MOLECULAR AND CELLULAR BIOLOGY, Nov. 2006, p. 8109–8121 Vol. 26, No. 21
0270-7306/06/$08.00+0 doi:10.1128/MCB.00404-06

Isoform-Specific Ras Activation and Oncogene Dependence during MYC- and Wnt-Induced Mammary Tumorigenesis▽

Joanne W. Jang, Robert B. Boxer, and Lewis A. Chodosh*

Departments of Cancer Biology, Cell and Developmental Biology, and Medicine and Abramson Family Cancer Research Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6160

Received 8 March 2006/Returned for modification 10 April 2006/Accepted 10 August 2006

We have previously shown that c-MYC-induced mammary tumorigenesis in mice proceeds via a preferred secondary pathway involving spontaneous activating mutations in *Kras2* (C. M. D'Cruz, E. J. Gunther, R. B. Boxer, J. L. Hartman, L. Sintasath, S. E. Moody, J. D. Cox, S. I. Ha, G. K. Belka, A. Golant, R. D. Cardiff, and L. A. Chodosh, Nat. Med. 7:235-239, 2001). In contrast, we now demonstrate that Wnt1-induced mammary tumorigenesis proceeds via a pathway that preferentially activates *Hras1*. In addition, we find that expression of oncogenic forms of *Kras2* and *Hras1* from their endogenous promoters has markedly different consequences for the progression of tumors to oncogene independence. Spontaneous activating *Kras2* mutations occurring in either MYC- or Wnt1-induced tumors were strongly associated with oncogene-independent tumor growth following MYC or Wnt1 downregulation. In contrast, *Hras1*-mutant Wnt1-induced tumors consistently remained oncogene dependent. Additionally, *Kras2*-mutant tumors exhibited substantially higher levels of ras-GTP, phospho-Erk1/2, and phospho-Mek1/2 compared to *Hras1*-mutant tumors, suggesting the involvement of the ras/mitogen-activated protein kinase (MAPK) pathway in the acquisition of oncogene independence. Consistent with this, by use of carcinogen-induced *ras* mutations as well as knock-in mice harboring a latent activated *Kras2* allele, we demonstrate that Kras2 activation strongly synergizes with both c-MYC and Wnt1 in mammary tumorigenesis and promotes the progression of tumors to oncogene independence. Together, our findings support a model for tumorigenesis in which c-MYC and Wnt1 select for the outgrowth of cells harboring mutations in specific *ras* isoforms and that these secondary mutations, in turn, determine the extent of ras/MAPK pathway activation and the potential for oncogene-independent growth.

A wealth of experimental evidence indicates that the development and progression of human cancers require multiple genetic events that occur in a stepwise fashion. Although few genetic alterations have been definitively placed in the sequence of events that contribute to breast cancer in humans, several genetic pathways have been implicated in determining breast cancer susceptibility, response to therapy, and prognosis.

c-MYC amplification occurs in 10 to 30% of human breast cancer (3, 20) and is correlated with aggressive tumor behavior and poor prognosis (9, 30). Wnt1, an upstream activator of c-MYC, was initially identified on the basis of its insertional activation by the mouse mammary tumor virus (MMTV) during the process of murine mammary tumorigenesis (27). Although mutations in Wnt family members are rare in human breast cancer, overexpression of Wnt family members and alterations in components of the Wnt signaling pathway are common (5). For example, β-catenin, which translocates to the nucleus when activated by the Wnt pathway, is localized in the nucleus in up to 60% of human breast cancers (21).

A third oncogene that has been extensively studied in human breast cancer is ras. Unlike the relatively high frequency of genomic alterations in c-MYC, ras family members are activated by mutation in less than 5% of human breast cancers (24, 29, 31). Nevertheless, like that of Wnt, ras pathway activation is extremely common in human breast cancer (7, 38). This likely results from the fact that regulatory molecules that signal through ras—such as HER-2/neu (1, 33), insulin-like growth factor, insulin-like growth factor receptor (17, 34), and c-Src (2)—are frequently amplified, overexpressed, or otherwise activated in human breast cancer.

While it is clear that multiple genetic alterations are required for the formation of breast cancers, relatively few such secondary alterations have been identified. In this regard, mouse models bearing defined initiating genetic alterations relevant to human cancer have been a valuable means to identify collaborating oncogenic events. For example, forced overexpression of c-MYC and v-Ha-ras in the mammary glands of bitransgenic mice results in strongly synergistic tumor formation (19, 32). Consistent with this, MYC-induced mammary tumors frequently harbor spontaneous activating point mutations in *Kras2* (8). Similarly, over 50% of MMTV-Wnt1 tumors contain activating point mutations in *Hras1* (28). These observations suggest that activation of the ras family pathway may contribute to MYC- and Wnt1-induced tumorigenesis.

To study mammary tumor progression, our laboratory has developed conditional bitransgenic systems for the doxycycline-inducible expression of c-MYC or Wnt1 in the murine mammary gland (8, 14). In these systems, the reverse tetracycline transcriptional activator (rtTA) is specifically expressed in the mammary epithelium of transgenic mice under the control of the MMTV promoter and, in the presence of doxycycline, induces expression of c-MYC or Wnt1 from a tetracycline-dependent promoter. Using these models, we have

* Corresponding author. Mailing address: University of Pennsylvania School of Medicine, 612 BRB II/III, 421 Curie Boulevard, Philadelphia, PA 19104-6160. Phone: (215) 898-1321. Fax: (215) 573-6725. E-mail: chodosh@mail.med.upenn.edu.

▽ Published ahead of print on 14 August 2006.

previously reported that expression of c-MYC in bitransgenic MMTV-rtTA/TetO-MYC (MTB/TOM) female mice leads to the development of mammary adenocarcinomas with an average latency of 22 weeks (8). Nearly half of these tumors harbor spontaneous activating point mutations in *Kras2* or *Nras*, with the majority found in *Kras2*. Upon downregulation of c-MYC in tumors by doxycycline withdrawal, 50% of MYC-induced tumors regress to a nonpalpable state whereas the remaining tumors resume growth in the absence of c-MYC overexpression or MYC pathway activation (4, 8). Notably, in MYC-induced mammary tumors, activating mutations in *Kras2*—but not *Nras*—are strongly associated with the acquisition of oncogene independence (8). This suggests that Kras2 may contribute both to the development of MYC-induced mammary tumors and to their progression to oncogene independence.

Similar to c-MYC, conditional expression of Wnt1 in bitransgenic MMTV-rtTA/TetO-Wnt1 (MTB/TWNT) mice leads to mammary tumor formation with an average latency of 20 weeks (14). In contrast to MYC-induced tumors, however, abrogation of Wnt1 expression by doxycycline withdrawal results in the regression of virtually all tumors to a nonpalpable state (14). Of note, it has recently been reported that more than half of MMTV-Wnt1 tumors harbor activating mutations in *Hras1*, but not in *Kras2* or *Nras* (28), raising the question of whether MYC and Wnt1 preferentially synergize with different ras family members.

We now report that the majority of Wnt1-induced tumors in MTB/TWNT mice harbor activating point mutations in *Hras1*, whereas a far smaller percentage of tumors harbor activating point mutations in *Kras2*. Nevertheless, treatment of MYC and Wnt1 transgenic mice with the carcinogen MNU (*N*-methyl-*N*-nitrosourea) demonstrated that activation of either Hras1 or Kras2 can strongly synergize with c-MYC or Wnt1 in mammary tumor formation. However, tumors bearing *Hras1* mutations remain oncogene dependent, whereas tumors bearing *Kras2* mutations uniformly progress to oncogene independence. Oncogene independence, in turn, is strongly associated with high levels of ras and mitogen-activated protein kinase (MAPK) pathway activity, suggesting a biochemical basis for the differential oncogene dependence exhibited by tumors bearing mutations in different *ras* isoforms. In aggregate, our findings suggest a model for tumorigenesis in which c-MYC and Wnt1 select for the outgrowth of cells harboring mutations in specific *ras* isoforms, which in turn determines the extent of MAPK pathway activation and the potential for oncogene-independent growth.

MATERIALS AND METHODS

Animals and tissues. MTB, TOM, and TWNT transgenic mice were maintained on an FVB background and have previously been described (8, 13, 14). *K-ras*^{LA2} mice (18) were obtained from Jax. MTB/TOM and MTB/TWNT mice were bred to K-ras^{LA2} mice to generate MTB/TOM/K-ras^{LA2} and MTB/TWNT/K-ras^{LA2} tritransgenic mice on a mixed FVB/C57Bl6/129sv background. Expression of the c-MYC or Wnt1 transgenes was induced by the administration of 2.0 mg/ml doxycycline with 5% sucrose in drinking water.

For MNU treatment, 1.0 g of MNU (Sigma-Aldrich) was dissolved in 100 ml of 0.85% NaCl adjusted to pH 7.0 with acetic acid. Animals were treated with 50 mg of MNU/kg of body weight by intraperitoneal injection.

For all studies, mice were monitored at least once per week for tumor formation. Tumor size was measured using calipers. Mice were withdrawn from doxycycline when tumors reached ~10 by 10 mm in size and were monitored for regression. Tissue for biochemical analysis was obtained by biopsy prior to doxycycline withdrawal.

***ras* mutation analysis.** Total RNA was harvested as previously described (23). cDNA was synthesized using a First Strand cDNA synthesis kit (Amersham Biosciences Corp.) and PCR amplified using primers specific for *Hras1*, *Nras*, or *Kras2*. PCR products were purified with a GeneClean III kit (Qbiogene Inc.) and sequenced to detect point mutations at codons 12, 13, and 61 as previously described (4, 8).

Western analysis and ras activity assays. Snap-frozen mammary and tumor tissues were homogenized using EBC buffer (0.5% NP-40, 50 mM Tris [pH 8.0], 0.12 M NaCl, 25 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 tablet protease inhibitors/50 ml EBC buffer) and a Dounce homogenizer. Equal amounts of protein from each extract were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gels and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S to confirm equal protein loading and efficient transfer. Membranes were blocked in phosphate-buffered saline–0.1% Tween 20–5% nonfat dry milk (PBST-MILK) for 1 h and incubated with primary antibody diluted in PBST-MILK at room temperature (RT) for 1 h or at 4°C overnight. Membranes were washed three times with PBST at RT for 5 min each and then incubated with secondary antibody diluted in PBST-MILK at RT for 1 h. Blots were washed three times with PBST and twice with phosphate-buffered saline, developed using the ECL Plus system (Amersham Biosciences), and exposed to film (Kodak XAR-5).

Ras activity assays were carried out using a ras activation assay kit (Upstate Biotechnology) according to the manufacturer's specifications. Briefly, the ras binding domain of human RAF1 fused to glutathione *S*-transferase was used to precipitate GTP-bound ras from protein lysates. Precipitated ras-GTP was visualized by Western blotting.

