Oncogene cooperation in tumor maintenance and tumor recurrence in mouse mammary tumors induced by *Myc* and mutant *Kras*

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Most, if not all, cancers are composed of cells in which more than one gene has a cancer-promoting mutation. Although recent evidence has shown the benefits of therapies targeting a single mutant protein, little attention has been given to situations in which experimental tumors are induced by multiple cooperating oncogenes. Using combinations of doxycycline-inducible and constitutive *Myc* and mutant *Kras* transgenes expressed in mouse mammary glands, we show that tumors induced by the cooperative actions of two oncogenes remain dependent on the activity of a single oncogene. Deinduction of either oncogene individually, or both oncogenes simultaneously, led to partial or complete tumor regression. Prolonged remission followed deinduction of *Kras^G12D* in the context of continued *Myc* expression, deinduction of a *MYC* transgene with continued expression of mutant *Kras* produced modest effects on life extension, whereas simultaneous deinduction of both *MYC* and *Kras^G12D* transgenes further improved survival. Disease relapse after deinduction of both oncogenes was associated with reactivation of both oncogenic transgenes in all recurrent tumors, often in conjunction with secondary somatic mutations in the tetracycline transactivator transgene, *MMTV-rtTA*, rendering gene expression doxycycline-independent. These results demonstrate that tumor viability is maintained by each gene in a combination of oncogenes and that targeted approaches will also benefit from combination therapies.

inducible | mammary gland | ras

Recent successes in the treatment of several human cancers using agents directed against the products of single oncogenes (1) and the prospect of many more such agents becoming available (2) raise important questions about long-term outcomes of interfering with certain mutations in cancer cells. Should targeted therapies be directed against all of the genetic damage in a cancer cell? Or do cancer cells display a differential dependence on mutations, and can that differential be used to select the most potent targeted therapies?

Experimental murine models of human cancer that depend on regulated oncogenic transgenes mimic hypothetical therapeutic situations in human cancers, for which highly effective targeted therapies might be used to block the action of one or more oncogenes. These models are indispensable for testing the effects of blocking two common human oncogenes, *Myc* and *Kras*, for which no effective therapies currently exist. By using this approach, tumor regression in response to oncogene withdrawal has been observed across multiple tumor types induced by either *Myc* or mutant *Kras* transgenes—lymphomas, leukemias, insulinomas, lung, bone, liver, and breast tumors (3, 4). However, inactivation of the same oncogene in different tumor types produced a range of long-term outcomes, from complete cure to invariable relapse.

To reduce the effects of genetic variability on tumor regression and long-term remission after selective oncogene inactivation, we took advantage of the cooperative behavior of *Myc* and mutant *Kras* oncogenes in tumorigenesis. Using constitutive and doxycycline-inducible alleles of these oncogenes expressed in the mammary gland (MG) of transgenic mice (5–9), we first confirmed the synergistic effects of *Myc* and mutant *Kras* oncogenes on mammary tumorigenesis, leading to rapid autochtonous tumor formation, whether expressed from inducible or constitutive promoters. We then compared the effects of deinducing either one or both oncogenes on cell growth, cell death, tumor regression, and tumor relapse. We observed a hierarchy of responses to deinduction of one or both oncogenes, revealing the existence of oncogene cooperation in tumor maintenance and tumor recurrence, not only in tumor initiation.

Results

Synergistic Pairs of Mammary Oncogenes. Our goals in this work were to determine whether primary cancer cell growth and viability depend on one or both members of pairs of oncogenes that cooperate during mammary tumorigenesis and whether long-term outcomes may be determined by selective inactivation of either one or both oncogenes. Because we planned to do this by using a combination of doxycycline-inducible and constitutive oncogenic transgenes, it was first important to show that the relevant combinations of inducible and constitutive oncogenic transgenes worked synergistically to cause mammary cancers.

Constitutively expressed transgenes encoding Myc and mutant Hras proteins were shown two decades ago to cooperate during mammary tumorigenesis (10). Our choice of *Myc* and *Ras* transgenes for these studies was influenced in part by these early observations, in part by subsequent work implicating spontaneous mutations of *Kras* genes in the maintenance of tumors induced by regulated Myc (11–13) and in part by the availability of doxycycline-inducible transgenes encoding Myc and mutant Kras (7, 8).

