Combined Inactivation of MYC and K-Ras Oncogenes Reverses Tumorigenesis in Lung Adenocarcinomas and Lymphomas

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Abstract

Background: Conditional transgenic models have established that tumors require sustained oncogene activation for tumor maintenance, exhibiting the phenomenon known as “oncogene-addiction.” However, most cancers are caused by multiple genetic events making it difficult to determine which oncogenes or combination of oncogenes will be the most effective targets for their treatment.

Methodology/Principal Findings: To examine how the MYC and K-rasG12D oncogenes cooperate for the initiation and maintenance of tumorigenesis, we generated double conditional transgenic tumor models of lung adenocarcinoma and lymphoma. The ability of MYC and K-rasG12D to cooperate for tumorigenesis and the ability of the inactivation of these oncogenes to result in tumor regression depended upon the specific tissue context. MYC-, K-rasG12D-, or MYC/K-rasG12D-induced lymphomas exhibited sustained regression upon the inactivation of either or both oncogenes. However, in marked contrast, MYC-induced lung tumors failed to regress completely upon oncogene inactivation; whereas K-rasG12D-induced lung tumors regressed completely. Importantly, the combined inactivation of both MYC and K-rasG12D resulted more frequently in complete lung tumor regression. To account for the different roles of MYC and K-rasG12D in maintenance of lung tumors, we found that the down-stream mediators of K-rasG12D signaling, Stat3 and Stat5, are dephosphorylated following conditional K-rasG12D but not MYC inactivation. In contrast, Stat3 becomes dephosphorylated in lymphoma cells upon inactivation of MYC and/or K-rasG12D. Interestingly, MYC-induced lung tumors that failed to regress upon MYC inactivation were found to have persistent Stat3 and Stat5 phosphorylation.

Conclusions/Significance: Taken together, our findings point to the importance of the K-Ras and associated down-stream Stat effector pathways in the initiation and maintenance of lymphomas and lung tumors. We suggest that combined targeting of oncogenic pathways is more likely to be effective in the treatment of lung cancers and lymphomas.

Introduction

Cancer is largely caused by the summation of activated oncogenes and inactivated tumor-suppressors that occur in a permissive epigenetic milieu resulting in various pathologic features: autonomous proliferation, immortalization, blocked differentiation, the induction of angiogenesis, capacity for invasion, resistance to apoptosis and genomic instability [1]. Transgenic mouse models have been a valuable means to identify cooperating oncogenic events relevant to human tumorigenesis. A classic example is the forced coexpression of c-myc and v-Ha-ras oncogenes in vivo resulting in a strongly synergistic tumorigenesis phenotype [2]. MYC encodes a transcription factor that regulates the expression of a multitude of genes involved in regulating cellular proliferation and...
growth and when overexpressed results in the prototypical pathologic features of cancer as described above [3,4]. K-ras encodes a low-molecular weight GTP-binding protein responsible for transmitting signals from receptor tyrosine kinases to downstream modulators of cell growth and survival [5,6] and has been shown to stabilize the MYC protein [7]. Thus, MYC and ras cooperate to induce tumorigenesis through multiple mechanisms.

Conditional mouse models allowing temporal control of oncogene expression have become increasingly important for teasing apart the tumorigenesis pathways in adult tissue compartments [8]. Comparison of different transgenic systems would also suggest that tissue type plays a role on the ability of oncogenes to promote tumorigenesis [9,10]. Conditional transgenic tumor models have permitted the investigation of how oncogenes not only initiate but maintain tumorigenesis in different tissue and developmental contexts. Using these models, it has been established that many experimental mouse tumors exhibit the phenomenon of oncogene addiction [11,12], whereby the inactivation of a single oncogene has been shown to be sufficient to induce sustained tumor regression [13–30]. Human tumors also appear to exhibit oncogene addiction [31–33]. Most notably, chronic myelogenous leukemia (CML) [34,35] and gastrointestinal stromal tumor (GIST) are highly sensitive to treatment with the tyrosine kinase inhibitor, imatinib [36].

