G12V}$ were expressed in p53-null MEFs at passage 5 and cells stained for sa-β-galactosidase activity at passage 7. As previously reported (28), Ras$^{G12V}$-induced senescence was dependent on p53. Likewise, p53-null MEFs expressing either FGFR2$^{S252W}$ or FGFR2$^{K659E}$ failed to

undergo proliferation arrest (data not shown) and showed no sa-β-galactosidase activity (Fig. 3A). Consistence with the abrogated senescence response in p53-null MEFs, p21 and p16 levels were not increased by mutant FGFRs or oncogenic Ras (Fig. 3D). p19$^{ARF}$ levels were elevated in p53-null MEFs as expected (37) and were further increased through a p53-independent mechanism by the expression of mutant FGF receptors or Ras (Fig. 3D).

Another potentially important factor involved in senescence is c-Myc. c-Myc is required for sustained proliferation of primary MEFs (38), as well as for susceptibility of fibroblasts to transformation by Ras (39), and is downregulated during senescence (40). Indeed, we found c-Myc protein and RNA levels were downregulated in primary MEFs expressing FGFR mutants or Ras$^{G12V}$ (Fig. 3D and data not shown), a result consistent with the increased sa-β-galactosidase activity and decreased proliferation observed with these cells. In contrast, p53-null MEFs failed to downregulate c-Myc levels in response to mutant FGFRs or Ras (Fig. 3D).

To examine the role of c-Myc downregulation in mediating growth arrest and senescence caused by mutant FGFR, primary MEFs at passage 2 were first generated to express ectopic c-Myc, and subsequently infected with control virus or viruses expressing wild-type FGFR2, FGFR2 mutants or Ras$^{G12V}$ at passage 4. Drug-selected populations were replated, grown for 5 days and then stained for sa-β-galactosidase activity. Ectopic c-Myc expression (Fig. 3D) was found to be effective at reducing the otherwise elevated number of sa-β-galactosidase-positive cells caused by the expression of mutant FGFRs or Ras alone (Fig. 3A and B). Additionally, the sa-β-galactosidase-positive cells found in these populations generally did not exhibit the highly enlarged and often multinucleated morphology of senescent cells (Fig. 3A). Finally, when compared with vector control cells or cells expressing c-Myc alone, cells co-expressing c-Myc with either the mutant FGFRs or Ras showed elevated proliferation during a period when MEFs typically undergo senescence. However, the proliferated rate of these cells was reduced compared with pre-senescent wild-type MEFs (Fig. 3C), suggesting that ectopic Myc only partially overcomes senescence-associated proliferative arrest.

Similar to a previous report (41), forced expression of c-Myc alone in primary MEFs had an initial toxic effect, as reflected in a reduction in cell number compared with vector control cells (Fig. 3C), The continued proliferation of cells expressing c-Myc plus FGFR2 mutants or Ras$^{G12V}$ indicates that these proteins at least partially negate Myc-dependent toxicity. c-Myc expression in turn appears to facilitate senescence rescue of MEFs expressing FGFR2 mutants or Ras$^{G12V}$ by overriding the effects of p53, p21 and p16, which remain upregulated in these cells (Fig. 3D). However, continued p53 expression in MEFs expressing ectopic c-Myc appears to play a role in the Myc-dependent toxicity, since little or no toxicity was observed when c-Myc was overexpressed in p53-null MEFs (data not shown, also see 41).

Taken together, these results suggest that while c-Myc expression facilitates escape from growth arrest/senescence caused by the expression of mutant FGFRs or Ras$^{G12V}$, FGFR2 mutants or Ras$^{G12V}$ partially rescues p53-dependent toxicity caused by forced c-Myc expression.

## c-Myc and constitutively active FGFR2 cooperate in oncogenic transformation

The ability of c-Myc to promote escape from mutant FGFR2-induced senescence raised the possibility that c-Myc and mutant FGFRs may cooperate in oncogenic transformation. To test this, we first performed focus formation assays. MEFs at passage 5 previously infected with c-Myc retrovirus were infected with virus expressing vector alone, wild-type FGFR2, FGFR2$^{K659E}$ or FGFR2$^{S252W}$. Infected cells were plated at equal density and stained with methylene blue two weeks later. In contrast to vector control and wild-type FGFR2, MEFs infected with FGFR2$^{K659E}$ or FGFR2$^{S252W}$ both gave rise to dense foci of morphologically transformed cells. However, FGFR2$^{K659E}$ was consistently more effective than FGFR2$^{S252W}$ in cooperating with c-Myc in these assays (Fig. 4A and B). Whereas cells transformed with FGFR2$^{K659E}$ plus c-Myc formed colonies in soft agar assays (Fig. 4C) and formed tumors when injected into athymic mice (4/4), cells expressing FGFR2$^{S252W}$ plus c-Myc neither formed colonies in soft agar (Fig. 4C) or formed tumors in athymic mice (0/4). In addition, when introduced into primary p53-null MEFs, FGFR2$^{K659E}$ (and Ras$^{G12V}$) was more effective than FGFR2$^{S252W}$ or wild-type FGFR2 in causing morphological transformation, foci formation and colony-forming ability in soft agar (not shown). Finally, consistent with results showing that c-Myc partially rescued senescence caused by the FGFR2 mutants, morphologically transformed cells derived from focus formation assays showed no indications of senescence and proliferated continuously for greater than 10 passages at a high rate (not shown).