Because the inducible transgene encoding mutant Kras has not been tested for its ability to cause mammary tumors (MT), we first generated bitransgenic mice carrying *TetO-Kras^G12D* (*TOR*) and *MMTV-rtTA* (*MTB*) (5, 11); the latter transgene expresses the reverse tetracycline transactivator (rtTA) in mammary epithelial cells (5). When fed doxycycline after weaning, these mice developed mammary gland hyperplasia as early as 1 week after induction, progressing to hyperplastic alveolar nodules and then palpable oligofocal mammary tumors with a median latency of 22 weeks. No tumors occurred in bitransgenic animals not given the antibiotic ($n = 15$, data not shown) or in *TOR* mice lacking the regulator transgene but fed doxycycline
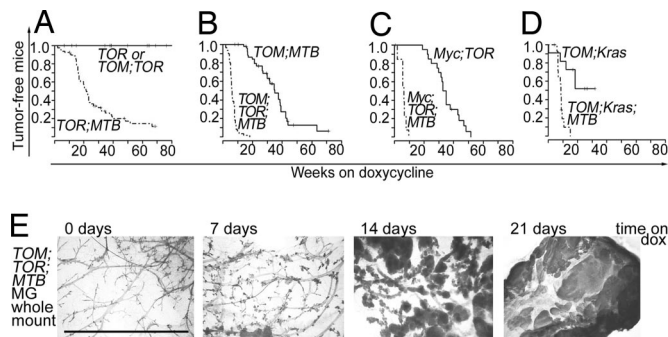
Fig. 1. Oncogenic Kras synergizes with Myc in mammary tumorigenesis. Tumor incidence in female mice from experimental and control lines from the beginning of doxycycline exposure. (*A*) *TOR; MTB* mice (dashed-dot line; *n* = 80) compared with *TOR* (*n* = 7) and *TOM;TOR* (*n* = 11) mice (solid line). (*B*) *TOM;TOR;MTB* mice (dashed-dot line; *n* = 60) compared with *TOM;MTB* mice (solid line; *n* = 61). (*C*) *Myc;TOR;MTB* mice (dashed-dot line; *n* = 14) compared with *Myc;TOR* mice (solid line; *n* = 20). (*D*) *TOM;Kras;MTB* mice (dashed-dot line; *n* = 15) compared with *TOM;Kras* mice (solid line; *n* = 11). (*E*) Diffuse tumor development in mature mouse mammary glands induced with doxycycline to coexpress *MYC* and mutant *Kras* transgenes. Whole-mount mammary gland preparations displayed are from tritransgenic *TOM;TOR;MTB* mice exposed to doxycycline for indicated periods of time. (Scale bar, 5 mm.)

(*n* = 7, see also Fig. 1*A*). We also confirmed previous studies (11–13) showing that bitransgenic animals with an inducible (human) *MYC* transgene (*TOM;MTB*) developed solitary mammary tumors; in our cohort the median latency was 26 weeks (Fig. 1*B*).

We then determined the potential of the doxycycline-inducible *Kras* and *MYC* oncogenes to collaborate with each other and with constitutively expressed transgenes, *MMTV-Myc* (*Myc*) and *MMTV-Kras^{G12V}* (*Kras*), in tritransgenic virgin females also expressing *MTB*. As shown in Fig. 1 *B–D*, expression of two transgenic oncogenes always induced mammary tumors much more efficiently than did a single transgene, regardless of whether either or both of the oncogenes was doxycycline-inducible. Each of the three combinations produced visible tumors in all mammary glands within 10 weeks of induction, with a median latency between 6 and 8 weeks, much shorter than that observed with any of the single inducible or constitutive oncogenic transgenes. Tumors in animals carrying all three combinations of inducible or constitutive *Myc* and *Ras* transgenes were phenotypically similar, with keratin 6-, keratin 8-, and smooth muscle actin-positive cell subsets, characteristic of tumors induced by *Wnt1*, β-*catenin*, and *Myc* oncogenes in our earlier study (14), indicating similar differentiation status of the tumors [supporting information (SI) Fig. S1].