The primary antibodies used were anti-ras clone RAS10 (Upstate Biotechnology) (1:1,000 dilution); anti-phospho-p44/42 MAPK (Thr202/Tyr 204) (anti-phospho-Erk1/2) (Cell Signaling) (1:1,000 dilution); anti-phospho-MEK1/2 (Ser217/221) (Cell Signaling) (1:1,000 dilution); anti-β-tubulin (Biogenex) (1:10,000 dilution); and anti-TFIIH p89, clone S-19 (Santa Cruz Biotechnology) (1:1,000 dilution). The secondary antibodies used were horseradish peroxidase-conjugated goat anti-mouse and horseradish peroxidase-conjugated goat anti-rat antibodies (Jackson ImmunoResearch) (1:10,000 dilution).

Quantitative PCR for *Kras2* amplification. For genomic DNA preparation, tissue was incubated with 500 μl digestion buffer (100 mM EDTA, 50 mM Tris [pH 8.0], 0.5% sodium dodecyl sulfate, 1.7 μl/ml proteinase K) overnight at 55°C. Following addition of 500 μl PCIA (50% phenol, 48% chloroform, 2% isoamyl alcohol), samples were vortexed and spun at maximum speed in a microcentrifuge for 10 min and 300 μl of the top layer was transferred to a fresh tube. A 600-μl volume of 100% EtOH was added to precipitate DNA, which was then washed with 70% EtOH and resuspended in 100 μl of water.

The *Kras2* probe (ACCCTACGATAGAGGTAACG) for quantitative PCR anneals to the intron 1-exon 1 junction of genomic *Kras2*, and the primers (K2651-4100F [GCCTTGACGATACAGCTAATTCAGAAT] and K2651-4100R [GCGCACGCAGACTGTAGA]) amplify a region spanning the intron 1-exon 1 junction. 18S RNA (assay identification number 4319413E-0412015) was used as an endogenous loading control. Quantitative PCRs were performed in a mixture consisting of 10 μl TaqMan Universal MasterMix, 1 μl TaqMan primer-probe mix, and 5 μl of DNA in a total volume of 20 μl. Reactions were performed in duplicate in 384-well microtiter plates in an ABI Prism 7900HT real-time PCR system (Applied Biosystems). Reaction conditions were as follows: 95°C denaturation for 10 min followed by 40 cycles consisting of 95°C denaturation for 15 s and 60°C annealing-extension for 1 min. *Kras2* amplification levels were normalized to 18S RNA amplification levels to control for differences in DNA concentration.

RESULTS

Isoform-specific *ras* mutations develop during MYC- and Wnt1-induced mammary tumorigenesis. We have previously reported that MYC-induced mammary tumorigenesis is commonly accompanied by spontaneous activating point mutations in *Kras2* and *Nras* (8). As c-*MYC* is a downstream target of the Wnt signaling pathway, we reasoned that Wnt1-induced tumors might also harbor activating *ras* mutations. In support of this hypothesis, Podsypanina et al. have shown that MMTV-Wnt1 mammary tumors frequently harbor activating *Hras1*

TABLE 1. Frequency of *ras* mutations in tumors from MTB/TWNT and MTB/TOM mice

Transgenic line	% *ras* mutations		
	Hras1	*Kras2*[a]	*Nras*[b]
MTB/TWNT ($n = 95$)	54	3	0
MMTV-Wnt1 ($n = 10$)	20	0	0

[a] *Kras2* versus *Hras1*; $P < 0.0001$.
[b] *Nras* versus *Hras1*; $P < 0.0001$.

mutations (28). Therefore, we examined mammary adenocarcinomas arising in MTB/TWNT mice for activating mutations in *Hras1*, *Kras2*, and *Nras* (Table 1). This analysis revealed that 54% (51/95) of Wnt1-induced tumors harbored activating point mutations in *Hras1* and 3% (3/95) harbored activating point mutations in *Kras2*, whereas none harbored activating point mutations in *Nras* (*Kras2* versus *Hras1*, $P < 0.0001$; *Nras* versus *Hras1*, $P < 0.0001$). No tumor had detectable mutations in more than one *ras* isoform. Consistent with these and previous findings, we further determined that 20% (2/10) of MMTV-Wnt1 tumors harbored activating *Hras1* mutations (28). These results demonstrate that, in contrast to the preferential association of MYC-induced mammary tumors with *Kras2* mutations, Wnt1-induced mammary tumors are preferentially associated with mutations in *Hras1*.

MNU synergizes with c-MYC and Wnt1 in mammary tumorigenesis and alters *ras* mutation frequency and distribution. The preferential occurrence of *Kras2* mutations in MYC-induced tumors and *Hras1* mutations in Wnt1-induced tumors suggested the possibility that c-MYC and Wnt1 may preferentially synergize with different ras isoforms during mammary tumorigenesis. Therefore, we hypothesized that c-MYC overexpression in the mammary gland might select for the outgrowth of *Kras2*-mutant cells, whereas Wnt1 might select for the outgrowth of *Hras1*-mutant cells. To address this hypothesis, we treated female MTB/TOM and MTB/TWNT mice with MNU, an agent with a 10-min biological half-life that induces mammary tumors harboring *Hras1* and *Kras2* mutations in mice and rats (15, 16, 25, 35). One week following MNU treatment, expression of either c-MYC or Wnt1 was induced in the mammary epithelium by doxycycline administration.

MNU treatment prior to oncogene induction decreased the median latency of MYC-induced tumor formation from 22 weeks to 8.3 weeks (Fig. 1A; $P < 0.0001$) and increased tumor multiplicity from 1.2 to 2.1 tumors per animal ($P = 0.0004$). Similarly, MNU decreased the median latency of Wnt1 tumorigenesis from 20 weeks to 3.5 weeks (Fig. 1B; $P < 0.0001$) and increased tumor multiplicity from 1.2 to 5.9 tumors per animal ($P < 0.0001$). These data suggest that mutations induced by MNU treatment strongly synergize with either c-MYC or Wnt1 in mammary tumor formation.

Analysis of mammary tumors harvested from MNU-treated c-MYC-induced mice revealed an increased frequency of activating mutations in *Hras1* (25% versus 0%; $P = 0.0021$) compared to tumors arising in non-MNU-treated animals (Table 2).

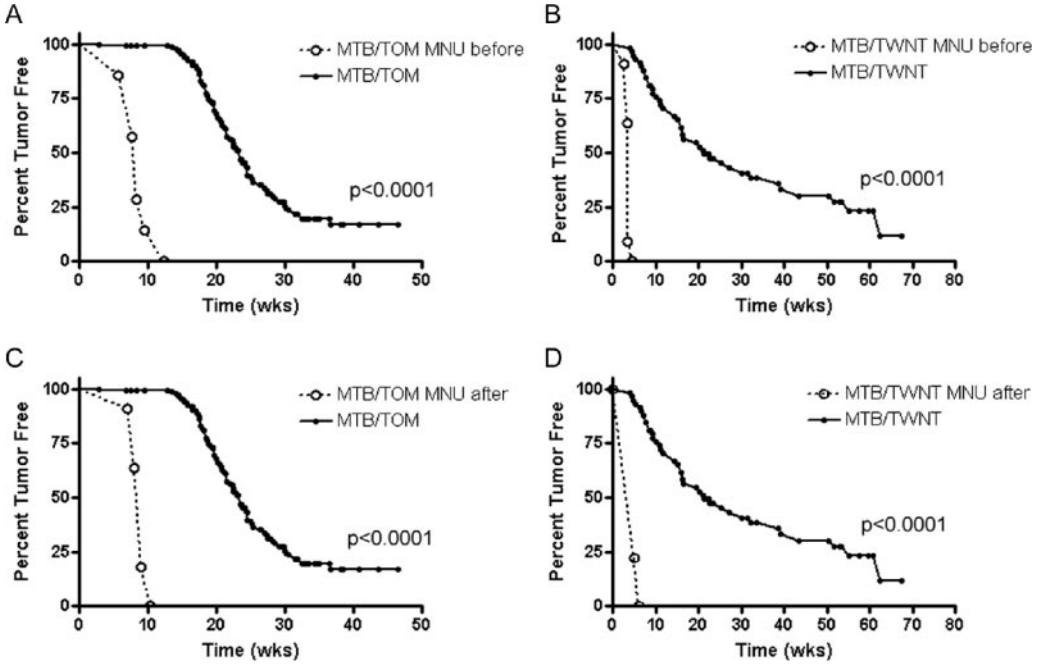

FIG. 1. MNU synergizes with Wnt1 and MYC in mammary tumorigenesis. (A) Mammary tumor-free survival curves for MTB/TOM mice ($n = 7$) injected intraperitoneally with 50 mg of MNU/kg of body weight at 6 weeks of age and induced with doxycycline at 7 weeks of age compared to untreated, doxycycline-induced MTB/TOM animals ($n = 148$). (B) Mammary tumor-free survival curves for MTB/TWNT animals ($n = 11$) subjected to the same MNU treatment as the animals represented in panel A compared to untreated, doxycycline-induced MTB/TWNT animals ($n = 58$). (C) Mammary tumor-free survival curves for MTB/TOM animals ($n = 11$) induced with doxycycline at 6 weeks of age and injected intraperitoneally with 50 mg/kg of MNU at 7 weeks of age compared to untreated, doxycycline-induced MTB/TOM animals ($n = 148$). (D) Mammary tumor-free survival curves for MTB/TWNT animals ($n = 9$) subjected to the same MNU treatment as the animals represented in panel C compared to untreated, doxycycline-induced MTB/TWNT animals ($n = 58$). Animals were palpated weekly for mammary tumors.

TABLE 2. Frequency of *ras* mutations in tumors from MNU-treated and untreated MTB/TWNT and MTB/TOM mice

Transgenic line and treatment	No. of mice	% *ras* mutations			
		Hras1[a]	*Kras2*[b]	*Nras*[c]	Total[d]
MTB/TOM					
None	85	0	34	11	45
MNU before Dox[e]	12	25	42	0	67
MNU after Dox	12	0	83	0	83
MTB/TWNT					
None	32	34	3	0	37
MNU before Dox	24	21	4	0	25
MNU after Dox	12	8	33	0	41

[a] *Hras1* mutation frequency. For MTB/TOM versus MTB/TOM, MNU before doxycycline treatment, $P = 0.0021$; for MTB/TWNT versus MTB/TWNT, MNU before doxycycline treatment, $P = 0.3701$; for MTB/TWNT versus MTB/TWNT, MNU after doxycycline treatment, $P = 0.3938$.

[b] *Kras2* mutation frequency. For MTB/TOM versus MTB/TOM, MNU before doxycycline treatment, $P = 0.3137$; for MTB/TOM versus MTB/TOM, MNU after doxycycline treatment, $P = 0.0034$; for MTB/TWNT versus MTB/TWNT, MNU before doxycycline treatment, $P = 0.9421$; for MTB/TWNT versus MTB/TWNT, MNU after dox, $P = 0.0194$.

[c] *Nras* mutation frequency. For MTB/TOM versus MTB/TOM, MNU before doxycycline treatment, $P = 0.3845$.