The differential abilities of FGFR2$^{S252W}$ and FGFR2$^{K659E}$ to cause malignant transformation in cooperation with c-Myc, or in the absence of p53, likely reflect the different mechanisms responsible for their activation; whereas FGFR2$^{K659E}$ is constitutively active, FGFR2$^{S252W}$ activation remains dependent on ligand binding, and FGF ligands may be limiting in our growth conditions.

## Cooperation between constitutively active FGFR2 and Myc relies on prosurvival activities of the mutant receptor

It is well-established that forced Myc expression can sensitize cells, particularly primary cells, to apoptosis (reviewed in 42,43). Therefore the ability of FGFR2 mutants to suppress toxicity caused by ectopic c-Myc expression (Fig. 3C) and cooperate with Myc in cell transformation (Fig. 4) raised the possibility that they do this at least in part by suppressing Myc-dependent apoptosis. To test this, MEFs coexpressing c-Myc alone or c-Myc plus the FGFR2 mutants, wtFGFR2, or Ras$^{G12V}$ were subjected to serum deprivation (0.1% fetal bovine serum—FBS), conditions in which forced c-Myc expression causes apoptosis. Simultaneous with serum deprivation, cells were treated with the FGFR inhibitor PD173074 (44) or DMSO vehicle control. Apoptosis was evaluated by fluorescence-activated cell sorting (FACS) of cells stained with AnnexinV antiserum and the DNA intercalator 7-AAD to distinguish early apoptotic cells (AnnnexinV-positive, 7-AAD-negative) and late apoptotic cells
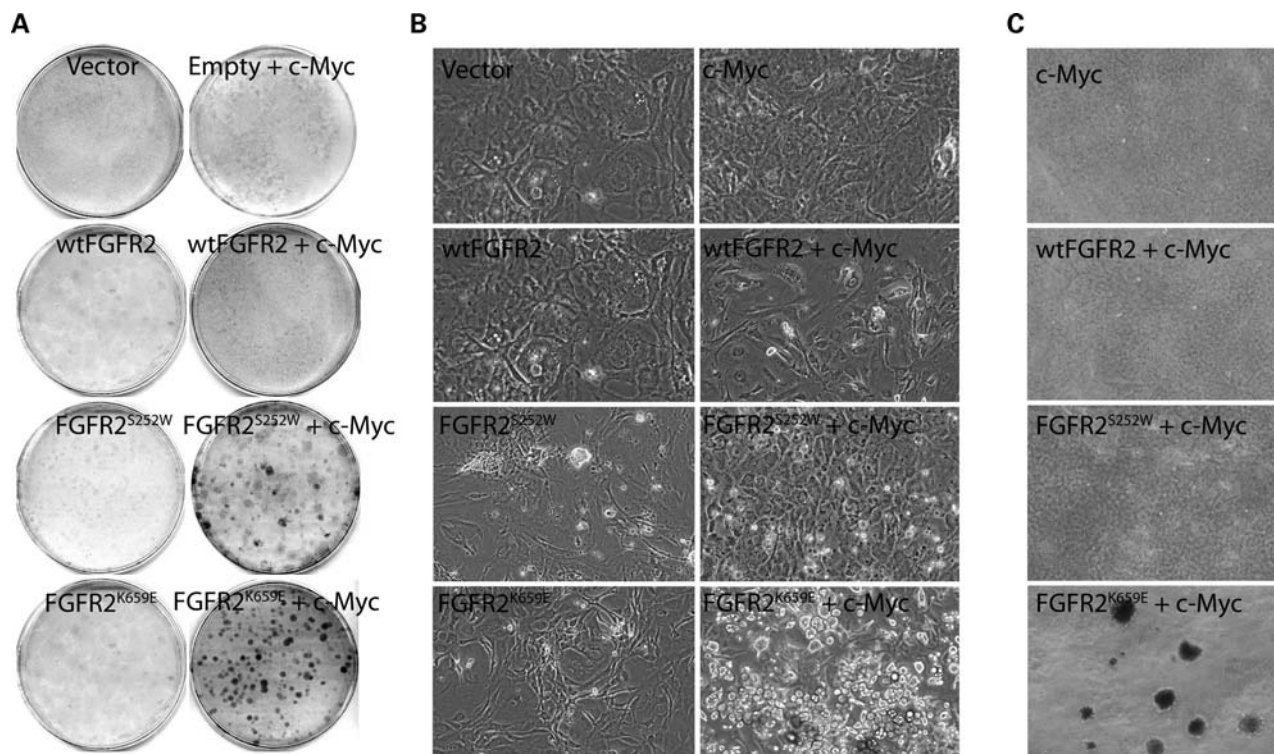
**Figure 4.** Constitutively activated FGFR2 cooperates with Myc in oncogenic transformation. (**A**) Focus formation assays, (**B**) live cell morphology and (**C**) colony formation assays in soft agar with primary MEFs infected with retroviruses expressing the indicated proteins or combinations of proteins.