The rapid pace of appearance and multifocal character of tumors caused by these combinations of oncogenes suggested that the tumors arose in a polyclonal fashion, implying that dysregulated expression of *Myc* and mutant *Kras* is sufficient for mammary transformation. This was further supported by examination of whole mounts of mammary fat pads from *TOM;TOR;MTB* mice (Fig. 1*E*). Diffuse mammary hyperplasia was apparent at 7 days after addition of doxycycline, numerous tumor nodules were evident throughout the glandular tree at 14 days, and confluent tumors were present at 21 days. Of the mice with cooperating oncogenes, only the mice with a constitutive *Kras^{G12V}* gene and inducible *MYC* showed distinct, multifocal growths. Sinn *et al.* (10) reported a similar phenotype in mice with two constitutive oncogenes, *Myc* and *MMTV-Hras*. It is possible that the result reflects mosaic expression of the *Kras* transgene or senescence of some mammary epithelial cells in response to early expression of a *Ras* oncogene, as described in mammary and other cell types (15–17).
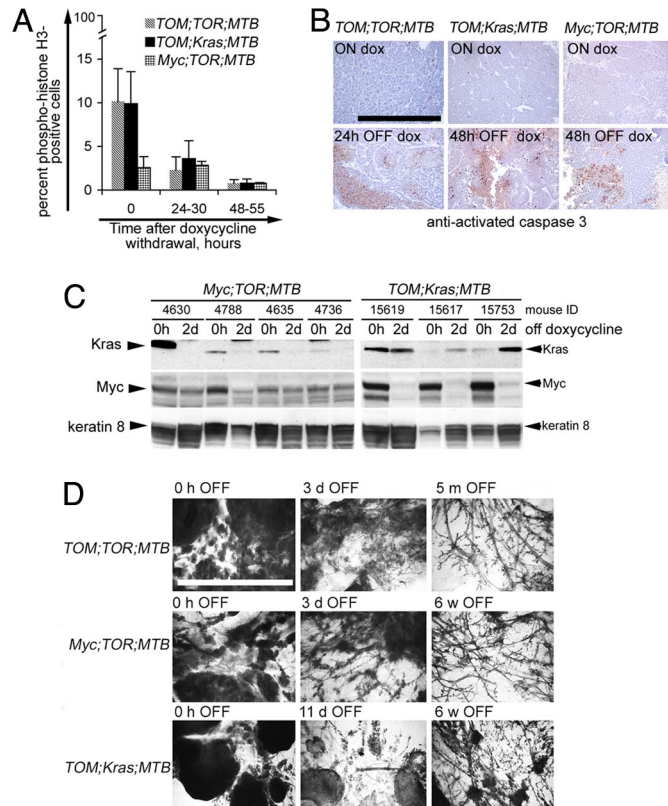
Fig. 2. Short-term responses to doxycycline withdrawal in mouse mammary tumors induced by *Myc* and mutant *Kras*. (*A*) Decrease in the number of proliferating cells in mammary tumors after doxycycline withdrawal in *TOM;TOR;MTB* mice (gray bars), *TOM;Kras;MTB* mice (black bars), and *Myc;TOR;MTB* mice (hatched bars). Proliferating cells were detected by immunostaining with anti-phosphorylated (Ser-10) histone H3 on paraffin tissue sections and counted as described in *Experimental Procedures*. (*B*) Increase in tumor cell death at 24–48 h after doxycycline withdrawal, assayed by immunodetection of anti-activated caspase-3 (Cell Signaling Technology) on paraffin tissue sections from *TOM;TOR;MTB* mice (*Left*), *TOM;Kras;MTB* mice (*Center*), and *Myc;TOR;MTB* mice (*Right*). The slides were counterstained with hematoxylin. Brown-red cells are positive for activated caspase 3. (*Upper*) Before removal of doxycycline. (*Lower*) At 24 or 48 h after removal of doxycycline. (Scale bar, 0.5 mm.) (*C*) Immunoblotting of total lysates from mammary tumors of tritransgenic *Myc;TOR;MTB* mice (*Left*) and *TOM;Kras;MTB* mice (*Right*) maintained on doxycycline for 7 weeks (0 h OFF) and contralateral tumors harvested 48–50 h after doxycycline removal (2 d OFF). The blot was probed with anti-Myc, anti-Kras, and anti-keratin-8 antibodies as described in *Experimental Procedures*. (*D*) Mammary whole mounts from tritransgenic animals taken off the doxycycline diet for the indicated periods of time. (Scale bar, 5 mm.)

Deinduction of Either MYC or Oncogenic Kras Individually, or both Oncogenes Simultaneously, Causes Apoptosis and Reduced Proliferation. We next asked whether the tumors depended on continued expression of either one or both of these oncogenes for continued growth or survival. As a first step in analyzing the response to the deinduction of oncogenes, we determined mitotic indices (by immunohistological detection of phosphorylated histone H3) and assayed tumor slices for apoptotic cells (by immunostaining for activated caspase-3) at several time points after withdrawal of doxycycline (Fig. 2). Deinduction of *TOM* and *TOR* transgenes in all three *Myc/Kras* combinations was accompanied by a drop in the proportion of mitotic cells (Fig. 2*A*), although a significantly smaller fraction of cells was initially cycling in tumors from mice with an inducible *Kras* and a constitutive *Myc* transgene than in the other two types of tritransgenic mice. Similarly, an increase in the proportion of

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apoptotic cells was observed in tumors from cohorts of all three tritransgenic mice sampled 1 or 2 days after removal of doxycycline from the diet (Fig. 2*B*). It was, however, difficult to measure apoptotic indices precisely, because discrete areas with many dying cells were often found surrounded by viable cells, perhaps reflecting the multifocal nature of these rapidly induced tumors.

To confirm our prediction that expression levels of the inducible transgenes rapidly decline after doxycycline withdrawal, irrespective of whether the cooperating oncogene is regulated, we measured levels of Myc and Kras proteins by immunoblots of extracts of mammary tumors from tritransgenic mice before and 2 days after removal of doxycycline from the diet (Fig. 2*C*). After 5–6 weeks on doxycycline, Myc and Kras proteins are detectable in mammary tumors from tritransgenic animals at variable levels (Fig. 2*C*). The variability may, in part, be due to histological heterogeneity of the tumors. After a 2-day deinduction, protein levels dropped to very low or undetectable levels when expressed from regulated alleles but not when expressed from constitutive alleles (Fig. 2*C*). These results indicate that we were able to achieve inducible transgene regulation in tumors coexpressing cooperating constitutive oncogenes and that changes observed in tumor proliferation and cell death upon doxycycline withdrawal can be attributed to loss of expression of the respective oncogenic transgenes.