[d] Total *ras* mutation frequency. For MTB/TOM versus MTB/TOM, MNU before doxycycline treatment, $P = 0.1538$; for MTB/TOM versus MTB/TOM, MNU after doxycycline treatment, $P = 0.0122$; for MTB/TWNT versus MTB/TWNT, MNU before doxycycline treatment, $P = 0.3216$; for MTB/TWNT versus MTB/TWNT, MNU after doxycycline treatment, $P = 0.3193$.

[e] Dox, doxycycline.

In addition, in MYC-induced tumors from MNU-treated mice, there was a modest increase in the frequency of activating mutations in *Kras2* (42% versus 34%; P = not significant [NS]) and a modest decrease in the frequency of *Nras* mutations (0% versus 11%; P = NS) compared to that seen with tumors arising in animals not treated with MNU (Table 2). Analysis of Wnt1-induced tumors arising in MNU-treated animals revealed a modest decrease in the frequency of activating *Hras1* mutations (21% versus 34%; P = NS) without change in the frequency of *Kras2* or *Nras* mutations compared to that seen with tumors in untreated animals (Table 2).

As the majority of *ras* mutations identified in Wnt1-induced tumors in MNU-treated mice were in *Hras1*, and the majority of *ras* mutations identified in MYC-induced tumors in MNU-treated mice were in *Kras2*, these findings were consistent with a model in which Wnt1 expression selects for the outgrowth of cells harboring *Hras1* mutations whereas c-MYC expression selects for the outgrowth of cells harboring *Kras2* mutations. However, our observation that up to 25% of MYC-induced tumors in MNU-treated mice harbored *Hras1* mutations suggested the possibility that Hras1 is able to synergize with c-MYC as well as Wnt1. Of note, we did not observe a significant increase in the overall frequency of *ras* mutations in either MYC- or Wnt1-induced tumors in MNU-treated mice. Given the striking synergy observed between MNU treatment and each of these oncogenes, these findings raise the possibility that MNU may induce genetic alterations other than *ras* mutation that synergize with c-MYC or Wnt1.

As an alternate approach to this question, oncogene expression in MTB/TOM and MTB/TWNT mice was induced with doxycycline 1 week prior to MNU treatment. Similar to the effects of MNU treatment prior to oncogene induction, MNU treatment following oncogene induction greatly accelerated mammary tumorigenesis and increased tumor multiplicity (Fig. 1C and D). MYC-induced tumor latency decreased from 22 to 9.0 weeks ($P < 0.0001$), and multiplicity increased from 1.2 to 4.1 tumors per animal ($P < 0.0001$). Similarly, Wnt1-induced tumor latency decreased from 20 to 5.0 weeks ($P < 0.0001$) and multiplicity increased from 1.2 to 6.9 tumors per animal ($P < 0.0001$). Thus, MNU treatment either before or after oncogene induction strongly synergizes with MYC- or Wnt1-induced tumorigenesis.

Analysis of *ras* mutation frequencies in mammary tumors revealed a marked increase in the percentage of MYC tumors bearing activating *ras* mutations in mice treated with MNU after oncogene induction compared to the results seen with untreated animals (83% versus 45%; $P = 0.012$) (Table 2). The distribution of *ras* mutations in MYC tumors was also altered, with 83% of MYC-induced tumors from mice treated with MNU after oncogene induction harboring *Kras2* mutations compared to 34% of MYC tumors in untreated mice ($P = 0.003$).

For Wnt1-induced tumors, while the overall frequency of *ras* mutations remained similar, the distribution of *ras* mutations between Wnt1 tumors in MNU-treated and untreated mice changed significantly (Table 2). Whereas 3% of Wnt1-induced tumors in untreated mice harbored activating *Kras2* mutations, 33% of tumors in MTB/TWNT mice treated with MNU after oncogene induction harbored *Kras2* mutations ($P = 0.019$). This increased frequency of *Kras2* mutation was accompanied by a corresponding decrease in *Hras1* mutation frequency (8% versus 34%; P = NS) in MNU-treated versus untreated animals. In aggregate, these findings provide additional support for the model that *ras* mutation is a rate-limiting step in both MYC- and Wnt1-induced tumorigenesis and further suggest that activated Kras2 and Hras1 are each able to synergize with Wnt1 or c-MYC in mammary tumorigenesis.

Loss of p53 in Wnt1-induced tumors partially abrogates selection pressure for *Hras1* mutation during mammary tumorigenesis. It has previously been shown that half of tumors arising in MMTV-Wnt1 transgenic mice bear activating point mutations in *Hras1* (28). Podyspanina and colleagues further demonstrated that mammary tumors arising in *p53* null MMTV-Wnt1 mice lacked activating *Hras1* mutations. Surprisingly, mammary tumors arising in MMTV-Wnt1 mice heterozygous for a null allele of *p53* exhibited an *Hras1* mutation frequency indistinguishable from that observed in *p53* wild-type mice, regardless of whether tumors displayed loss of heterozygosity (LOH) (28). These findings suggest that loss of p53 function early in tumor development may obviate the selective pressure for *Hras1* mutation.

To determine whether similar changes in selective pressure due to loss of p53 occurred in our conditional system, we analyzed *ras* mutation frequencies in 36 MTB/TWNT tumors that arose in mice heterozygous for a null allele of *p53*. Of 30 MTB/TWNT/p53$^{+/-}$ tumors that had not undergone LOH at the *p53* locus as evaluated by Southern hybridization, 11 (37%) harbored activating *Hras1* mutations. In contrast, of six MTB/TWNT/p53$^{+/-}$ tumors that had undergone *p53* LOH, none harbored *Hras1* mutations. Consistent with this difference and previous reports, no *Hras1* mutations were detected in four MTB/TWNT tumors arising in *p53* null mice. These data sug-

TABLE 3. *Kras2* mutations are associated with the progression of Wnt1-induced mammary tumors to oncogene independence

MTB/TWNT tumor *ras* status	% Tumors with indicated status	
	Full regression	Incomplete regression
Wild type ($n = 30$)[a]	93	7
Hras1 mutant ($n = 33$)[b]	94	6
Kras2 mutant ($n = 3$)	33	67

[a] For wild-type *ras* versus *Kras2*, $P = 0.0024$.
[b] For *Hras1* versus *Kras2*, $P = 0.0014$.

gest that reduced p53 pathway activity decreases the selective pressure for the outgrowth of cells bearing *Hras1* mutations during Wnt1-induced mammary tumorigenesis.

Activating *Kras2* mutations are associated with Wnt1 oncogene-independent tumor growth. We have previously demonstrated that the presence of spontaneous activating point mutations in *Kras2* in MYC-induced mammary tumors is tightly correlated with their ability to resume growth following doxycycline withdrawal and MYC downregulation (4, 8). To determine whether activating *ras* mutations in Wnt1-induced mammary tumors were also associated with their acquisition of oncogene-independent growth, tumors were subjected to biopsy while they were still expressing Wnt1 to establish their *ras* mutation status. Mice were then withdrawn from doxycycline and tumor sizes monitored to determine their regression behavior in response to Wnt1 downregulation (Table 3).

Analysis of tumor biopsies for *ras* mutations revealed that 93% (28/30) of Wnt1-induced tumors wild type for *Hras1*, *Kras2*, and *Nras* regressed to a nonpalpable state following doxycycline withdrawal. Similarly, 94% (31/33) of tumors with activating point mutations in *Hras1* also regressed to a nonpalpable state. In contrast, of three Wnt1-induced tumors identified with activating point mutations in *Kras2*, two grew in a Wnt1-independent manner following doxycycline withdrawal (*Hras1* versus *Kras2*; $P = 0.0014$). These findings are consistent with a model in which activating *Kras2* mutations in Wnt1-induced tumors correlate with the acquisition of oncogene-independent growth in a manner analogous to that observed for *Kras2*-mutant MYC-induced tumors. In contrast, *Hras1* mutation during Wnt1-induced mammary tumorigenesis was not associated with oncogene-independent growth. These data suggest that—in the context of Wnt1-induced tumorigenesis—activating *Kras2* mutations promote the progression of tumors to oncogene independence, whereas *Hras1* mutations do not.

Wnt1 tumors do not progress to oncogene independence despite repeated cycles of Wnt1 activation. We previously demonstrated that nearly all MYC-induced mammary tumors progress to oncogene independence following repeated cycles of MYC induction and deinduction (4) in part attributable to the emergence of novel *Kras2* mutations in recurring tumors. The fact that neither MYC- nor Wnt1-induced tumors have been identified with activating point mutations in more than one *ras* isoform, despite the fact that *ras* mutation is common in these tumors, implies that activation of one *ras* isoform relieves the selective pressure for activation of other *ras* isoforms. This, in turn, suggests that *Hras1*, *Kras2*, and *Nras* are to some extent functionally redundant in their ability to synergize with c-MYC and Wnt1 during mammary tumorigenesis. As a consequence, since Wnt1-induced mammary tumors are most commonly associated with activating mutations in *Hras1* rather than *Kras2*, and since *Hras1* mutations are not associated with oncogene-independent growth, we hypothesized that Wnt1 tumors would be more likely than MYC tumors to remain dependent upon oncogene expression for tumor growth.

To determine whether Wnt1-induced tumors could be induced to progress to oncogene independence in a manner similar to that observed for MYC-induced tumors, we subjected Wnt1-induced tumors to multiple cycles of oncogene induction and deinduction (Fig. 2A). Wnt1 was initially induced in the mammary gland until tumors developed and reached 1 cm^3 in size. Wnt1 expression was then abrogated by doxycycline withdrawal and tumor regression monitored. Tumors that regressed to a nonpalpable state were then induced to regrow by readministration of doxycycline. When tumors again reached 1 cm^3 in size, Wnt1 expression was once again terminated. This cycle of Wnt1 induction and deinduction was repeated three times.