(AnnexinV- positive and 7-AAD-positive). In the absence of FGFR inhibition (DMSO vehicle alone), c-Myc expression significantly increased apoptosis compared to vector-infected MEFs as expected, and apoptosis was further increased by suppression of endogenous FGFR signaling with PD173074 (Fig. 5A and C). Expression of wild-type FGFR2 or FGFR2$^{S252W}$ provided protection against Myc-dependent apoptosis that was lost upon treatment with PD173074 (Fig. 5A and C). FGFR2$^{K569E}$ also provided some protection against c-Myc-dependent apoptosis (that did not reach statistical significance), but strikingly, in the presence of PD173074, nearly all cells expressing c-Myc and FGFR2$^{K569E}$ had undergone apoptosis within 14 hours of serum deprivation (Fig. 5A–C). Expression of FGFR2$^{K569E}$ (or FGFR2$^{S252W}$) had little effect on apoptosis in the absence of ectopic c-Myc expression (Fig. 5A and data not shown), demonstrating that the high level of apoptosis that occurs in the presence of the FGFR2$^{K569E}$ plus c-Myc is dependent on both the mutant FGFR and c-Myc. Finally, Ras$^{G12V}$ provided modest, but not statistically significant, protection against Myc-dependent apoptosis, and apoptosis was not significantly increased when these cells were treated with PD173074 (Fig. 5A–C). The latter results are consistent with Ras operating downstream of FGFRs. Moreover, both the MEK inhibitor PD98059 and the PI3K inhibitor LY294002 were effective at abrogating the prosurvival activities of Ras$^{G12V}$ or FGFR2$^{K569E}$ in cells that coexpressed ectopic c-Myc (not shown).

To further investigate the effect of mutant FGFRs on c-Myc-dependent apoptosis, experiments were repeated under normal serum levels (10%) using two different concentrations of PD173074 (1.25 or 5 μM). MEFs expressing c-Myc alone showed relatively low levels of apoptosis in the presence of vehicle (Fig. 5D). wild-type and FGFR2$^{S252W}$ had a modest, but not statistically significant, protective effect against apoptosis that was lost with FGFR inhibition (Fig. 5D). The combination of FGFR2$^{K569E}$ plus c-Myc exhibited less apoptosis than c-Myc alone, but treatment with PD173074 induced a high level of apoptosis (Fig. 5D). FGFR inhibition in MEFs expressing Ras$^{G12V}$ plus c-Myc again had little or no effect on levels of apoptosis (Fig. 5D).

Taken together, these results indicate that mutant hyperactive FGFR2 signaling generally has a protective effect against Myc-dependent apoptosis and that MEFs oncogenically transformed by the combination of FGFR2$^{K569E}$ plus c-Myc appear to become highly addicted to the prosurvival activities of the mutant receptor and, as a result, highly susceptible to apoptosis when subjected to FGFR inhibition.