Complete Tumor Regression After Deinduction of Oncogenic Kras, With or Without Simultaneous Deinduction of a Cooperating MYC Oncogene. The reduced proliferation and appearance of apoptosis upon deinduction of oncogenes in our mouse mammary tumor models suggested that both *Myc* and mutant *Kras* play a role in maintaining tumor cell growth and viability. To determine whether deinduction also leads to tumor regression and sustained remission, we observed cohorts of the three types of tritransgenic mice for prolonged periods after removal of doxycycline from the diets of tumor-bearing animals. After doxycycline withdrawal, almost all of the grossly transformed mammary glands that developed in *TOM;TOR;MTB* mice completely regressed to a nonpalpable state within 2 weeks (Fig. 2*D Top*); at later time points, mammary glands often displayed normal ductal architecture. All of the mammary tumors that developed through the combined expression of an inducible mutant *Kras* transgene and a constitutively expressed *Myc* transgene also completely regressed to a nonpalpable size upon withdrawal of doxycycline, with restoration of a nearly normal architecture in all mammary glands, indicating that expression of mutant *Kras* is essential for tumor viability even when expression of the cooperating *Myc* oncogene is maintained (Fig. 2*D Middle*). However, hyperplastic changes, characteristic of glands expressing the *Myc* transgene alone, persisted despite deinduction of mutant *Kras* and the dramatic tumor clearance (data not shown).

Deinduction of MYC in the Presence of Continuous Expression of a Kras Oncogene Results in Widespread but Incomplete Regression and Swift Recurrence of Mammary Tumors. Loss of expression of a *Myc* oncogene causes tumor regression and extended survival in several experimental tumor models (7, 18, 19), including *TOM;MTB*-induced mammary tumors (11–13). Because we showed that deinduction of the *MYC* transgene in *TOM;Kras;MTB* mice was associated with a 10-fold reduction in proliferation (Fig. 2*A*) and increased tumor cell death (Fig. 2*B*), we expected to observe tumor regression after removal of doxycycline from the diet. In fact, all of the tumors in these animals became smaller within 5–7 days of doxycycline withdrawal (Fig. 2*D Bottom*). However, none of the palpable tumors regressed completely after 3 weeks of monitoring, at which point some of them began to regrow. Within 3–6 weeks after withdrawal of doxycycline, animals harbored multiple focal tumors.

Fig. 3. History of mammary tumors is associated with tumor recurrence and reduced survival. (*A*) Time of appearance of recurrent tumors in a cohort of mature female *TOM;TOR;MTB* mice exposed to doxycycline from 4 to 20 weeks of age (median, 8 weeks) until tumor burden reached an estimated 10% of body weight and then placed on a doxycycline-free diet (dashed-dot line; $n = 36$), compared with time of appearance of primary tumors in *TOM;TOR;MTB* control female mice never exposed to doxycycline (solid line; $n = 31$). (*B*) Time of appearance of recurrent tumors in a cohort of mature female *Myc;TOR;MTB* mice exposed to doxycycline from 4 to 20 weeks of age (median, 9 weeks) until total tumor burden reached an estimated 10% of body weight and then placed on a doxycycline-free diet (dashed-dot line; $n = 14$) compared with time of appearance of primary tumors in bitransgenic *Myc;TOR* control female mice on doxycycline (solid line; $n = 22$). (*C*) Survival of animals after doxycycline removal in *TOM;Kras;MTB* mice (solid line; $n = 12$), *Myc;TOR;MTB* (dashed-dot line; $n = 12$), and *TOM;TOR;MTB* (dotted line, $n = 36$). The animals were removed from doxycycline when the initial tumor burden reached an estimated 10% of body weight. Mice were euthanized when total recurrent tumor burden reached an estimated 10% of body weight, and these time points were used to calculate survival time after doxycycline withdrawal.

The incomplete regression and relatively rapid recurrence of tumors in these mice suggested that the tumor cells were less dependent for viability on *MYC* than on mutant *Kras*.

Tumors Also Recur After Deinduction of a Mutant *Kras* Oncogene in Tritransgenic Mice. In mice with the two other tritransgenic combinations (*TOM;TOR;MTB* and *Myc;TOR;MTB*), apparently complete regression of tumors occurred when mutant *Kras* or both *Kras* and *MYC* were deinduced (Fig. 2 *D*), and mice entered remission, defined by the absence of palpable tumors. However, tumors ultimately reappeared in most animals. We found that even simultaneous deinduction of *MYC* and mutant *Kras* oncogenes did not completely protect from relapse. Solitary mammary tumors developed after withdrawal of doxycycline in 20 of 36 of the *TOM;TOR;MTB* animals followed up to 80 weeks of age, at a median age of 53 weeks (Fig. 3*A*). The histological appearance of the recurrent tumors was similar to that of primary tumors, although 3 of the 20 tumors displayed morphologic hallmarks of an epithelial-to-mesenchymal transition (data not shown). Although it is not possible to determine directly whether these tumors arose from surviving cells from the regressed tumors, it is notable that only one mammary tumor was observed in 31 *TOM;TOR;MTB* mice that never received doxycycline (Fig. 3*A*).