Consistent with our prior observations, nearly all Wnt1-in-

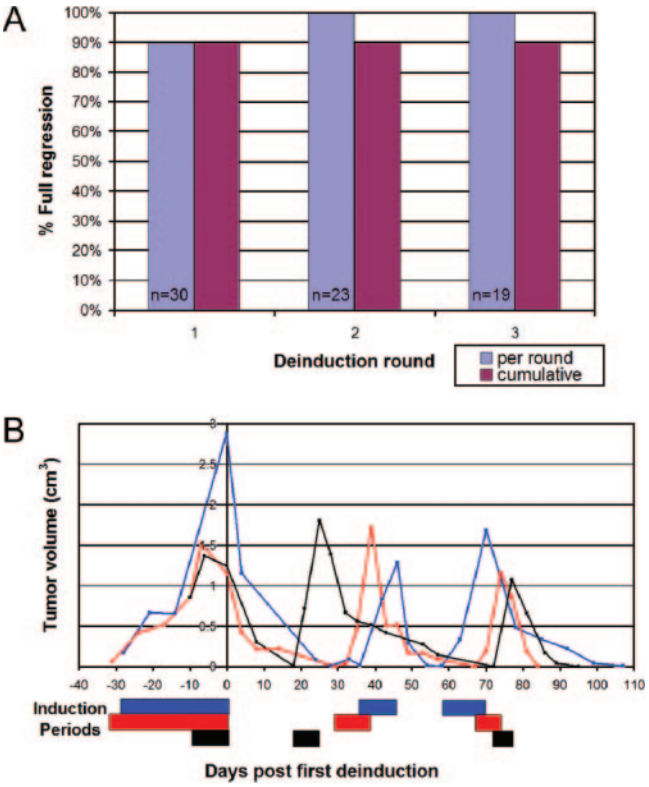

FIG. 2. Wnt1-induced mammary tumors remain stably dependent upon Wnt1 for tumor maintenance and growth. (A) Representative growth curves of three mammary tumors that underwent repeated cycles of Wnt1 induction and deinduction, showing progressing to Wnt1 oncogene independence during the first, second, or third cycle of oncogene induction. (B) Chart displaying the percentages of tumors that remained dependent upon Wnt1 for tumor maintenance and growth at each cycle of deinduction. Blue bars represent the percentages of tumors that remained Wnt1 dependent in each round of deinduction. Maroon bars represent the cumulative percentages of Wnt1-dependent tumors determined for by each deinduction round.

duced tumors (27/30) regressed to a nonpalpable state following the first round of doxycycline withdrawal (Fig. 2B). When mice harboring oncogene-dependent tumors were reinduced with doxycycline, in all cases tumors rapidly regrew at the site of the initial tumor. This indicates that residual tumor cells survive following oncogene deinduction and remain susceptible to the oncogenic effects of Wnt1. Similar to their behavior during the first round of Wnt1 downregulation, 100% of tumors (23/23) fully regressed during the second round of deinduction (Fig. 2B). This oncogene-dependent behavior persisted through a third round of deinduction, in which 100% (19/19) of the remaining tumors underwent regression to a nonpalpable state. Thus, in marked contrast to our findings in MYC-induced mammary tumors, Wnt1-induced mammary adenocarcinomas remain stably dependent upon Wnt1 for tumor maintenance and growth.

Differential ras pathway activation in *Kras2* and *Hras1*-mutant tumors. Given the differential oncogene-dependent behavior of tumors bearing *Kras2* versus *Hras1* mutations, we wished to investigate potential differences in the biochemical consequences of *Kras2* and *Hras1* activation in vivo. Specifically, we considered the possibility that *Kras2* mutation activates the ras pathway to a different extent than *Hras1* mutation. To explore this possibility, we analyzed the extent of ras and MAPK pathway activation by determining levels of ras-GTP, phosphorylated Erk1/2, and phosphorylated Mek1/2 in protein lysates prepared from MYC- and Wnt1-induced mammary tumors harboring mutations in different *ras* isoforms (Fig. 3 and 4).

Compared to MYC-induced mammary tumors without detectable *ras* mutations, MYC-induced tumors harboring activating *Kras2* mutations exhibited significantly higher levels of ras-GTP as well as higher levels of phospho-Erk1/2 and phospho-Mek1/2 (Fig. 3A). Notably, Wnt1 tumors with activating *Hras1* mutations had significantly, but only modestly, elevated levels of ras-GTP compared to Wnt1-induced tumors without *ras* mutations (Fig. 3B). Moreover, no elevations in phospho-Erk1/2 or phospho-Mek1/2 were observed in *Hras1*-mutant Wnt1 tumors. Direct comparison of *Hras1*-mutant Wnt1 tumors to *Kras2*-mutant MYC tumors revealed markedly higher levels of ras-GTP, phospho-Erk1/2, and phospho-Mek1/2 in *Kras2*-mutant MYC tumors (Fig. 4A). This comparison also demonstrated that phosphorylation of the MAPK pathway targets p90RSK and Elk1 was elevated in MYC tumors harboring *Kras2* mutations compared to *Hras1*-mutant Wnt1 tumors (Fig. 4A). Moreover, consistent with the inference that *Kras2* mutation is a more potent activator of the MAPK pathway than *Hras1* mutation in mammary tumors, Wnt1-induced tumors with activating *Kras2* mutations displayed high levels of ras-GTP that were comparable to those observed in MYC tumors harboring *Kras2* mutations (Fig. 4B).

In addition to MAPK pathway activation, we also addressed the possibility that other downstream ras effector pathways, such as the RalGDS or PI3K pathways, might be differentially activated in MYC- and Wnt1-induced mammary tumor bearing mutations in different *ras* family members. To study the RalGDS pathway, we examined the levels of Ral-GTP in *Hras1*-mutant Wnt1 tumors, *Kras2*-mutant MYC tumors, and MYC- and Wnt1-induced tumors wild type for *ras* (data not shown). Analogously, to study the PI3K pathway, we compared

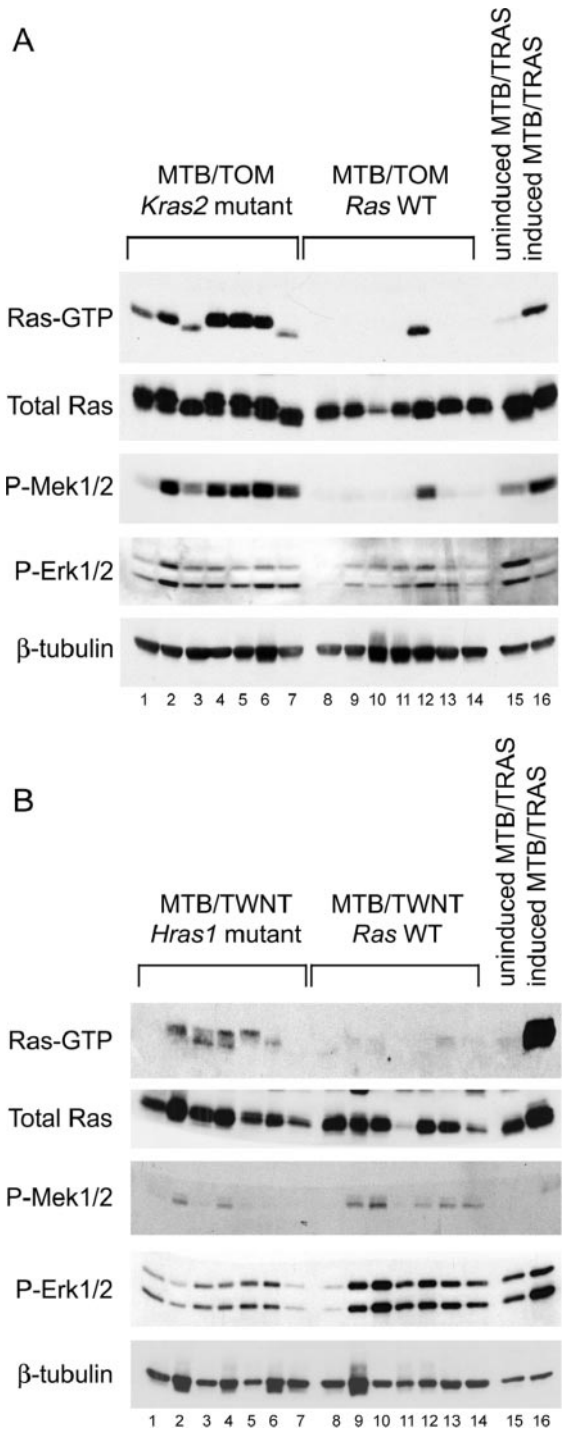

FIG. 3. Ras and MAPK pathway activities in Wnt1- and MYC-induced mammary tumors differ according to *ras* mutation status. (A) Immunoblots showing levels of ras-GTP, ras, phospho-Mek1/2, and phospho-Erk1/2 in *Kras2* mutant MTB/TOM tumors compared to the levels seen with MTB/TOM tumors without detectable *ras* mutations. The results for mammary glands from doxycycline-induced and uninduced MTB/TRAS mice that permit expression of v-Ha-ras in the mammary gland are shown as negative and positive controls. β-Tubulin is shown as a loading control. (B) Immunoblots showing levels of ras-GTP, ras, phospho-Mek1/2, and phospho-Erk1/2 in *Hras1* mutant MTB/TWNT tumors compared to the levels obtained with MTB/TWNT tumors without detectable *ras* mutations. Uninduced and induced mammary glands from MTB/TRAS mice were used as negative and positive controls. β-Tubulin is shown as a loading control.

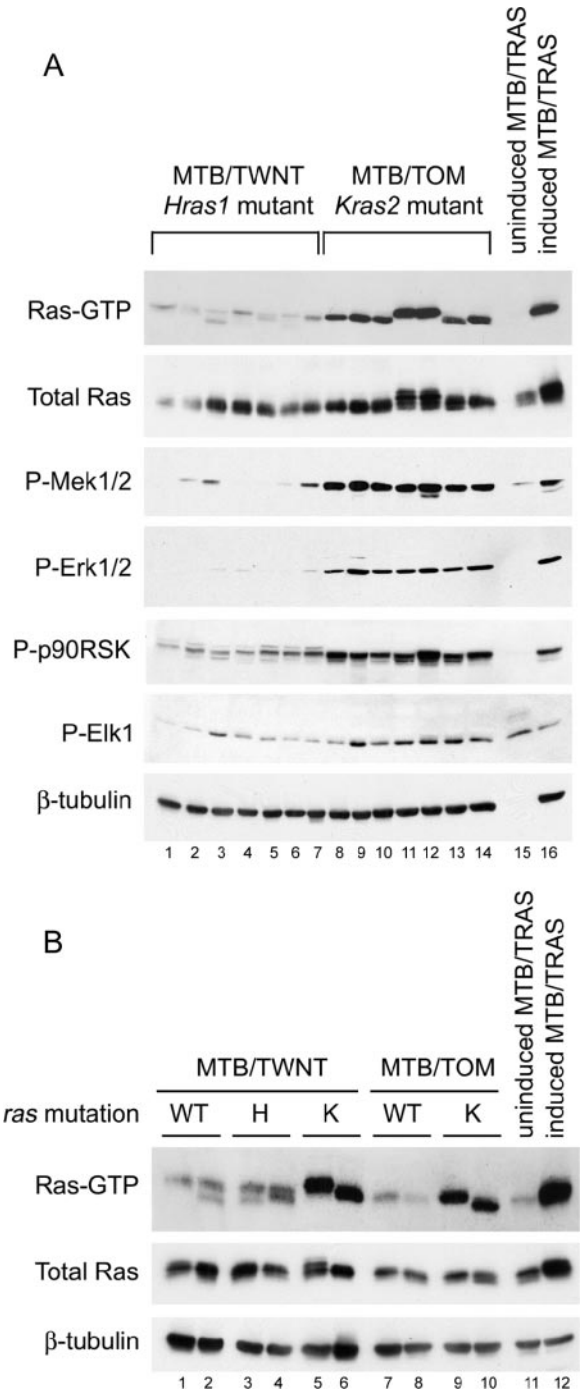

FIG. 4. Ras and MAP kinase pathway activity in Wnt1- and MYC-induced mammary tumors bearing mutations in different *ras* family members. (A) Immunoblots showing levels of ras-GTP, ras, phospho-Mek1/2, and phospho-Erk1/2 in *Hras1*-mutant MTB/TWNT tumors compared to the levels seen with *Kras2*-mutant MTB/TOM tumors. Uninduced and induced mammary glands from MTB/TRAS mice were used as negative and positive controls. β-Tubulin is shown as a loading control. (B) Immunoblots showing levels of ras-GTP and ras for MTB/TOM and MTB/TWNT tumors without detectable *ras* mutations or with *Kras2* mutations and for MTB/TWNT tumors with *Hras1* mutations. Uninduced and induced mammary glands from MTB/TRAS mice were used as negative and positive controls. β-Tubulin is shown as a loading control.

the levels of phospho-Akt in a series of Western blot analyses that included *Hras1*-mutant Wnt1 tumor samples, *Kras2*-mutant MYC tumor samples, and Wnt1- and MYC-induced tumors samples without detectable *ras* mutations (data not shown). These experiments failed to reveal any consistent differences in the levels of Ral-GTP or in the levels of phospho-Akt between MYC- and Wnt1-induced tumors harboring different *ras* mutations or MYC- and Wnt1-induced tumors wild type for *ras*.