Since most human cancers are genetically complex and are associated with the activation of more than one oncogene, strategies targeting multiple oncogenes appear to be a logical approach for the treatment of human cancers [1,37,38]. Notably, elegant studies illustrated that breast adenocarcinomas induced by conditional MYC overexpression but that also subsequently develop mutations in K-Ras fail to undergo sustained regression upon MYC inactivation [16,17]. These results suggest that the combined inactivation of both MYC and mutant Ras may be more effective in inducing sustained tumor regression. However, to date it has not been directly examined if the coordinate inactivation of both MYC and mutant Ras would be more effective in inducing sustained tumor regression.

To study how MYC and K-rasG12D cooperate for the initiation and maintenance of tumorigenesis, we have generated double conditional transgenic mouse models of lymphoma and lung adenocarcinoma. MYC- K-rasG12D- or MYC/ K-rasG12D-induced lymphomas exhibited sustained regression upon single or double oncogene inactivation. Interestingly, in contrast to most MYC-induced tumor models, MYC-induced lung tumors were not oncogene-addicted; whereas K-rasG12D inactivation did induce complete tumor regression in K-rasG12D-induced lung tumors. Furthermore, the combined inactivation of MYC and K-rasG12D was associated with reversible lung tumorigenesis. In addition, we observed that downstream K-Ras effector, Stat3, was down-regulated upon oncogene inactivation in lung tumors and lymphomas that regressed. However, non-regressing MYC-induced lung tumors were found to have aberrantly active Stat3 signaling. These data have important implications for treatment strategies where use of multiple targeted agents is being considered and highlight the significance of the K-Ras and Stat pathways for tumorigenesis and tumor maintenance.

Results

MYC inactivation alone fails to induce regression of lung cancer

To examine the role of MYC in the initiation and maintenance of tumorigenesis, transgenic mice were generated that exhibit conditional expression of the human c-MYC oncogene (referred to as MYC from now on) by crossing TetO-MYC transgenic mice [15] with the CCSP-rtTA transgenic line [39] generating CCSP-rtTA/ TetO-MYC mice (now termed CM; see Figure 1A). The CCSP-rtTA mouse line contains the Clara cell secretory protein (CCSP or CC10) promoter which drives expression of the reverse tetracycline transactivating protein (rtTA) in lung Clara cells, alveolar Type II pneumocytes and some other non-iliated bronchial and bronchiolar epithelial cells [23,39]. To verify conditional regulation, CM mice were examined for MYC expression using quantitative real-time polymerase chain reaction (qRT-PCR).

The addition of doxycycline induced expression of MYC transcripts 380-fold in the lung with no appreciable expression in non-induced lung tissue or induced liver (Figure 1B). Similar to previous reports using the CCSP-rtTA line, the kinetics of inactivation revealed background MYC expression by 3-days after doxycycline withdrawal [23,39]. Examination for MYC protein revealed similar robust inducible regulation by western blotting (Figure 1C) and immunohistochemical (IHC) analysis in CM lung tissue (Figure 1D–E). Notably, two target genes of MYC, ornithine decarboxylase (ODC) and nucleolin [3], were found to exhibit expression that was coordinately regulated in a conditional manner as expected from a functional MYC protein (Figure S1). Thus, we have developed a conditional model for the expression of MYC in the lung.