We made similar observations with tritransgenic mice in which tumors that developed on doxycycline regressed fully after deinduction of the *TOR* transgene, whereas the *Myc* transgene remained constitutively expressed (Fig. 3*B*). Again, animals that carried tumors and were apparently successfully treated by doxycycline withdrawal developed recurrent tumors at a much

younger age than animals that lacked the regulator transgene developed primary tumors (33 vs. 46 weeks, $P = 0.04$). Thus, a history of tumor growth and regression predisposes these animals to recurrent mammary tumorigenesis, suggesting that regrowth of residual tumor cells, rather than spontaneous primary tumors, was responsible for the tumors arising late in life.

Deinduction of both MYC and Mutant Kras Improves Survival More than Deinduction of Either Oncogene Alone. The experimental situations we have created with the three kinds of tritransgenic mice mimic hypothetical therapeutic situations in human cancer, in which highly effective targeted therapies might be used to block the action of one or both of a pair of cooperating oncogenes. Because deinduction of *TOR* was clearly superior to the deinduction of *TOM* for tumor regression, we tested whether combined deinduction would produce a more stable remission than that achieved by loss of *TOR* alone.

We compared the results of deinducing either one or both oncogenes by using a common measure of therapeutic effectiveness, extension of life. We considered the time when the estimated tumor burden becomes large enough (after 7 weeks of doxycycline exposure, on average) to require euthanasia of the animal to be approximately equivalent to the end of life. Because doxycycline was withdrawn when the total size of primary tumors was large enough to require euthanasia, extension of life was measured as the time from doxycycline withdrawal to the time when tumors recurring in the absence of inducer again became large enough to require euthanasia.

In the comparison of the three tritransgenic lines (Fig. 3*C*), it is apparent that inactivation of the *TOM* oncogene by doxycycline withdrawal conferred only a few additional weeks of life on "treated" mice. Inactivation of the *TOR* transgene added ≈14 weeks of life, a significant increase over what is achieved by deinducing *TOM*. Finally, inactivation of both *TOR* and *TOM* oncogenes added approximately another 33 weeks of life beyond what was achieved by inactivation of *TOR* alone, another significant increase (*P* value overall <0.001). The analysis thus suggests that, whereas dramatic immediate responses, including reduction in tumor size, are observed with all three "therapies," targeting *Kras* is preferable to targeting *MYC*, and targeting both oncogenes is superior for achieving extended remissions.

Tumor Recurrence in *TOM;TOR;MTB* and *Myc;TOR;MTB*, but Not *TOM;Kras;MTB* Mice, Is Associated with Reactivation of Oncogenic Transgenes and Somatic Mutations in the *rtTA* Transgene. Acquired resistance to targeted therapies in human cancer is often associated with the emergence of secondary mutations in the targeted oncogenes that interfere with drug binding, suggesting continued dependence of the tumor on the initiating oncogenic lesion (20–23). Similarly, doxycycline-independent reexpression of the transgenic oncogene is found in many (for example, refs. 24–26) but not all (refs. 11, 13, and 27) tumors that recur in inducible-oncogene-driven tumor models. To determine whether recurrent tumors in our transgenic lines were associated with doxycycline-independent reexpression of the transgenes, we tested recurrent tumor and tumor-free mammary gland samples from the three models for transgenic transcripts by RT-PCR (Fig. 4*A*). The *MMTV-rtTA* transcript was detectable in the majority of the samples, both tumors and tumor-free glands, but no inducible transgene expression was observed in any of the tumor-free mammary gland preparations from aged mice from any of the three lines. Consistent with published data (11, 13), no reexpression of the *TOM* transgene was found in recurrent tumors from the *TOM;Kras;MTB* mice, although it could be induced in the recurrent tumors (and mammary glands) when mice were returned to a doxycycline diet (Fig. 4*A Upper Left*).