In aggregate, our findings demonstrate that tumors bearing spontaneous activating *Kras2* mutations in which *Kras2* is expressed from its endogenous promoter exhibit higher levels of ras and MAPK pathway activation than tumors without *ras* mutations or tumors bearing *Hras1* mutations. This, in turn, suggests that the oncogene-independent growth observed in tumors bearing activating *Kras2*—but not *Hras1*—mutations could be due to increased activation of the ras and MAPK pathways selectively conferred by *Kras2* mutation.

Activated *Kras2* synergizes with c-MYC and Wnt1 in mammary tumorigenesis. The high frequency of spontaneous activating *ras* mutations that we observed in MYC- and Wnt1-induced mammary tumors, as well as the increased *Kras2* mutation frequency and synergy observed between MNU treatment and MYC and Wnt pathway activation, suggested that expression of activated *Kras2* from its endogenous promoter can synergize with MYC and Wnt1 in mammary tumor development. However, in each of the above-described experimental contexts, multiple genetic alterations in addition to *Kras2* mutation likely occur during the process of tumorigenesis, thereby complicating assessment of the contribution of *Kras2* activation to tumor growth and the acquisition of oncogene independence. Moreover, while v-Ha-ras has been shown to synergize with MYC in carcinogenesis in several model systems, including the mammary gland (19, 32), to date synergy between c-MYC and Kras2—or between Wnt1 and any ras isoform—has not been demonstrated in vivo.

In light of these observations, we analyzed mice bearing a latent activated *Kras2* allele knocked into the endogenous locus (K-ras^{LA2}) (18) to determine directly whether expression of activated *Kras2* from its endogenous promoter could synergize with c-MYC or Wnt1 in mammary tumorigenesis and confer oncogene-independent growth. In this strain, spontaneous recombination of the transcriptionally inactive K-ras^{LA2} allele results in the expression of an activated K-ras^{LA2} allele from the endogenous *Kras2* locus. We reasoned that if *Kras2* activation was selected for during the process of MYC- or Wnt1-induced tumorigenesis, the presence of cells bearing a spontaneously rearranged—and activated—K-ras^{LA2} allele would accelerate the tumorigenic process. Therefore, K-ras^{LA2} mice were bred to MYC- and Wnt1-inducible mice to generate tritransgenic MTB/TOM/K-ras^{LA2} and MTB/TWNT/K-ras^{LA2} mice. Oncogene expression was induced in these tritransgenic mice, along with littermate controls, by doxycycline administration.

MTB/TOM/K-ras^{LA2} tritransgenic mice developed mammary tumors far faster (median latency, 8.6 weeks versus 25.9 weeks; $P < 0.0001$) (Fig. 5A and Table 4) and had higher tumor multiplicities (2.9 versus 1.2; $P = 0.0002$) (Table 4) than control doxycycline-induced MTB/TOM bitransgenic mice. Similarly, MTB/TWNT/K-ras^{LA2} mice exhibited markedly

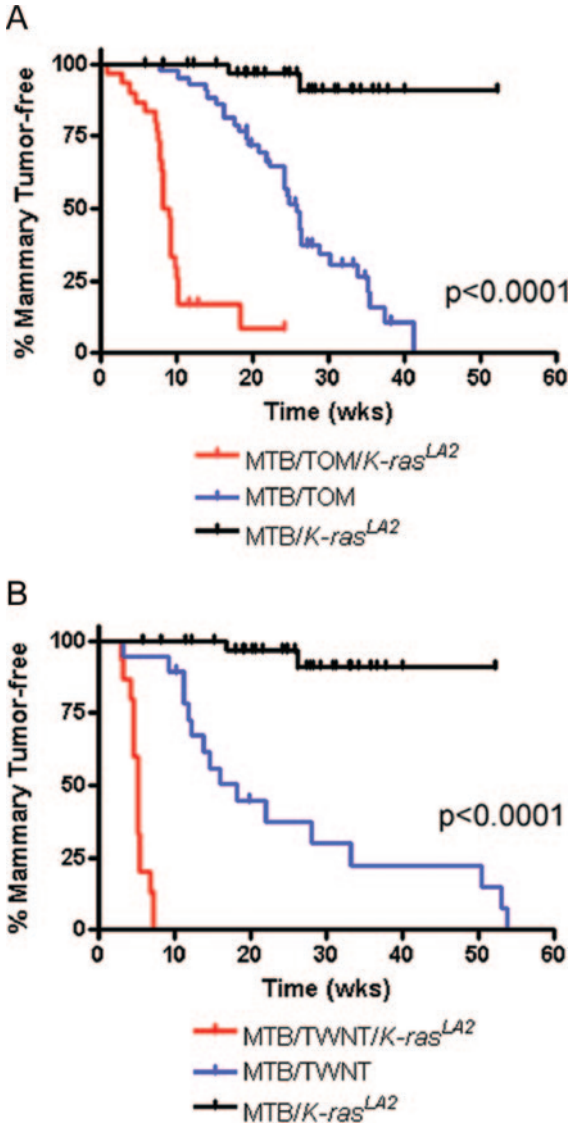

FIG. 5. A latent K-*ras*^{LA2} allele synergizes with MYC- and Wnt1-induced mammary tumorigenesis. (A) Mammary tumor-free survival curves for doxycycline-induced MTB/TOM/K-*ras*^{LA2} mice ($n = 30$). Results for doxycycline-induced MTB/TOM ($n = 43$) and MTB/K-*ras*^{LA2} ($n = 33$) littermates are shown as controls. (B) Mammary tumor-free survival curves for doxycycline-induced MTB/TWNT/K-*ras*^{LA2} mice ($n = 15$). Results for doxycycline-induced MTB/TWNT ($n = 19$) and MTB/K-*ras*^{LA2} ($n = 33$) littermates are shown as controls.

shorter tumor latencies (5.3 weeks versus 18.3 weeks; $P < 0.0001$) and higher tumor multiplicities (2.8 versus 1.2; $P = 0.0004$) than MTB/TWNT controls (Fig. 5B and Table 4). MTB/K-*ras*^{LA2} mice treated with doxycycline only rarely developed mammary tumors (Fig. 5). Finally, analysis of genomic DNA from tumors arising in MTB/TOM/K-*ras*^{LA2} and MTB/TOM/K-*ras*^{LA2} mice indicated that all tumors had rearranged and activated the latent *Kras2* allele (data not shown). These data demonstrate that activated *Kras2* expressed from its endogenous locus can strongly synergize with either the MYC or Wnt1 pathway in mammary tumorigenesis.

TABLE 4. A latent K-*ras*^{LA2} allele synergizes with c-MYC and Wnt1 in mammary tumorigenesis

Transgenic line	Median latency (wks)	Average multiplicity (tumors/mouse)
MTB/TWNT/K-*ras*^{LA2} ($n = 30$)[a]	5.3	2.8
MTB/TWNT ($n = 18$)	18.3	1.2
MTB/TOM/K-*ras*^{LA2} ($n = 41$)[b]	8.6	2.9
MTB/TOM ($n = 28$)	25.9	1.2

[a] For MTB/TWNT/K-*ras*^{LA2} versus MTB/TWNT, latency, $P < 0.0001$; multiplicity, $P = 0.0004$.
[b] For MTB/TOM/K-*ras*^{LA2} versus MTB/TOM, latency, $P < 0.0001$; multiplicity, $P = 0.0002$.

Activated *Kras2* promotes the progression of Wnt1- and c-MYC-induced mammary tumors to oncogene independence. If activation of an endogenous *Kras2* allele by point mutation were sufficient to confer MYC- or Wnt1-independent growth in mammary tumors, we reasoned that tumors arising in MTB/TOM/K-*ras*^{LA2} and MTB/TWNT/K-*ras*^{LA2} tritransgenic mice would continue to grow following abrogation of *MYC* or *Wnt1* transgene expression. To test this hypothesis, we removed doxycycline from the drinking water of tumor-bearing tritransgenic MTB/TOM/K-*ras*^{LA2} and MTB/TWNT/K-*ras*^{LA2} mice and bitransgenic MTB/TOM and MTB/TWNT mice and monitored tumors for regression (Table 5).

Consistent with our prior observations, 0 of 18 tumors arising in MTB/TWNT mice were able to grow in an oncogene-independent manner. In contrast, 27% (8/30) of tumors in MTB/TWNT/K-*ras*^{LA2} mice grew in an oncogene-independent manner following downregulation of Wnt1 overexpression ($P = 0.016$). Thus, Wnt1-induced tumors bearing an activated K-*ras*^{LA2} allele were more than sixfold more likely to progress to Wnt1 oncogene independence than those that did not. Nevertheless, the majority of Wnt1-induced tumors bearing activated *Kras2* alleles regressed to a nonpalpable state following Wnt1 downregulation. These findings demonstrate that while an activated *Kras2* allele is not sufficient for Wnt1-independent growth, it is strongly associated with the progression of tumors to oncogene independence.

Similarly, of MYC-induced tumors in MTB/TOM mice that were wild type for *Kras2*, 20% (2/10) were able to grow following the downregulation of MYC expression, whereas 59% of tumors (24/41) arising in MTB/TOM/K-*ras*^{LA2} mice grew in an oncogene-independent manner following doxycycline with-

TABLE 5. A latent K-*ras*^{LA2} allele promotes the progression of MTB/TWNT and MTB/TOM tumors to oncogene independence

Transgenic line	No. of tumors with indicated status	
	Full regression	Incomplete regression
MTB/TWNT/K-*ras*^{LA2} ($n = 30$)[a]	22	8
MTB/TWNT ($n = 18$)	18	0
MTB/TOM/K-*ras*^{LA2} ($n = 41$)[b]	17	24
MTB/TOM, *Kras2* wild type ($n = 10$)	8	2

[a] For MTB/TWNT/K-*ras*^{LA2} versus MTB/TWNT, $P = 0.0182$; incomplete regression odds ratio > 6.18.
[b] For MTB/TOM/K-*ras*^{LA2} versus MTB/TOM, $P = 0.0385$; incomplete regression odds ratio = 5.65.

drawal ($P = 0.029$). Thus, MYC-induced tumors with an activated K-*ras*^{LA2} allele were 5.6-fold more likely to progress to oncogene independence than MYC-induced tumors wild type for *Kras2*. This indicates that, as observed for Wnt1, expression of an activated *Kras2* allele from its endogenous promoter is strongly associated with MYC-independent growth. Notably, however, activation of the K-*ras*^{LA2} latent allele in tumors arising in either MTB/TWNT/K-*ras*^{LA2} or MTB/TOM/K-*ras*^{LA2} mice was not sufficient in and of itself to confer oncogene-independent growth.