Induction of MYC in the lung epithelium by the administration of doxycycline in the drinking water of CM mice uniformly resulted in tumorigenesis (Figure 2A) that on histologic examination were consistent with adenomas or adenocarcinomas (Figure 2C–D) [40]. Tumors were composed of cuboidal to columnar cells lining alveoli frequently containing vacuolated tumor cells, multiple nuclei and mitoses. Using the consensus classification system as developed by Yu and colleagues, these tumors would be classified as adenoma-mixed subtype (1.2,1.2,3) and adenocarcinoma–NOS (1.2,3,2.5) [40]. Activated tumor cells stained intensely for MYC protein by IHC analysis (Figure 2F) and were TTF-1 positive as expected (data not shown). To enhance detection and allow serial monitoring of lung tumors during growth and following interventions in our study, micro-computed tomography (µCT) was performed on cohorts of mice for the detection of millimeter sized lesions (Figure 2B). CM mice developed tumors with a median latency of 52 weeks as detected by µCT screening usually well before clinical signs developed. CM mice usually developed 1–2 dominant tumors that were located more centrally in the mediastinum (Figure 2B). Thus, MYC induction by the CCSP promoter is sufficient to induce lung adenocarcinomas.

To simulate MYC targeted treatment and evaluate if MYC inactivation was sufficient to reverse lung tumorigenesis, doxycycline treatment was removed to suppress expression of the transgene. Surprisingly, 98% (n = 51) of tumor bearing CM mice did not exhibit complete tumor regression following doxycycline withdrawal as demonstrated by gross examination on necropsy, radiographically and/or histologically (Figure 2E–H). Only 1 out of 8 CM mice demonstrated volumetric tumor regression greater than 60% by radiographic exam following 6 weeks of doxycycline withdrawal (and see below). A trivial explanation for doxycycline-independent tumor viability could be either the aberrant expression of MYC independent of doxycycline or endogenous upregulation of murine c-Myc. To address this possibility qRT-PCR and IHC were performed on tissue from mice in which MYC was inactivated but the tumors had not regressed. No transgene or protein expression of MYC were detected (Figure 2I–K and data not shown, n = 6). Since the anti-MYC antibody used for IHC in our study also cross reacts with murine c-Myc, we
concluded that these tumors also had not upregulated endogenous c-Myc (Figure 2J–K). Taken together these results suggest continuously activated CM mice develop lung tumors that become independent of MYC for tumor maintenance.

**MYC and K-ras**

On the basis of these results, we were surprised to find that CM tumors were independent of MYC, since a multitude of previous studies have demonstrated that MYC-induced tumors exhibit complete tumor regression upon MYC inactivation [13–15,17,21,22]. Studies demonstrated a subset of breast tumors induced by MYC overexpression that fail to undergo sustained regression upon MYC inactivation have mutations in K-Ras [16,17]. To evaluate in our conditional lung model if MYC and K-rasG12D cooperate, we utilized a conditional mutant K-rasG12D line previously described, CCSP-rtTA/TetO-K-rasG12D (now called CR) [23]. CM and CR were then used to produce the bi-conditional animals CCSP-rtTA/TetO- MYC/TetO-K-rasG12D (or CMR), which upon doxycycline administration simultaneously overexpress both oncogenes under the control of the CCSP promoter in lung and as described below in lung tumors (Figure 3A). At 3–4 weeks of age cohorts of CM, CR and CMR mice were treated with doxycycline and screened using physical exam and microcomputed tomography (µCT) screening. As described above, CM mice developed lung tumors with a median latency of 52 weeks (Figure 3A). Upon doxycycline treatment, CR mice developed lung adenocarcinomas with a median latency of 26 weeks. Surprisingly, CMR mice developed lung adenomas and adenocarcinomas (Figure 4A) with a latency of 36 weeks similar to the CR mice (Figure 3A; not significantly different by log-rank analysis, $p>0.05$). Thus, in the setting of adult lung epithelium MYC and K-rasG12D failed to cooperate to induce accelerated tumorigenesis.