Consistent with the powerful pro-apoptotic effect that PD173074 had on cells transformed by FGFR2$^{K659E}$ plus c-Myc, FGFR signaling was strongly and rapidly suppressed by PD173074 treatment (2 h) in these cells, as indicated by the activation state of ERK1/2 and, to a lesser extent, Akt (Fig. 6). In contrast, PD173074 had only a weak inhibitory effect on ERK and Akt activation in cells expressing FGFR2$^{K659E}$ alone, consistent with the notion that when expressed together with Myc, cells develop an increased dependence on the mutant receptor for ERK activation and survival. PD173074 also had only a weak inhibitory effect on ERK and Akt activation in cells expressing wtFGFR2 or
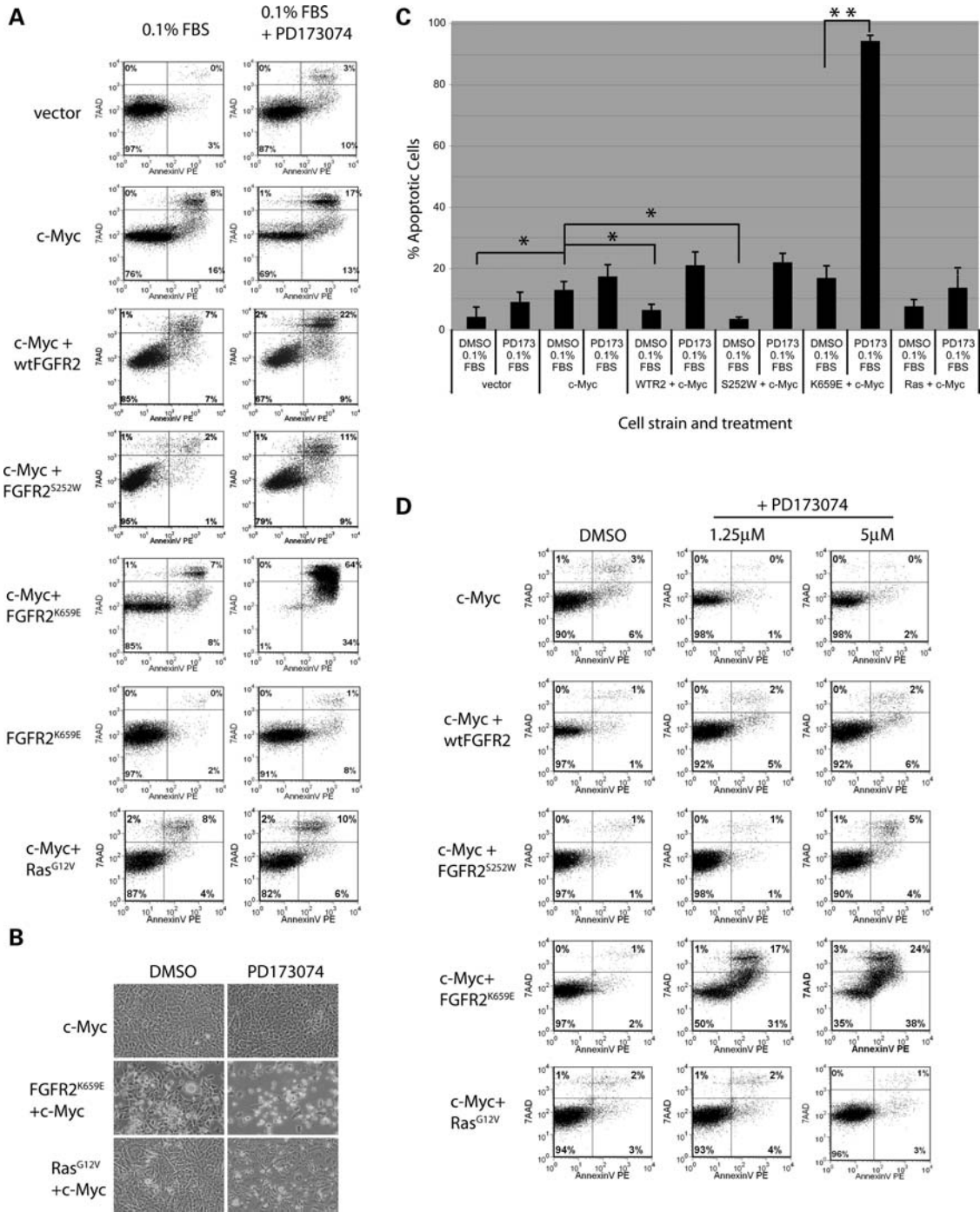
**Figure 5.** Prosurvival activity of FGF receptor mutants. (**A**) MEFs expressing the indicated proteins were subjected to serum deprivation (0.1% FBS) plus DMSO vehicle or FGFR inhibitor PD173074 for 14 h. Cells were stained with AnnexinV and 7AAD and the percentage of AnnexinV-positive apoptotic cells (right-side quadrants) and 7AAD stained cells (lysed/dead cells—upper quadrants) determined by FAC sorting. Data shown are representative of at least two independent experiments performed in triplicate. (**B**) Live cell images showing the response of the indicated cell lines to serum deprivation with and without PD173074. (**C**) Bar graph showing mean percentage of AnnexinV-positive cells present following serum-deprivation and either DMSO vehicle or PD173074. *$P < 0.005$ and **$P < 0.0005$ for the specific comparisons indicated. Note that the percentage of apoptotic cells observed with the FGFR2$^{K659E}$ plus c-Myc strain treated with PD173074 was significantly greater than in any of the other cell strains tested in this experimental set. (**D**) MEFs expressing the indicated proteins grown in 10% FBS were subjected to DMSO vehicle or FGFR inhibitor PD173074 treatment at either 1.25 or 5 μM and analyzed for apoptosis as described above. The number of apoptotic cells in the expressing FGFR2$^{K659E}$ plus c-Myc strain was significantly greater ($P < 0.0005$) than any of the other experimental settings shown in (D).
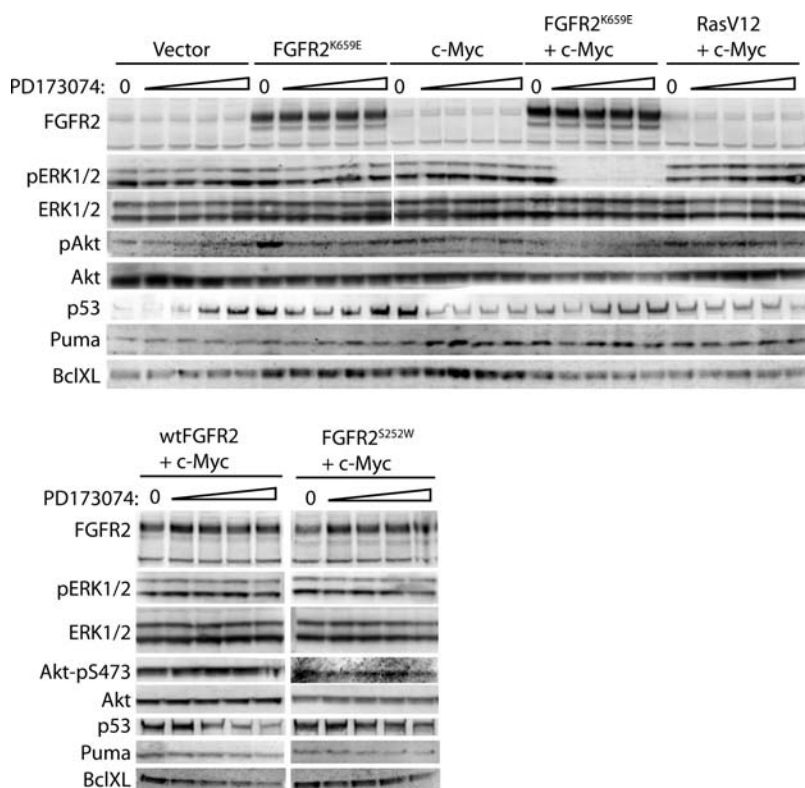
**Figure 6.** Immunoblot analysis of FGFR signaling in cells treated with PD173074. MEFs expressing the indicated proteins were treated with DMSO vehicle (O) or a titration of PD173074 (1.25, 2.5, 5 and 10 μM) for 2 h. Cell lysates were analyzed for expression of the indicated proteins. pAKT–pSer473.