Half of the recurrent tumors from the *Myc;TOR;MTB* mice and all of those from *TOM;TOR;MTB* mice, however, reex-
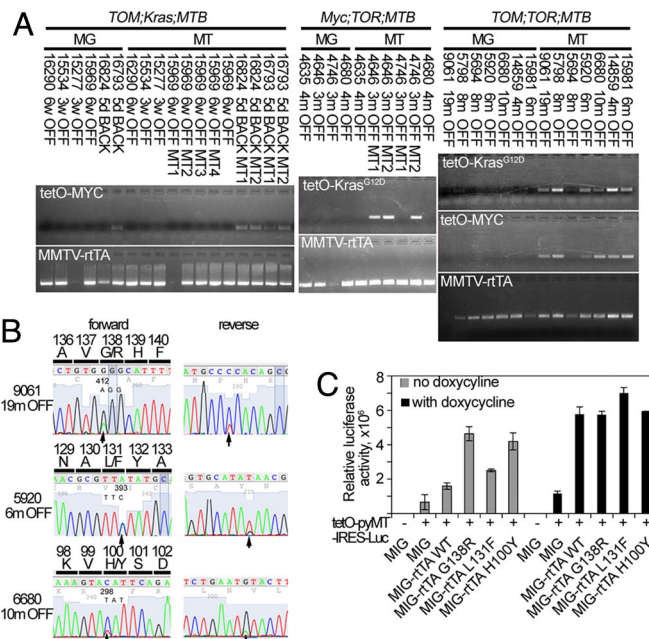
Fig. 4. Loss of doxycycline dependence in tumors from *TOM;TOR;MTB* and *Myc;TOR;MTB* mice is associated with somatic mutations in the *MMTV-rtTA* transgene. (*A*) Analysis of transgene reactivation in recurrent tumors from *TOM;Kras;MTB* (*Left*), *Myc;TOR;MTB* (*Center*), and *TOM;TOR;MTB* (*Right*) animals. Paired RNA samples from recurrent tumor and non-tumor-bearing mammary gland were collected after removal OFF doxycycline or after placing the animals BACK on doxycycline as indicated and assayed as described in *Experimental Procedures*. (*B*) Examples of forward and reverse sequencing traces from tumor samples: 9061, 19 m OFF; 5920, 6 m OFF; 6680 10 m OFF by direct sequencing of *rtTA* amplification products with nucleotide and predicted amino acid changes indicated in the forward sequencing panel. (*C*) Luciferase activity of the three mutant *rtTA* clones is increased compared with wild-type *rtTA* and to mock-transfected controls with and without doxycycline addition. Wild-type and mutant *rtTA* was cloned into a modified MigR1 retroviral vector (33) and cotransfected into 293T cells with luciferase reporter *pTetO-PyMT-IRES-Luc* [Nancy Y.-C. Du (Memorial Sloan–Kettering Cancer Center) and H.E.V., unpublished work]. At 16 h after transfection, doxycycline was added to appropriate samples, and, 24 h later, cells were harvested, and luciferase activity for each sample was compared with the mock-transfected control to calculate the increase in luciferase activity.

pressed their inducible transgenes. Consistent reexpression of both transgenes in the recurrent tumors from *TOM;TOR;MTB* mice suggested continued dependence of tumor growth on both oncogenes in this model. To ask whether there might be a common origin of doxycycline independence, we sequenced the 5′ portion (codons 13–210) of the *rtTA* transgene cDNA; this portion is reported to contain residues that govern binding to tet operators and thus the activation of the promoters (28). Five of seven samples contained nucleotide changes predicted to change amino acids (Fig. 4*B*), and the nucleotide substitution G412A was found in three independent tumor relapses. This mutation was also found in one of three relapses in *Myc;TOR;MTB* mice with *TOR* transgene reexpression. No mutations were found in the *rtTA* cDNA from tumor-free mammary glands of the same animals ($n = 7$) or in *rtTA* cDNA from primary tumors from animals receiving doxycycline ($n = 8$). Because the amino acid changes (H100Y, L131F, G138R) were distributed close to the region that differs between the rtTA and tTA (amino acids 71–102), they were likely to alter the ability of rtTA to bind and activate the *tetO* promoter.

To test directly whether mutant rtTA proteins were doxycycline-independent activators, we cloned mutant and wild-type *rtTA* sequences into a modified MSCV-based vector MigR1 and

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tested them in a doxycycline-responsive luciferase assay by cotransfection into 293T cells with a reporter plasmid (*TetO-PyMT-IRES-Luc*, provided by Nancy Y.-C. Du and H.E.V., unpublished work). All three mutants produced 1.5- to 3-fold-higher luciferase activity in the absence of doxycycline than did wild-type rtTA (Fig. 4*C*). These levels were comparable with 3.6-fold activation observed after addition of doxycycline in the cells transfected with the wild-type rtTA clone. Exposure to doxycycline further increased reporter activity in the mutants. These results are consistent with the observed doxycycline-independent expression of the inducible transgenes in recurrent tumors. Thus, somatic mutations in the *MMTV-rtTA* transgene appear to have a major role in tumor relapse in *TOM;TOR;MTB* mice and some of the *Myc;TOR;MTB* mice.

Because we and others have shown that tumors induced by dysregulated expression of *Myc* often contain somatic activating mutations in the endogenous *Ras* genes (11, 29), we screened recurrent tumors from the *Myc;TOR;MTB* mice with no evident reexpression of *TOR* for activating mutations in the endogenous *Hras*, *Kras*, and *Nras* genes. One of three tumors had a GGC13GTC mutation in the *Hras* gene (data not shown), reflecting continued requirement for activated Ras signaling in these tumors.