We were surprised that the conditional MYC and K-rasG12D oncogenes did not cooperate to induce lung tumorigenesis. To evaluate if these transgenes would cooperate in another tissue setting, we induced expression of either oncogene alone or together in lymphocytes utilizing an Em-SR-tTA line (data not shown). In contrast to what we observed in the lung, we found that MYC (LM) was a much more potent oncogene than K-rasG12D (LR) at inducing lymphomas with a median latency of tumor onset of 13 weeks versus more than 100 weeks (Figure 3B; $p<0.0001$ by log rank). Moreover, we found that MYC and K-rasG12D cooperate to induce tumorigenesis with a reduced median latency of 5 weeks (all curves different, $p<0.0001$ by log rank). Thus, MYC and K-rasG12D cooperate to induce lymphoma but not lung adenocarcinoma.

**Combined MYC/K-ras**

Inactivation induces complete tumor regression

We speculated that in our MYC-induced lung tumors, activation of the Ras signaling pathway may provide a means to bypass the requirement for MYC, as has been previously suggested [16,17]. To directly test this hypothesis, we simulated double targeted treatment of MYC and K-rasG12D using dual conditional CMR tumor laden mice by inactivating both oncogenes (Figure 4B–F) and then comparing similarly to the single CM and CR mice. Serial µCT imaging was performed on cohorts of...
CR, CM and CMR lung tumor bearing mice prior to and following oncogene-inactivation was performed (Figure 5A). CR mouse tumors demonstrated complete lung tumor regression following oncogene inactivation, as has been described previously (n = 11; Figure 5A–B) [23]. In marked contrast, CM tumors failed to regress completely, as described above (n = 8; Figure 5A–B). Tumor bearing CMR mice on the whole exhibited tumor regression intermediate to that of the CR and CM mice following dual oncogene-inactivation of MYC and K-rasG12D oncogenes (n = 10; Figure 5A–B). However as predicted, 40% of the individual CMR-induced lung tumors analyzed showed complete tumor regression equal to those from CR mice following doxycycline withdrawal (Figure 5B). By qRT-PCR and IHC, we confirmed that MYC, transgenic K-rasG12D and endogenous murine MYC protein were not expressed in the inactivated CMR lung tumors and CMR tumors were indeed inactivated for both oncogenes (Figure 4B–F). Altogether, these data suggest that activation of the K-Ras signaling pathway is an essential rate-limiting event during lung tumorigenesis. Moreover, our observation that the combined inactivation of both MYC and K-rasG12D induced lung tumor regression more effectively suggests that the K-Ras pathway may be an important target for the treatment of lung cancer.

From these results, we speculated that K-Ras may be mutated in MYC-induced lung tumors, as has been observed in MYC-induced breast tumors [16,17,20]. To address this possibility, we sequenced three MYC-inactivated CM tumors (or derived cell lines) for mutations in K-Ras but were unable to detect any hotspot activating mutations (Figure S3). Therefore, activating mutations of K-ras were not a common occurrence and was not an explanation for MYC independence in our lung model, in contrast

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to what has been previously reported for breast cancer [16,17,20]. Other components of the K-Ras effector pathway were obvious next candidates and were investigated as described below.

Notably, in the lymphoma model, inactivation of MYC (LM Off), K-rasG12D (LR Off) or MYC/K-rasG12D together (LMR Off) were each able to induce complete regression of lymphomas and extend tumor free survival (Figure 5C; no difference by log rank analysis, \(p<0.05\)). For lymphomas, inactivation of MYC or K-rasG12D alone were fed water and never developed lung tumors \((n=8)\). Tumor Free Survival was scored by serial CT imaging of animals following addition of doxycycline at 3–4 weeks of age. (B) Kaplan-Meier analysis of Tumor Free Survival for oncogene-induced lymphomagenesis. MYC-induced lymphomas \(\text{(LM, } n=26\) arose with a median latency of 13 weeks after conditional oncogene activation. In contrast, less than half of the mice developed lymphoma after 100 weeks of conditional K-rasG12D activation \((LR, n=25)\). The double conditional oncogene animals \(\text{(LMR, } n=22\) had a median latency that was significantly different than the single CR animals by log-rank analysis suggesting that K-rasG12D was epistatic to MYC for lung tumorigenesis. A syngenic control cohort consisting of wildtype mice, those with MYC/K-rasG12D \(\text{(without CCSP), CCSP alone, or K-rasG12D alone were fed water and never developed lung tumors \((n=6)\). Tumor Free Survival was scored when animals were moribund with tumor.