FGFR2$^{S252W}$ plus c-Myc (Fig. 6), a result consistent with the low levels of apoptosis induced in these cells. As expected, phosphorylation of Erk and Akt was not altered by PD173074 in MEFs expressing Ras$^{G12V}$ plus c-Myc (Fig. 6).

In addition to inactivation of ERK, the high level of apoptosis caused by PD173074 treatment of cells expressing FGFR2$^{K659E}$ plus c-Myc was associated with modestly increased levels of Puma, a pro-apoptotic protein encoded by a p53 target gene and lower levels of anti-apoptotic Bcl$_{XL}$ protein than in cells expressing either FGFR2$^{K659E}$ or c-Myc alone (Fig. 6). Although p53 levels appeared to not directly correspond to levels of apoptosis, the unique combination of elevated Puma, low Bcl$_{XL}$ and loss of ERK in cells expressing FGFR2$^{K659E}$ plus c-Myc may contribute to the high level of apoptosis observed with these cells.

### Apoptosis of MEFs transformed by FGFR2$^{K659E}$ plus c-Myc triggered by PD173074 is p53-dependent

We next directly tested whether the robust apoptosis caused by PD173074 treatment of cells expressing FGFR2$^{K659E}$ plus c-Myc was p53-dependent. p53-null MEFs were infected with c-Myc retrovirus, drug selected, then infected with FGFR2$^{K659E}$ retrovirus and drug selected. Similar to wild-type MEFs expressing FGFR2$^{K659E}$ plus c-Myc, these cells were capable of forming foci of morphologically transformed cells and growth in soft agar (data not shown). However, in contrast to wild-type MEFs transformed by FGFR2$^{K659E}$ plus c-Myc, p53-null MEFs transformed by FGFR2$^{K659E}$

plus c-Myc showed little or no apoptosis following PD173074 treatment, either in medium containing 10% FBS or under serum-deprived conditions (Fig. 7A and B). The loss of sensitivity to PD173074 in these cells occurred despite the presence of elevated levels of c-Myc and FGFR2$^{K659E}$ (Fig. 7C).