Discussion

Regression of tumors in response to inactivation of an initiating oncogene in experimental systems and in human tumors suggests that a single oncogene may play a crucial role in the maintenance of genetically complex tumors. However, in the context of a tumor induced by multiple known oncogenes, it has been unclear whether one or a subset of the genetic lesions is essential for tumor maintenance and whether anything can be gained by interfering with cooperating genetic lesions simultaneously.

Using combinations of doxycycline-inducible and constitutively expressed *Myc* and mutant *Kras* transgenes in the mouse mammary gland and observing tumor growth, regression, and recurrence, we have established a hierarchy of responses to deinduction of one or both oncogenes. In brief, whereas all three "treatments" produce benefits, deinduction of mutant *Kras* is significantly more effective than deinduction of *Myc*, and deinduction of both transgenic oncogenes produces the most durable remissions. However, in all three conditions, tumors ultimately recur in the absence of the inducer, doxycycline, although, in many recurrent tumors, doxycycline regulation was bypassed as a result of secondary somatic mutations in the *rtTA* transgene.

Why Is Loss of Kras Sufficient for Tumor Regression Despite Continued Expression of Myc? Our findings with tritransgenic mice indicate that coexpression of *MYC* and mutant *Kras* in the mouse mammary gland is sufficient for full and rapid malignant transformation and that loss of mutant *Kras* expression alone is sufficient for dramatic, but not permanent, regression of the resulting tumors, with arrest of proliferation and increased tumor cell death (Fig. 2).

Why might genetically complex tumors with multiple oncogenic lesions respond to inactivation of a single oncogene? One explanation might be that certain oncogenic events are uniquely important for tumor viability because complementary mutations depend on them. The action of the initiating oncogene violates physiological checkpoints by inducing changes in gene regulation or secondary mutations, which would normally trigger cell death unless neutralized by the effects of another oncogenic mutation. This model predicts that an oncogenic mutation represents an attractive target for therapeutic intervention if it protects tumor cell viability. In fact, down-regulation of antiapoptotic BCL-2 in Myc-induced mouse leukemia resulted in remission of the disease and prolonged survival of the mice (30). Alternatively, the direct

participation of one cooperating oncogene, such as *Myc*, in death signaling could explain why loss of its partner, mutant *Kras*, alone induces cell death and clinical remission in our dual-oncogene tumor models. In short, the mammary tumor models described here support the view that, although several genetic events may be necessary to confer the traits associated with oncogenic transformation (31), inactivation of a single oncogenic partner may undermine tumor maintenance, leading to tumor regression and temporary disease remission. Continued requirement for activated Ras signaling is reflected by frequent reactivation of the *TOR* transgene or somatic mutation in the endogenous *Hras* gene in the recurrent tumors of the *Myc;TOR;MTB* mice (Fig. 4A and data not shown).

Why Is Loss of MYC Insufficient for the Complete Regression of Tumors? MYC has been shown to be essential for maintenance of several tumor types (7, 11, 19). We do not know whether the incomplete regression upon deinduction of the *MYC* transgene in our *TOM;Kras;MTB* mice is the result of primary resistance, i.e., preexisting tumor cells insensitive to the inactivation of the *MYC* transgene. Alternatively, recurrence could depend on secondary resistance, with the rapid emergence of recurrent tumors from rare clones that acquired independence through additional oncogenic genetic or epigenetic events. Irrespective of the mechanism, down-regulation of *MYC*, although itself a poor mediator of tumor regression compared with deinduction of mutant *Kras*, extended the length of remission almost 3-fold when combined with deinduction of *TOR*. This provides a strong argument for developing combination therapies against multiple targets to postpone disease recurrence.

Why Does Simultaneous Deinduction of both MYC and Mutant Kras Fail to Prevent Relapse? Despite the deinduction of even both of two cooperating oncogenes, we found that tumors eventually reappeared in almost all animals maintained on doxycycline-free diets. These tumors likely represent the outgrowth of cells from the original, doxycycline-induced tumors, because tumors were not observed in animals that had not experienced oncogene expression (Fig. 3A). It is expected that tumor cells giving rise to recurrent tumors become independent of the transgenic oncogenes by acquiring genetic lesions or epigenetic changes that substitute for the requirement of *MYC* and/or mutant *Kras*, as was demonstrated in other models of doxycycline-independent relapses (7, 11, 25, 27, 34). The high prevalence of doxycycline-bypassing mutations in the *MMTV-rtTA* transgene that confer doxycycline-independence in the recurrent tumors from *TOM;TOR;MTB* mice reflects the essential role of oncogene cooperation in tumor relapse, because none of the recurrent tumors displayed reinduction of one oncogene alone. Molecular mechanisms of resistance to doxycycline withdrawal in this model may be analogous to forms of acquired resistance in human tumors treated with targeted therapies.