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Figure 3. Cooperation during tumorigenesis by conditional MYC and K-rasG12D oncogenes. (A) Kaplan-Meier analysis of Tumor Free Survival for oncogene-induced lung tumorigenesis. Single MYC- \((\text{CM, } n=51)\) and K-rasG12D- \((\text{CR, } n=41)\) lung tumors arose with a median latency of 52 and 26 weeks, respectively, after conditional oncogene activation. The double conditional oncogene animals \(\text{(CMR, } n=25\) had a median latency that were no different than the single CR animals by log-rank analysis suggesting that K-rasG12D was epistatic to MYC for lung tumorigenesis. A syngenic control cohort consisting of wildtype mice, those with MYC/K-rasG12D \(\text{(without CCSP), CCSP alone, or K-rasG12D alone were fed water and never developed lung tumors \((n=6)\). Tumor Free Survival was scored when animals were moribund with tumor.

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Tumor regression is associated with the dephosphorylation of Stat3

Our results suggested that the K-Ras signaling pathway is crucial for both the initiation and maintenance of lung tumorigenesis. Important upstream and downstream regulators of the K-Ras pathway are the epidermal growth factor receptor (EGFR) and Erk1/2, Akt1 and Stat3/5, respectively [27,41–50]. The upstream effector, EGFR, was not found to be phosphorylated in any of our MYC- or K-rasG12D- induced lung tumors by IHC (data not shown) and as described previously for K-Ras-induced lung tumors [27]. As expected, we observed phosphorylation of Erk1/2 in K-rasG12D-induced tumors decrease upon K-rasG12D inactivation \((CR; \text{Figure 6A})\). However, there was no evidence for phosphorylated-Erk1/2 staining by IHC in MYC-induced lung tumors. Analogously we observed phospho-Akt1 staining in the CR lung tumors decrease upon K-rasG12D inactivation \((Figure 6B)\). We observed only minimal changes in the phosphorylation of Akt1 in CM lung tumors upon inactivation of MYC. K-rasG12D-induced lung tumors exhibited robust conditional Stat3 and Stat5 phosphorylation that was dependent on K-rasG12D activation \((Figure 6C–D)\). In contrast, MYC-induced tumors had cells that stained highly positive for both phospho-Stat3 and phospho-Stat5, and a large proportion of highly positive phospho-Stat5 and phospho-Stat3 cells remained after MYC inactivation \((Figure 6C–D)\).

Surprisingly, whereas single K-rasG12D-induced lung tumors exhibited a high degree of Stat5 phosphorylation, dual MYC/K-rasG12D-induced lung tumors did not \((compare \text{Figures 6C and 7A)\). CMR lung tumors did show a high degree of Stat3 phosphorylation that decreased upon simultaneous inactivation of both MYC/K-rasG12D in persistent lung tumors \((Figure 7B)\). Thus, K-rasG12D- or dual MYC/K-rasG12D-initiated lung tumors demonstrated a decrease in Stat3 phosphorylation upon oncogene inactivation that was associated with reversible tumorigenesis.

Next, we examined the consequences of MYC and/or K-rasG12D inactivation in lymphoma. LM, LR, and LMR lymphoma cells all exhibited phosphorylation of Stat3 that decreased upon inactivation of MYC and/or K-rasG12D \((Figure 6A)\). In contrast, despite the fact that LM, LR, and LMR lymphomas all regress upon MYC and/or K-rasG12D inactivation, phospho-Stat5 decreased upon MYC, but not K-rasG12D- or dual MYC/K-rasG12D inactivation. Collectively our results illustrate that for both MYC/K-rasG12D-induced lung tumors and lymphomas dephosphorylation of Stat3 is correlated with the ability of oncogene inactivation to induce tumor regression.