## DISCUSSION

Our results further define the oncogenic activities of mutant activated FGFRs by showing that they promote DNA-damage-associated senescence and that events such as loss of p53 or Myc deregulation suppress their senescence-promoting activity and promote oncogenic conversion. Previous studies using immortal and cancer-derived cell lines indicated that the oncogenic activity of cancer-associated FGFR mutants is associated with both their ability to promote cell proliferation and to exert prosurvival activity (6,7, data not shown). The intrinsic ability of mutant FGFRs to stimulate cell proliferation, together with our finding that they promote DNA-damage signaling, is consistent with models of oncogene-induced 'hyperproliferative stress', in which excessive proliferation signaling is thought to compromise the integrity of DNA replication and lead to the development of DNA strand breaks (45). However, although generally consistent with this model, it remains to be shown that hyperproliferation signaling mediated by mutant FGFRs leads to DNA strand breaks. In addition to DNA-damage signaling, senescence has been linked to several other mechan-
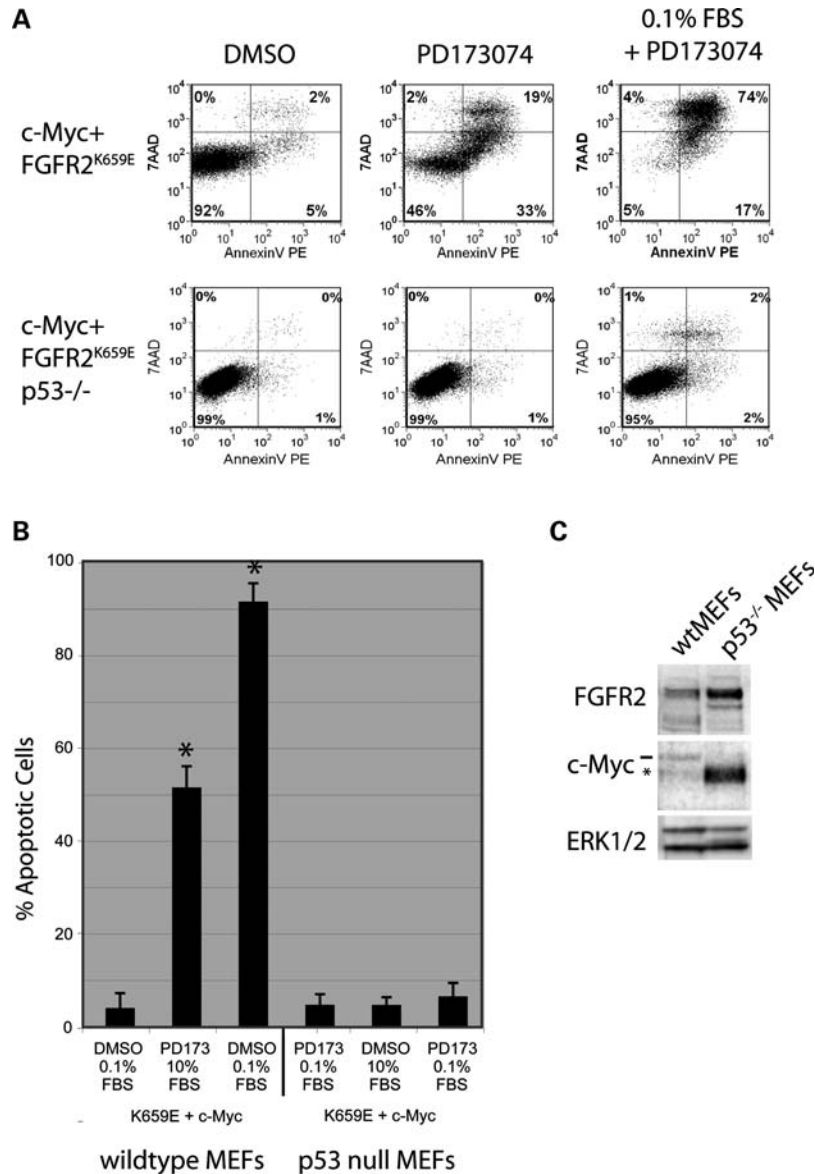
**Figure 7.** Apoptosis caused by FGFR inhibition in MEFs expressing exogenous c-Myc and FGFR2$^{K569E}$ is p53 dependent. (**A**) Primary MEFs and p53-null MEFs, both transformed by co-expression of c-Myc and FGFR2$^{K569E}$, were treated with DMSO vehicle or 1.25 μM PD173074 in medium containing 10% FBS or in medium containing 0.1% FBS for 14 h. The percentage of AnnexinV and 7AAD positive cells was determined by FACS analysis. Assays were performed in triplicate and mean percentage of total apoptotic cells with standard deviation are shown in (**B**). *$P < 0.0005$ when compared with comparably treated p53-null MEFs. (**C**) Western blot showing expression of FGFR2$^{K569E}$ and c-Myc in the indicated cell lines. The dash indicates endogenous c-Myc and the asterisk indicates the slightly smaller human c-Myc protein that was introduced.

isms (which may not be mutually exclusive), including the induction of ER stress signaling (46), Sprouty upregulation and negative feedback signaling in the MAPK pathway (47), senescence-associated heterochromatin formation (SAHF), production of reactive oxygen species (ROS) and telomere erosion (reviewed in 36). Although we cannot rule out that ROS and telomere erosion may be involved in DNA-damage signaling and senescence observed in this study, we did not consistently observe induction of Sprouty or ER stress markers in MEFs or IMR90 fibroblasts expressing mutant FGFR2 (data not shown). However, we did observe increased levels of trimethylated (K9) Histone H3, a potential indication

that SAHF cooperates with, or is a byproduct of activation of DNA-damage signaling by these oncogenic proteins.