Activation of the latent K-*ras*^{LA2} allele is not sufficient for high levels of ras or MAPK pathway activation. A potential explanation for the continued oncogene-dependence of some MTB/TWNT/K-*ras*^{LA2} and MTB/TOM/K-*ras*^{LA2} tumors, despite having activated the K-*ras*^{LA2} allele, is that these tumors do not activate ras or the MAPK pathway to the same extent as MTB/TWNT and MTB/TOM tumors bearing spontaneous activating *Kras2* mutations.

Biochemical analysis of biopsies from mammary tumors in MTB/TWNT/K-*ras*^{LA2} mice that were subsequently shown to be oncogene dependent following doxycycline withdrawal demonstrated that, despite expressing the activated K-*ras*^{LA2} allele from the endogenous locus, these tumors did not universally display the high levels of ras-GTP, phosphorylated Mek1/2, and phosphorylated Erk1/2 seen in MTB/TWNT tumors harboring spontaneous *Kras2* mutations (Fig. 6A). Ras-GTP was undetectable in two of nine tumors from MTB/TWNT/K-*ras*^{LA2} mice, and in six of the remaining seven tumors, ras-GTP levels—though elevated—were somewhat lower than those observed in *Kras2*-mutant MTB/TWNT tumors. Consistent with this, in many tumors the levels of phospho-Mek1/2 and phospho-Erk1/2 were lower than those observed in *Kras2*-mutant MTB/TWNT tumors.

In contrast to the biochemical characteristics of Wnt1-dependent tumors in MTB/TWNT/K-*ras*^{LA2} mice, nearly all Wnt1-independent tumors from such mice were found to have ras-GTP levels at least as high as those observed in *Kras2*-mutant MTB/TWNT tumors (Fig. 6B). Analogously, biochemical analysis demonstrated that nearly all tumors arising in MTB/TOM/K-*ras*^{LA2} mice that were subsequently demonstrated to be oncogene independent following doxycycline withdrawal had high levels of ras-GTP, phospho-Mek1/2, and phospho-Erk1/2 comparable to those found in *Kras2*-mutant MTB/TOM tumors (Fig. 7).

These findings indicate that rearrangement and activation of a latent K-*ras*^{LA2} allele in MYC- and Wnt1-induced mammary tumors does not uniformly result in the high levels of ras-GTP, phospho-Mek1/2, and phospho-Erk1/2 typically found in MTB/TWNT or MTB/TOM tumors bearing spontaneous *Kras2* mutations. This, in turn, implies that genetic alterations in addition to *Kras2* mutation may be required for high levels of ras pathway activation and progression of MYC- and Wnt1-induced tumors to oncogene independence. Nevertheless, our observation that virtually all oncogene-independent MTB/TWNT/K-*ras*^{LA2} and MTB/TOM/K-*ras*^{LA2} tumors displayed levels of activated ras, Mek1/2, and Erk1/2 comparable to those found in oncogene-independent MTB/TWNT and MTB/TOM tumors harboring spontaneous *Kras2* mutations supports a model in which high levels of ras pathway activation promote

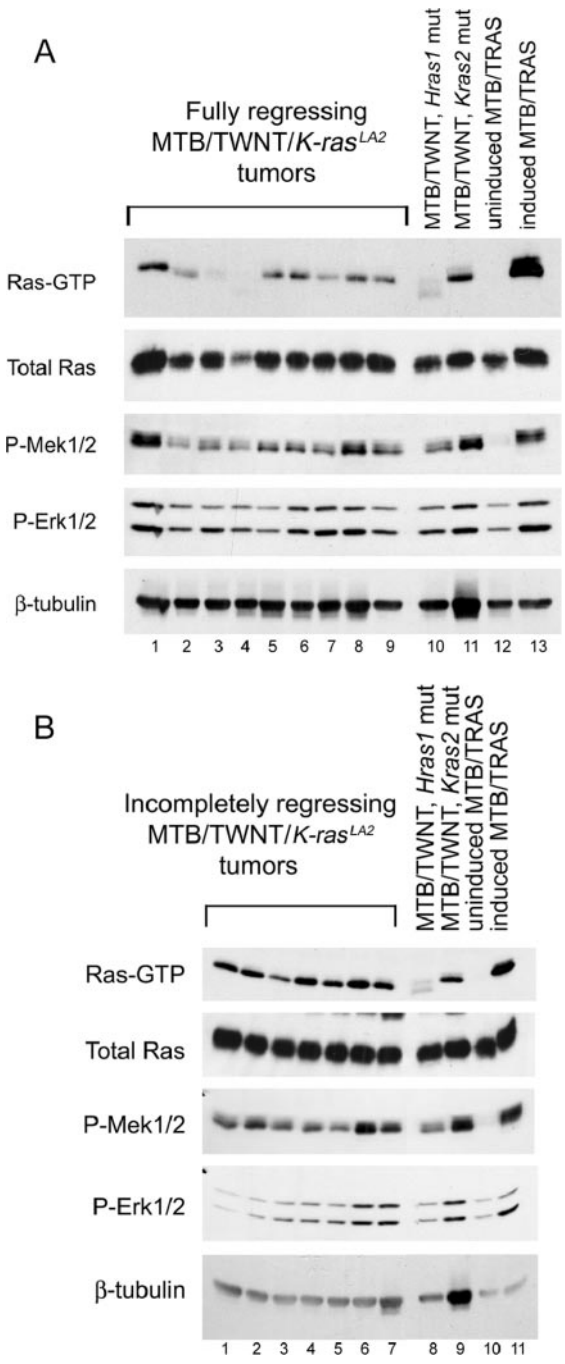

FIG. 6. Ras and MAPK activity in tumors arising in MTB/TWNT/K-*ras*^{LA2} mice. MTB/TWNT/K-*ras*^{LA2} mice were induced with doxycycline until tumors reached 1 cm^3. Tumors were subjected to biopsy, deinduced, and monitored weekly for regression behavior. (A) Immunoblots showing the levels of ras-GTP, phospho-Mek1/2, and phospho-Erk1/2 in biopsy samples from nine tumors from MTB/TWNT/K-*ras*^{LA2} mice that were later demonstrated to be Wnt1 dependent following doxycycline withdrawal. An *Hras1*-mutant MTB/TWNT tumor, a *Kras2*-mutant MTB/TWNT tumor, and uninduced and induced mammary glands from MTB/TRAS mice were used as controls. β-Tubulin is shown as a loading control. (B) Immunoblots showing the levels of ras-GTP, phospho-Mek1/2, and phospho-Erk1/2 in biopsy samples from seven tumors from MTB/TWNT/K-*ras*^{LA2} mice that were later demonstrated to be oncogene independent following doxycycline withdrawal. An *Hras1*-mutant MTB/TWNT tumor, a *Kras2*-mutant MTB/TWNT tumor, and uninduced and induced mammary glands from MTB/TRAS mice were used as controls. β-Tubulin is shown as a loading control.

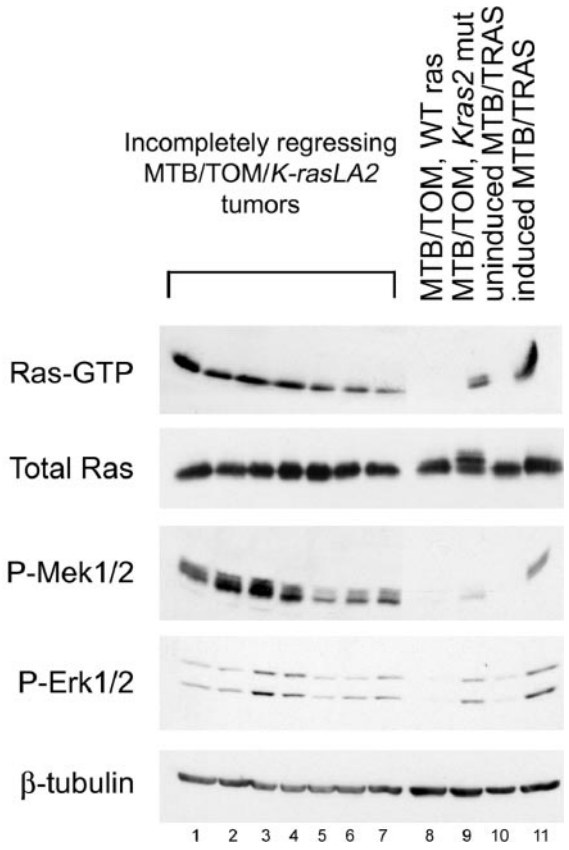

FIG. 7. Ras and MAPK activity in tumors arising in MTB/TOM/K-ras^{LA2} mice. Immunoblots showing the levels of ras-GTP, phospho-Mek1/2, and phospho-Erk1/2 in biopsy samples from seven tumors from MTB/TOM/K-ras^{LA2} mice that were later demonstrated to be oncogene independent following doxycycline withdrawal are presented. An MTB/TOM tumor lacking detectable *ras* mutations, a *Kras2*-mutant MTB/TOM tumor, and uninduced and induced mammary glands from MTB/TRAS mice were used as controls. β-Tubulin is shown as a loading control.

the progression of MYC- and Wnt1-induced tumors to an oncogene-independent state.

High levels of ras activation and oncogene independence are not due to *Kras2* amplification in MYC- or Wnt1-induced tumors. It has previously been reported that amplification of the *Kras2* locus can lead to activation of the MAPK pathway (22). We considered this as a potential explanation for the high levels of MAPK pathway activation observed in *Kras2*-mutant MTB/TOM tumors and for the observation that some tumors with rearranged latent K-ras^{LA2} alleles exhibited high levels of MAPK pathway activation whereas others did not. Therefore, we assessed *Kras2* genomic copy numbers by quantitative PCR to determine whether the *Kras2* locus was amplified in MTB/TOM, MTB/TOM/K-ras^{LA2}, or MTB/TWNT/K-ras^{LA2} tumors.