Discussion

Targeting single oncogenes is not likely to be effective in all cases for the treatment of human cancers [38,51]. Murine models provide a preclinical strategy to identify which combination of oncogenes are most likely to be effective [30,52]. To our knowledge, our study is the first to examine experimentally using conditional transgenic model systems if the combined inactivation of two oncogenes is more likely to be effective in the treatment of cancer in situ. Using our models we interrogate the role of MYC and K-rasG12D alone or in combination for the initiation and maintenance of lung and hematopoietic tumorigenesis. The inactivation of K-rasG12D but not MYC could induce complete tumor regression in lung adenocarcinomas; whereas in marked contrast, single K-rasG12D- or MYC-inactivation both succeeded in inducing sustained regression in lymphomas. However, the combined inactivation of both K-rasG12D and MYC was capable

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**Dual-Oncogene Inhibition Model**

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of inducing complete regression in both lung tumors and lymphomas.

Our data highlight two important considerations in targeted therapeutics: first, initiation of tumorigenesis by a specific oncogene does not mean inactivation of that specific oncogene will be sufficient to induce tumor regression; and second, that the consequences of the inactivation of a particular oncogene are pointedly dependent on tissue context. Specifically, we have demonstrated that the K-Ras pathway and its down-stream effector Stat3 are correlated with the ability of K-ras\textsuperscript{G12D} or MYC to initiate lung tumorigenesis and that down regulation of the K-Ras/Stat pathway is strongly correlated with lung tumor and lymphoma

Figure 4. Conditional expression of MYC/K-ras\textsuperscript{G12D} in the lung predisposes to bronchiogenic adenocarcinomas. (A) Double MYC/K-ras\textsuperscript{G12D} (CMR)-induced tumors have histology consistent with adenomas/adenocarcinomas similar to MYC- and K-ras\textsuperscript{G12D}-induced tumors on H&E. (B) To rule out the possibility that the double oncogene-induced lung tumors had developed doxycycline (or TetO)-dysregulated MYC or K-ras\textsuperscript{G12D} expression, inactivated double oncogene-induced lung tumors were examined for spurious expression of MYC and K-ras\textsuperscript{G12D} at the mRNA and/or protein level. qRT-PCR analysis of double oncogene-induced lung tumors from CMR mice that had been inactivated (doxycycline removed from drinking water) for 2–9 weeks demonstrated lack of expression of the MYC transgene in contrast to a MYC-induced tumor that had never been inactivated. Immunohistochemical analysis (performed like Figure 1D) on similar (C) inactivated double oncogene-induced tumors also showed lack of MYC transgene product and endogenous murine MYC protein compared to a (D) MYC-activated tumor. qRT-PCR analysis of double oncogene-induced lung tumors from CMR mice that had been inactivated demonstrated no expression of the (E) K-ras\textsuperscript{G12D} transgene or upregulation of (F) endogenous murine K-ras (\( \geq 3–9 \) tumors for \( \geq 3 \) mice per experiment).

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regression. We conclude that the K-Ras/Stat3 pathways have a dominant role in the initiation and maintenance of lung tumors.

Our experimental model system re-examines the classic experiments first demonstrating the cooperation between c-myc and v-Ha-ras for malignant transformation in vivo [2]. Identical to previous results using conventional transgenic models [2], MYC and K-rasG12D cooperated to induce tumorigenesis in lymphocytes (compare LM, LR and LMR mice; Figure 3B). In contrast, MYC