Another mechanism that might contribute to the induction of senescence is c-Myc downregulation. Senescence induced by the expression of FGFR2 mutants, as well as Ras$^{G12V}$, led to c-Myc downregulation, and coexpression of Myc with these proteins facilitated senescence escape. The decreased levels of c-Myc in primary cells may be an indirect consequence of cells exiting the cell cycle and becoming senescent. Alternatively, DNA-damage signaling or other signaling events activated by oncogenic Ras or FGFR2 mutants in primary cells may serve to more directly downregulate

c-Myc. Since c-Myc is essential for sustained proliferation of primary fibroblasts (38,48), its downregulation in this setting likely directly contributes to cell cycle exit and senescence. Our results therefore raise the possibility that the diminished or abrogated senescence response typical of immortal and cancer cells may in part reflect the acquisition of oncogenic events that prevent proper downregulation of Myc. This idea is consistent with results showing that conditional inactivation of Myc in tumors triggers senescence (49,50). Moreover, the finding that c-Myc levels are elevated in early passage MEFs lacking p53 (Fig. 3D), raises the possibility that c-Myc upregulation in this setting constitutes a critical and perhaps general mechanism underlying senescence escape and oncogenic progression associated with loss of p53.

Although suppression of mutant FGFR2-induced senescence by exogenous Myc expression appears to be an important mechanism contributing to their oncogenic cooperation, suppression of Myc-driven apoptosis by hyperactive FGFR signaling may be equally important. A number of different oncogenic proteins that are strongly mitogenic sensitize cells to apoptosis, with Myc being the prototypical member of this group (42,43). Both the mitogenic and pro-apoptotic activity of Myc is enhanced by constitutive and/or high-level expression of c-Myc protein, a common feature of tumor cells. Accordingly, the mitogenic and oncogenic activities of deregulated Myc expression are linked to events that function to suppress Myc's pro-apoptotic activity, such as loss of p53. Our results suggest that the constitutively active FGFR2$^{K569E}$ mutant cooperates with forced Myc expression in oncogenic transformation in part by suppressing Myc-dependent apoptosis. Further, the striking sensitivity of these cells to apoptosis induced by FGFR inhibition, which was far greater than that caused by c-Myc expression alone, suggests that they develop an addiction to the prosurvival activities of the mutant receptor. Interestingly, the sensitivity of cells expressing FGFR2$^{K659E}$ plus c-Myc to apoptosis was linked to robust inactivation of ERK1/2, which did not occur in cells expressing FGFR2$^{K569E}$ alone. One possible explanation for this result is that constitutively active FGF receptor signaling may be prone to co-op, replace or otherwise downregulate the activities of overlapping signaling pathways that contribute to ERK activation and normally provide pro-survival signaling. Such a tendency would be predicted to make cells more dependent on the mutant receptor for ERK activation and associated prosurvival signaling, but would be driven and manifest only in the presence of strong pro-apoptotic activity, such as that caused by constitutively high Myc expression. Although the underlying mechanisms responsible for the development of oncogene addiction or co-dependency in general, and in cells expressing Myc and FGFR2$^{K569E}$ in particular, remain unclear, these cells may offer a useful model for dissecting this phenomenon.

The combination of FGFR2$^{K569E}$ and c-Myc created highly oncogenic cells susceptible to apoptotic elimination by FGFR inhibition, whereas cells expressing FGFR2$^{S252W}$ plus c-Myc were neither tumorigenic nor vulnerable to apoptosis. This difference in susceptibility is likely due to FGFR2$^{K569E}$ being constitutively activated and FGFR2$^{S252W}$ being largely dependent on ligand binding. Interestingly, endometrial tumor cells containing an FGFR2$^{S252W}$ mutation and gastric tumors with FGFR2 amplification were highly susceptible to apoptosis when treated with PD173074 (6,7,51). Although it is not clear why these tumor cells are more sensitive to FGFR inhibition than MEFs expressing FGFR2$^{S252W}$ plus c-Myc, it may be related to cell type-specific differences in endogenous levels of FGF ligand(s) that the mutant receptor requires for full activation. Because aberrant activation of FGFR2$^{S252W}$ is related primarily to its broadened ligand-binding specificity and increased ligand-binding affinity, its pathological activities must necessarily be linked to local or cell-intrinsic ligand levels. Therefore, the ligand-dependent nature of Apert syndrome mutations, like FGFR2$^{S252W}$ and FGFR2$^{S253W}$, together with a requirement for additional cooperating events like Myc deregulation or loss of p53 that suppress senescence, suggests that their oncogenic potential is less likely to be realized than constitutively activated mutant receptors. Moreover, the multiple events needed to elicit oncogenic activity from FGFR2$^{S252W}$ may explain why Apert syndrome is not associated with a strong predisposition to cancer.