Experimental Procedures

Animal Husbandry and Genotyping. All animals were kept in specific pathogen-free housing with abundant food and water under guidelines approved by the Memorial Sloan–Kettering Cancer Center Institutional Animal Care and Use Committee and Research Animal Resource Center. *Myc*, *MTB*, *TOR*, *Kras*, and *TOM* transgenes have been described (5–9). Doxycycline was administered by feeding mice with doxycycline-impregnated food pellets (625 ppm; Harlan–Teklad). Mice were placed on doxycycline at various ages. The median age at the beginning of doxycycline exposure was 8 weeks. The median time of tissue sampling before doxycycline withdrawal was 7 weeks. Tail DNA was isolated by using the DNeasy 96 Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. Detection of the transgenes was performed as described (5–9). Animals were inspected for tumors weekly and monitored for tumor size according to institutional guidelines. Regressing tumors that were nonpalpable on at least two consecutive occasions were considered to have fully regressed.

Histology and Immunohistochemistry. Mammary tissues were obtained by either excision biopsy or postmortem according to institutional guidelines. Mammary glands and tumors were excised; some were flash-frozen in liquid nitrogen for molecular analyses, and some were fixed in 10% buffered formalin for 16–48 h at room temperature, placed in 70% ethanol, and sent for paraffin embedding and sectioning (Histoserv). Whole-mount preparations were done as described (http://mammary.nih.gov/tools/histological/wholemounts/index.html).

Immunohistochemistry was performed with Vectastain ABC and M.O.M. kits (Vector Laboratories) according to the manufacturer's instructions. The following antibodies were used: anti-phospho (Ser-10) histone H3 (1:200 dilution, cat no. 9701; Cell Signaling Technology) and anti-cleaved (Asp-175) caspase-3 (clone 5A1, 1:200 dilution, cat no. 9661; Cell Signaling Technology). To determine proliferation index, phosphohistone H3-positive and -negative cells were counted in three representative fields for at least three tumors at each time point. More than 150 nuclei per field were counted for tumors with the regulated *MYC* allele, and 700–1,100 nuclei per field were counted for tumors with constitutive *Myc* and regulated mutant *Kras* transgenes.

Antibodies and Immunoblotting. Tissue samples were crushed and lysed in lysis buffer [50 mM Tris·HCl (pH 8.0), 150 mM sodium chloride, 1 mM EDTA, 1% Triton X-100, 0.25% sodium deoxycholate, 1% Nonidet P-40, 40 mM sodium fluoride, 1 mM sodium orthovanadate, and 100 nM okadaic acid] containing protease inhibitors (Roche Diagnostics). After quantitation by Bio-Rad protein assays, 25–50 mg of each sample was separated by gel electrophoresis on 12% Tris-Glycine precast gels (Invitrogen) and transferred to nitrocellulose. Specific proteins were detected by using enhanced chemiluminescence (Amersham Pharmacia) or SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and the following antibodies: anti-Myc (1:1,000 dilution, cat no. 9402; Cell Signaling Technology), anti-Kras (clone F234, 1:500 dilution, cat. no. sc-30; Santa Cruz Biotechnology), anti-keratin-8 (TROMA, 1:500 dilution; Developmental Studies Hybridoma Bank, Iowa City, IA), HRP-conjugated anti-rabbit Ig (1:5,000 dilution; Amersham Pharmacia), and HRP-conjugated anti-mouse IgG (1:2,000 dilution; Roche Applied Science).

Transgene Reexpression Studies, Mutation Analysis, and Luciferase Assays. Frozen tissue samples were crushed in liquid nitrogen and total RNA extracted according to standard protocols. After DNase I treatment (Invitrogen), 250 ng of RNA were reverse transcribed with random hexamer primers by using the SuperScript III first-strand synthesis system (Invitrogen), omitting the enzyme for the control (RT) set.

Transgenes were amplified by using the following primers: rtTA.F 5′-tgattaacagcgcattagagc-3′; rtTA.R 5′-ctgtacgcggacccacttt-3′ (the same primers were used for sequencing); tor.F: 5′-aaggacaaggtgtacagttatgtga-3′; tor.R: 5′-ctccgtctgcgacatcttc-3′, and, as reported in ref. 32) full-length wild-type and mutant rtTA sequences were amplified by using primers rtTAclone.F: 5′-gcgcgaattcaccatgtctagattagataaaaag-3′, rtTAclone.R 5′-cgcgctcgagctacccac-cgtactcgtcaattc-3′, cloned into a modified MigR1 retroviral vector (33). For luciferase assays, 293T cells were grown to 50% confluence in 12-well plates and cotransfected by using FuGENE 6 (Roche) according to the manufacturer's protocol with MigR1 constructs and a luciferase reporter *pTetO-PyMT-IRES-Luc* (provided by Nancy Y.-C. Du and H.E.V., unpublished work). At 16 h after transfection, doxycycline or mock treatment was added to samples, and 24 h later, cells were harvested, and luciferase activity was measured by using the luciferase reporter assay system (Promega). Luciferase activity for each sample was compared with the mock-transfected control to calculate the increase in luciferase activity.

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