Of the tumors arising in MTB/TOM mice, all *Kras2*-mutant tumors examined exhibited high levels of ras-GTP and MAPK pathway activity and were able to grow in an oncogene-independent manner. Nevertheless, *Kras2*/*18S* genomic ratios for *Kras2*-mutant, oncogene-independent MYC-induced tumors did not differ significantly from those observed for MYC-induced tumors that were wild type for *ras* and oncogene dependent (1.13 versus 1.01; $P = 0.447$). Similarly, *Kras2*/*18S* genomic ratios for MTB/TOM/K-ras^{LA2} or MTB/TWNT/K-ras^{LA2} tumors that were oncogene independent or that had high levels of ras-GTP were not significantly different from those of tumors that were oncogene dependent or that had lower levels of ras-GTP (data not shown). These results indicate that in MYC- and Wnt1-induced tumors, either with or without a latent K-ras^{LA2} allele, amplification of the *Kras2* locus does not appear to account for high levels of ras pathway activity or the acquisition of oncogene independence.

DISCUSSION

Our findings demonstrate that MYC-induced mammary tumorigenesis proceeds via a preferred pathway involving *Kras2* mutation, whereas Wnt1-induced tumorigenesis proceeds via a preferred pathway involving *Hras1* mutation. Furthermore, our observation that MNU treatment strongly synergizes with c-MYC and Wnt1 in mammary tumorigenesis, and significantly alters the frequency and distribution of *ras* mutations, suggests that *ras* mutations directly contribute to—and represent a rate-limiting step in—MYC- and Wnt1-induced mammary tumorigenesis. Consistent with this, expression of a latent activated K-ras^{LA2} allele from the endogenous *Kras2* locus strongly synergized with both c-MYC and Wnt in mammary tumor formation. Additionally, we have shown that alterations in the *p53* tumor suppressor pathway can alter secondary pathways in tumorigenesis, as Wnt1-induced tumors lacking functional p53 exhibited significantly fewer *Hras1* mutations than tumors arising in *p53* wild-type mice. In aggregate, our data suggest a model in which c-MYC induction preferentially selects for the outgrowth of cells harboring *Kras2* mutations, whereas Wnt1 induction preferentially selects for the outgrowth of cells harboring *Hras1* mutations.

The experiments described above suggest that a specific synergistic interaction exists between c-MYC and Kras2, and between Wnt1 and Hras1, during mammary tumorigenesis. However, the finding that *Kras2* mutations do arise in Wnt1-induced tumors, and that Wnt1 induction in K-ras^{LA2} mice dramatically accelerates mammary tumorigenesis and increases tumor multiplicity, suggests that it is possible for Wnt1 to synergize with Kras2 as well as with Hras1 in mammary tumorigenesis. Similarly, *Hras1* mutations were found in a substantial fraction of MYC-induced mammary tumors in the setting of carcinogen treatment, providing evidence that—like Wnt1—c-MYC can synergize with Hras1 as well as with Kras2 in mammary tumorigenesis.

Notably, while *ras* mutation frequently accompanies both MYC- and Wnt1-induced mammary tumorigenesis, the behaviors of these two tumor types following oncogene downregulation were found to be markedly different. Our data show that *Kras2* mutation—whether in the context of MYC- or of Wnt1-induced tumorigenesis—is strongly associated with the acquisition of oncogene-independent tumor growth. In contrast, *Hras1* mutation was not associated with oncogene-independent growth. Our further observation that ras-GTP, phosphorylated Erk1/2, and phosphorylated Mek1/2 levels were all significantly elevated in tumors harboring *Kras2* mutations compared to the levels seen in tumors with *Hras1* mutations suggests a biochemical basis for the differential oncogene independence exhibited

by *Kras2*-mutant MYC and *Hras1*-mutant Wnt1 mammary tumors.

Several in vitro studies have demonstrated differential activation of ras effector pathways by different ras family members. In a variety of cell types, activated Kras2 stimulates the Raf-1/MAPK pathway more strongly than does activated Nras or Hras1 (37, 40). Conversely, activated Hras1 stimulates the PI3K/Akt pathway more strongly than activated Kras2 (6, 40). Overexpression of specific ras isoforms has also been shown to result in differential cellular behavior, in part through activation of different downstream ras effector pathways (6, 26). Consistent with these in vitro experiments, we observed that spontaneous activating *Kras2* mutations in MYC- and Wnt1-induced mammary tumors are associated with a level of activation of the Raf-1/MAPK pathway greater than that seen with spontaneous activating mutations in *Hras1*. Moreover, tumors harboring spontaneous point mutations in *Kras2* behave differently than tumors bearing *Hras1* mutations, as demonstrated by the ability of *Kras2*-mutant MYC- and Wnt1-induced mammary tumors to progress to oncogene independence. As such, these models for MYC- and Wnt1-induced mammary tumorigenesis provide a novel in vivo demonstration of differential activation of ras effector pathways depending on the *ras* isoform mutated.

Notably, a subset of MYC-induced tumors exhibit high levels of Ras-GTP, phospho-Mek1/2, and phospho-Erk1/2 despite harboring wild-type *ras* alleles (Fig. 3A and data not shown). Similarly, levels of phospho-Mek1/2 and phospho-Erk1/2 were higher in some *ras* wild-type Wnt1-induced tumors than in *Hras1*-mutant Wnt1-induced tumors (Fig. 3B). This raises the possibility that in both MYC- and Wnt1-induced tumors, mechanisms other than activating point mutation in *ras* likely impinge on ras and the MAPK pathway. These alternative mechanisms could include an increase in Ras-GEF function or a decrease in Ras-GAP function.

To more directly address the contribution of Kras2 activation to oncogene-independent growth, MYC- and Wnt1-inducible mice were bred to mice bearing a latent activated K-ras^{LA2} allele at the endogenous *Kras2* locus. Surprisingly, in contrast to tumors arising in MTB/TOM and MTB/TWNT mice bearing spontaneous activating point mutations in *Kras2*, a subset of K-ras^{LA2} tumors remained oncogene dependent despite having recombined and activated the endogenous latent K-ras^{LA2} allele. Nevertheless, Wnt1- and MYC-induced tumors bearing rearranged and activated latent K-ras^{LA2} alleles were five- to sixfold more likely to be able to grow in a transgene-independent growth. This suggests that expression of an activated *Kras2* allele from its endogenous promoter contributes to, but is not be sufficient for, progression of Wnt1- and MYC-induced tumors to an oncogene-independent state.

Remarkably, analysis of over 600 tumors indicated that once a *ras* allele has been activated by mutation in a tumor, mutations in other *ras* family members either do not occur or are not selected for during tumorigenesis. This implies that the specific *ras* mutation selected for during tumor formation ultimately determines the extent of oncogene independence that will be observed in the resulting tumor. In this model, c-MYC induction in the mammary gland preferentially selects for cells harboring activating *Kras2* mutations during tumorigenesis. *Kras2* activation, in turn, leads to high levels of MAPK pathway activation that ultimately enable tumors to grow in an oncogene-independent manner following MYC downregulation. In contrast, Wnt1 induction preferentially selects for cells harboring activating *Hras1* mutations, which do not result in high levels of ras or MAPK pathway activity. As a consequence, the majority of Wnt1-induced tumors regress to a nonpalpable state following Wnt1 downregulation. This model further predicts that Wnt1 tumors do not become oncogene independent following repeated cycles of Wnt1 induction and deinduction as a consequence of the apparent ability of *Hras1* mutation to preclude the subsequent acquisition of mutations in other *ras* isoforms.

As predicted by this model, a significantly higher fraction of tumors arising in MTB/TOM/K-ras^{LA2} and MTB/TWNT/K-ras^{LA2} tritransgenic mice grew in an oncogene-independent manner compared to tumors arising in MTB/TOM and MTB/TWNT littermates. However, a substantial number of MYC and Wnt1 tumors remained oncogene dependent despite having rearranged and activated the latent K-ras^{LA2} allele. This was unexpected based upon our previous finding that virtually all *Kras2*-mutant tumors arising in MTB/TOM mice grow in an oncogene-independent manner following MYC downregulation. A potential explanation for this differential behavior may be found in our observation that although each of the tumors in tritransgenic animals had rearranged and activated the latent K-ras^{LA2} allele, tumors did not uniformly exhibit high levels of activated ras, Mek, and Erk. Potentially, subtle differences in expression may exist between the rearranged latent K-ras^{LA2} allele and endogenous activated *Kras2* alleles that contribute to their differential biochemical activation of ras. An alternate explanation, however, is that mutation and activation of endogenous *Kras2* may not be sufficient for high levels of ras or MAPK pathway activation. This, in turn, would suggest that alterations that arise nearly universally in MTB/TOM and MTB/TWNT tumors that spontaneously activate endogenous *Kras2* do not occur universally in MYC- and Wnt1-induced mammary tumors bearing a latent K-ras^{LA2} allele.

Consistent with this inference, it has recently been demonstrated that activation of *Kras2* at its endogenous locus is not sufficient to confer high levels of MAPK pathway activation (12, 36). In this regard, reports that wild-type Kras2 may inhibit *Kras2* alleles activated by mutation raise the possibility that loss of wild-type *Kras2* may contribute to tumor progression and MAPK pathway activation in *Kras2*-mutant tumors (10, 41). Conversely, other studies have implicated amplification of the *Kras2* locus in the upregulation of MAPK pathway activity (22). While our results suggest that amplification of the *Kras2* locus does not commonly play a role in ras pathway activation in MYC-induced mammary tumors, they also suggest that alterations in addition to *Kras2* mutation are required for tumor cells to exhibit high levels of ras and MAPK pathway activation.

Notably, the origins of the *Hras1* and *Kras2* mutations found in MYC- and Wnt1-induced mammary tumors remain obscure. One possibility is that MYC or Wnt1 induction results in the outgrowth of rare mammary epithelial cells that harbor spontaneous activating *ras* mutations. With the expression of an additional oncogene, these cells may have a proliferation or survival advantage over cells lacking such mutations. Alterna-

tively, MYC or Wnt1 induction could lead to molecular changes within mammary cells that predispose them to the acquisition of additional oncogenic mutations. For example, MYC dysregulation has been shown to cause genetic instability in several systems (11, 39). Genetically unstable cells that sustain activating mutations in *ras* oncogenes may possess a growth advantage over other cells, eventually producing tumors that harbor *ras* mutations.

Taken in concert, our data demonstrate that the nature of a particular initiating oncogenic event plays a major role in determining the specific secondary genetic alterations that are subsequently selected for during the process of tumorigenesis. These secondary events, in turn, play a major role in determining the likelihood that tumors will progress to oncogene independence. Whether such mechanisms of selection are relevant to human tumorigenesis is as yet unclear. However, the fact that preferentially associated sets of mutations have been identified in human cancers and the fact that a significant number of specific genetic alterations in human breast cancer—including c-*MYC* amplification—have been correlated with aggressive tumor behavior and poor prognosis suggest that these mechanisms of selection are operative during mammary tumorigenesis in both mice and humans. As such, elucidating the genetic events that contribute to the progression of tumors to oncogene independence may provide insights into the processes by which human breast cancers develop and progress to more aggressive states.

ACKNOWLEDGMENTS

This research was supported in part by grants CA92910, CA93719, CA98371, and CA105490 from the National Cancer Institute, grants W81XWH-05-1-0405, DAMD17-03-1-0327 (J.W.J.), and DAMD17-00-1-0397 (R.B.B.) from the U.S. Army Breast Cancer Research Program, and a grant from the Susan G. Komen Breast Cancer Foundation.