Although it is clear that FGFR inhibition can induce apoptosis in cells derived from some tumors containing FGFR2 (and FGFR3) mutations or gene amplification (6,7,51), the underlying mechanism remains unclear. Our results suggest that their vulnerability is likely dependent on an additional event(s) that has latent pro-apoptotic activity, such as Myc deregulation. Interestingly, not all gastric tumor-derived cell lines containing amplified FGFR2 respond to FGFR inhibition by undergoing apoptosis (50). Instead, some respond by exiting from the cell cycle, a result consistent with the tumor cells becoming dependent on the proliferation-associated activities of the mutant or amplified receptors. Thus, it might be informative to screen gastric and endometrial tumor cells that have FGFR2 mutations or gene amplification for alterations at Myc loci (e.g. gene amplification), and then determine whether their sensitivity to apoptosis (versus blocked proliferation) by FGFR inhibition is related to, or dependent on, Myc deregulation. Defining these relationships may more precisely identify tumors particularly susceptible to apoptotic elimination by small molecule FGFR inhibitors.

## MATERIALS AND METHODS

### Expression plasmids

Retroviral expression plasmids used included pFBneo-wtFGFR2c (Bek), pFBneo-FGFR2c$^{K659E}$, pFBneo-Ras$^{G12V}$, pFBneo-GFP, pLXSN-FGFR2c$^{S252W}$, pBABEpuro-c-Myc, pBABEpuro-H-Ras$^{G12V}$. Ecotropic retroviruses were produced in Ecopack 2 293 cells (Clonetech), and amphotropic retroviruses were generated in Phoenix cells following the manufacturers' protocols.

### Cells, cell culture and retroviral infection

Primary MEF cultures were derived from E13.5 mouse embryos and cultured in DMEM containing 10% fetal calf serum as described previously (39). IMR-90 (American Type Culture Collection) and Ecopack 2 293 cells (Clontech) were cultured in DMEM (Invitrogen) supplemented with

10% FBS (Hyclone). Stable retroviral infections of IMR-90 (ATCC), C3H10T1/2 (ATCC) and MEFs were performed as previously described (38). For sequential infections, cells were infected with the second virus 2–3 days following the initial infection. Selection with puromycin (1.5 µg/ml) or geneticin (1 mg/ml) was done after all rounds of infection.

### Senescence, transformation, tumor formation and apoptosis assays

Staining for sa-β-galactosidase activity was performed as described Lin *et al.* (29). Soft agar and focus formation assays were performed as previously described (39). For tumor formation assays, 8-week-old male nude mice (*Foxn1$^{nu}$* Jackson Labs) were injected subcutaneously into their shoulder flank with $2 \times 10^6$ cells. Tumors were not allowed to get $> 2$ cm in diameter as required by OHSU IACUC guidelines.

For apoptosis assays, subconfluent cells were plated at 200 000 cells per 35 mm dish into growth media containing (10% FBS) for 24 h. FGFR inhibitor PD173074 (Calbiochem) was added at concentrations ranging from 1.25 to 10 µM (in DMSO vehicle) in either 10% FBS medium or in medium replaced to contain 0.1% FBS. PI3K inhibitor LY294002 (Cell Signaling Technology) and MEK inhibitor PD98059 (Calbiochem) were used at 50 µM. Cells were harvested 14 h following addition of inhibitors and/or serum deprivation, stained with annexin-5PE (BD) antibody and 7AAD (BD) and flow-sorted using FACS Caliber and *De Novo* FCS Express V3 software.

### Western blot and immunofluorescence

For western blots, cells were lysed with RIPA buffer (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1.0% NP-40, 0.5% sodium deoxycholate) containing COMPLETE Protease Inhibitor cocktail (Roche). Equal amounts of protein were run on 4–12% Novex Bis-Tris gels in MOPs buffer. Western blotting was done as previously described (39) using the following antibodies: c-Myc (N-262), BEK (C-17), p16 (M-156), p21 (sc-1207) ERK1 (K-23) and pERK (E-4) from Santa Cruz, p53BP1 (S25) from Bethyl Labs, p53, p53-pS15, pChk2 (Thr68), Akt and pAkt from Cell Signaling Technologies and p19$^{ARF}$, Chk2, Histone H3, phospho-Histone H3 (ser10) and 53BP1 from Abcam and Trimethyl Histone H3 K9 from Millipore. For immunoflourescence, cells were fixed on coverslips and antibody staining performed as previously described (52). Antibodies used for immunofluorescence included γHistone H2AX (pSer139) (Cell Signaling Technologies) pATM (Ser1981) (Upstate) and p53BP1 (S25) (Bethyl Labs). Cy3-conjugated Donkey Anti-Mouse (Jackson Immuno Research) secondary antibody was used. DAPI staining was used to visualize nuclei.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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