We thank C. Sterner and J. Gutnick for assistance with animal husbandry and E. Gunther for contributing mouse tissue and initial observations in synergy between MNU treatment and Wnt1 pathway activation.

REFERENCES

1. **Berger, M. S., G. W. Locher, S. Saurer, W. J. Gullick, M. D. Waterfield, B. Groner, and N. E. Hynes.** 1988. Correlation of c-erbB-2 gene amplification and protein expression in human breast carcinoma with nodal status and nuclear grading. Cancer Res. **48:**1238–1243.
2. **Biscardi, J. S., R. C. Ishizawar, C. M. Silva, and S. J. Parsons.** 2000. Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. Breast Cancer Res. **2:**203–210.
3. **Blancato, J., B. Singh, A. Liu, D. J. Liao, and R. B. Dickson.** 2004. Correlation of amplification and overexpression of the c-myc oncogene in high-grade breast cancer: FISH, in situ hybridisation and immunohistochemical analyses. Br. J. Cancer **90:**1612–1619.
4. **Boxer, R. B., J. W. Jang, L. Sintasath, and L. A. Chodosh.** 2004. Lack of sustained regression of c-MYC-induced mammary adenocarcinomas following brief or prolonged MYC inactivation. Cancer Cell **6:**577–586.
5. **Brown, A. M.** 2001. Wnt signaling in breast cancer: have we come full circle? Breast Cancer Res. **3:**351–355.
6. **Choi, J. A., M. T. Park, C. M. Kang, H. D. Um, S. Bae, K. H. Lee, T. H. Kim, J. H. Kim, C. K. Cho, Y. S. Lee, H. Y. Chung, and S. J. Lee.** 2004. Opposite effects of Ha-Ras and Ki-Ras on radiation-induced apoptosis via differential activation of PI3K/Akt and Rac/p38 mitogen-activated protein kinase signaling pathways. Oncogene **23:**9–20.
7. **Clark, G. J., and C. J. Der.** 1995. Aberrant function of the Ras signal transduction pathway in human breast cancer. Breast Cancer Res. Treat. **35:**133–144.
8. **D'Cruz, C. M., E. J. Gunther, R. B. Boxer, J. L. Hartman, L. Sintasath, S. E. Moody, J. D. Cox, S. I. Ha, G. K. Belka, A. Golant, R. D. Cardiff, and L. A. Chodosh.** 2001. c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous Kras2 mutations. Nat. Med. **7:**235–239.
9. **Deming, S. L., S. J. Nass, R. B. Dickson, and B. J. Trock.** 2000. C-myc amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. Br. J. Cancer **83:**1688–1695.
10. **Devereux, T. R., W. Holliday, C. Anna, N. Ress, J. Roycroft, and R. C. Sills.** 2002. Map kinase activation correlates with K-ras mutation and loss of heterozygosity on chromosome 6 in alveolar bronchiolar carcinomas from B6C3F1 mice exposed to vanadium pentoxide for 2 years. Carcinogenesis **23:**1737–1743.
11. **Felsher, D. W., and J. M. Bishop.** 1999. Transient excess of MYC activity can elicit genomic instability and tumorigenesis. Proc. Natl. Acad. Sci. USA **96:**3940–3944.
12. **Guerra, C., N. Mijimolle, A. Dhawahir, P. Dubus, M. Barradas, M. Serrano, V. Campuzano, and M. Barbacid.** 2003. Tumor induction by an endogenous K-ras oncogene is highly dependent on cellular context. Cancer Cell **4:**111–120.
13. **Gunther, E. J., G. K. Belka, G. B. Wertheim, J. Wang, J. L. Hartman, R. B. Boxer, and L. A. Chodosh.** 2002. A novel doxycycline-inducible system for the transgenic analysis of mammary gland biology. FASEB J. **16:**283–292.
14. **Gunther, E. J., S. E. Moody, G. K. Belka, K. T. Hahn, N. Innocent, K. D. Dugan, R. D. Cardiff, and L. A. Chodosh.** 2003. Impact of p53 loss on reversal and recurrence of conditional Wnt-induced tumorigenesis. Genes Dev. **17:**488–501.
15. **Guzman, R. C., R. C. Osborn, S. M. Swanson, R. Sakthivel, S. I. Hwang, S. Miyamoto, and S. Nandi.** 1992. Incidence of c-Ki-ras activation in N-methyl-N-nitrosourea-induced mammary carcinomas in pituitary-isografted mice. Cancer Res. **52:**5732–5737.
16. **Hwang, S. I., R. C. Guzman, S. M. Swanson, and S. Nandi.** 1996. Hormone dependent and independent mammary tumor development form N-methyl-N-nitrosourea-treated rat mammary epithelial cell xenografts in the nude mouse: multiple pathways and H-ras activation. Cancer Lett. **101:**123–134.
17. **Ibrahim, Y. H., and D. Yee.** 2005 Insulin-like growth factor-I and breast cancer therapy. Clin. Cancer Res. **11:**944s–950s.
18. **Johnson, L., K. Mercer, D. Greenbaum, R. T. Bronson, D. Crowley, D. A. Tuveson, and T. Jacks.** 2001. Somatic activation of the K-ras oncogene causes early onset lung cancer in mice. Nature **410:**1111–1116.
19. **Land, H., L. F. Parada, and R. A. Weinberg.** 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature **304:**596–602.
20. **Liao, D. J., and R. B. Dickson.** 2000. c-Myc in breast cancer. Endocr. Relat. Cancer **7:**143–164.
21. **Lin, S. Y., W. Xia, J. C. Wang, K. Y. Kwong, B. Spohn, Y. Wen, R. G. Pestell, and M. C. Hung.** 2000. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc. Natl. Acad. Sci. USA **97:**4262–4266.
22. **Liu, M. L., F. C. Von Lintig, M. Liyanage, M. A. Shibata, C. L. Jorcyk, T. Ried, G. R. Boss, and J. E. Green.** 1998. Amplification of Ki-ras and elevation of MAP kinase activity during mammary tumor progression in C3(1)/SV40 Tag transgenic mice. Oncogene **17:**2403–2411.
23. **Marquis, S. T., J. V. Rajan, A. Wynshaw-Boris, J. Xu, G. Y. Yin, K. J. Abel, B. L. Weber, and L. A. Chodosh.** 1995. The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues. Nat. Genet. **11:**17–26.
24. **Miyakis, S., G. Sourvinos, and D. A. Spandidos.** 1998. Differential expression and mutation of the ras family genes in human breast cancer. Biochem. Biophys. Res. Commun. **251:**609–612.
25. **Miyamoto, S., S. Sukumar, R. C. Guzman, R. C. Osborn, and S. Nandi.** 1990. Transforming c-Ki-ras mutation is a preneoplastic event in mouse mammary carcinogenesis induced in vitro by *N*-methyl-*N*-nitrosourea. Mol. Cell. Biol. **10:**1593–1599.
26. **Moon, A., M. S. Kim, T. G. Kim, S. H. Kim, H. E. Kim, Y. Q. Chen, and H. R. Kim.** 2000. H-ras, but not N-ras, induces an invasive phenotype in human breast epithelial cells: a role for MMP-2 in the H-ras-induced invasive phenotype. Int. J. Cancer **85:**176–181.
27. **Nusse, R., and H. E. Varmus.** 1982. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell **31:**99–109.
28. **Podsypanina, K., Y. Li, and H. E. Varmus.** 2004. Evolution of somatic mutations in mammary tumors in transgenic mice is influenced by the inherited genotype. BMC Med. **2:**24.
29. **Rochlitz, C. F., G. K. Scott, J. M. Dodson, E. Liu, C. Dollbaum, H. S. Smith, and C. C. Benz.** 1989. Incidence of activating ras oncogene mutations associated with primary and metastatic human breast cancer. Cancer Res. **49:**357–360.
30. **Schlotter, C. M., U. Vogt, U. Bosse, B. Mersch, and K. Wassmann.** 2003. C-myc, not HER-2/neu, can predict recurrence and mortality of patients with node-negative breast cancer. Breast Cancer Res. **5:**R30–R36.
31. **Schondorf, T., A. Andrack, D. Niederacher, A. Scharl, M. Becker, H. Engel, and U. J. Gohring.** 1999. H-ras gene amplification or mutation is not common in human primary breast cancer. Oncol. Rep. **6:**1029–1033.
32. **Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and P. Leder.** 1987. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell **49:**465–475.

33. **Slamon, D. J., G. M. Clark, S. G. Wong, W. J. Levin, A. Ullrich, and W. L. McGuire.** 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science **235:**177–182.
34. **Surmacz, E.** 2000. Function of the IGF-I receptor in breast cancer. J. Mammary Gland Biol. Neoplasia **5:**95–105.
35. **Swanson, S. M., R. C. Guzman, K. Christov, S. Miyamoto, and S. Nandi.** 1994. Pituitary-isografted mice are highly susceptible to MNU-induced mammary carcinogenesis irrespective of the level of alveolar differentiation. Carcinogenesis **15:**1341–1346.
36. **Tuveson, D. A., A. T. Shaw, N. A. Willis, D. P. Silver, E. L. Jackson, S. Chang, K. L. Mercer, R. Grochow, H. Hock, D. Crowley, S. R. Hingorani, T. Zaks, C. King, M. A. Jacobetz, L. Wang, R. T. Bronson, S. H. Orkin, R. A. DePinho, and T. Jacks.** 2004. Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. Cancer Cell **5:**375–387.
37. **Voice, J. K., R. L. Klemke, A. Le, and J. H. Jackson.** 1999. Four human ras homologs differ in their abilities to activate Raf-1, induce transformation, and stimulate cell motility. J. Biol. Chem. **274:**17164–17170.
38. **von Lintig, F. C., A. D. Dreilinger, N. M. Varki, A. M. Wallace, D. E. Casteel, and G. R. Boss.** 2000. Ras activation in human breast cancer. Breast Cancer Res. Treat. **62:**51–62.
39. **Wu, Y., C. A. Renard, F. Apiou, M. Huerre, P. Tiollais, B. Dutrillaux, and M. A. Buendia.** 2002. Recurrent allelic deletions at mouse chromosomes 4 and 14 in Myc-induced liver tumors. Oncogene **21:**1518–1526.
40. **Yan, J., S. Roy, A. Apolloni, A. Lane, and J. F. Hancock.** 1998. Ras isoforms vary in their ability to activate Raf-1 and phosphoinositide 3-kinase. J. Biol. Chem. **273:**24052–24056.
41. **Zhang, Z., Y. Wang, H. G. Vikis, L. Johnson, G. Liu, J. Li, M. W. Anderson, R. C. Sills, H. L. Hong, T. R. Devereux, T. Jacks, K. L. Guan, and M. You.** 2001. Wildtype Kras2 can inhibit lung carcinogenesis in mice. Nat. Genet. **29:**25–33.