Figure 5. Regression of tumors following dual MYC/K-rasG12D-oncogene inactivation. (A) Representative serial mCT images of single and double-oncogene-induced lung tumors following withdrawal of doxycycline for 6 weeks. CR (CCSP-rtTA/TetO-K-rasG12D, n = 11) animals demonstrated rapid tumor regression within ~2 weeks following inactivation of the oncogene. In contrast, CM (CCSP-rtTA/TetO-c-MYC, n = 8) mice did not demonstrate full tumor regression even after 6 weeks following oncogene inactivation. Interestingly, CMR (CCSP-rtTA/TetO-c-MYC/TetO-K-rasG12D, n = 10) animals on a whole exhibited an intermediate level of tumor regression, with some tumors regressing completely, compared to the single oncogene-induced lung tumors. (B) Left panel shows the mean with standard deviation of normalized tumor volumes from (A) at 6 weeks following oncogene-inactivation. The relative genotype order for mean tumor regression: CR (n = 11)>CMR (n = 8)>CM (n = 10); all pair-wise comparisons were p<0.0013. The right panel demonstrates same data as the left panel but in a scatter plot form with the mean denoted as a horizontal line. (C) Kaplan-Meir analysis of tumor-free survival of conditional lymphoma mice. Oncogene inactivation in MYC-induced lymphoma resulted in sustained regression in more than half the mice (LM OFF, n = 15). Oncogene inactivation in mice with K-rasG12D-induced lymphoma resulted in tumor regression and increased median survival by 12 weeks (LR OFF, n = 5). Inactivating both MYC/K-rasG12D together also resulted in tumor regression and increased median survival by 5 weeks. There is no significant difference between inactivating MYC/K-rasG12D together vs K-rasG12D alone (log rank analysis p = 0.4849). Relapse free survival was scored when mice were moribund with tumor burden.

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failed to cooperate with K-rasG12D to induce lung adenocarcinomas (compare CM, CR and CMR mice; Figure 3A). Thus, whether or not MYC and K-rasG12D functionally cooperate to activate critical tumor promoting pathways appears to depend upon the specific tissue context. In lung adenocarcinomas induced by MYC and/or K-rasG12D, tumors exhibited activation of the K-Ras/Stat3 signaling pathway. Apparently, MYC activation is not capable of initiating lung tumorigenesis without activation of the mediators of the K-Ras/Stat3 pathway, perhaps accounting for why MYC does not appear to cooperate with K-rasG12D to induce lung tumorigenesis. For lung tumorigenesis, there must be an essential role for activation of the K-Ras pathway or downstream mediators such as the Stat pathway. Similarly, the combined inactivation of both the MYC and K-Ras pathways in these breast tumor models will also result in complete tumor regression.

In our lung tumor model system other genes are likely to be somatically activated in the EGFR/BRAF/KRAS pathway or parallel pathways that may also contribute to the escape from the requirement of MYC expression. This is evidenced in our study by the inactivated CM (Figure 6) and CMR (Figure 7) lung tumors that did not demonstrate aberrant signaling in any of the pathways we examined. Possible candidates to undergo such mutations include a multitude of gene products described in studies of human lung tumors [53–62], some of these studies have implicated the EGFR/IL-6/Stat3 pathway in the pathogenesis of lung adenocarcinomas [43,49,50,61–63]. Stat3 and Stat5 transcription factors have been widely implicated in the pathogenesis of tumors [64,65] and are known to be downstream targets of K-Ras [27,44,48–50]. Phosphorylation of Stat3/5 was found in the majority of our inactivated MYC-induced lung tumors, as evidenced by elevated phosphorylation and nuclear localization by IHC (Figure 6C–D). Consistent with a role of Stat3/5 in oncogene-addiction, phosphorylation of Stat3/5 has been shown to diminish in tumor cells undergoing apoptosis upon oncogene inactivation in vitro [41,42]. Phosphorylated Stat3/5 appears to be particularly important for survival of human lung adenocarcinoma

![Figure 6. Persistent activation of downstream Ras signaling pathways after MYC inactivation.](image-url)
cells harboring certain EGFR mutations [43,49,62]. Similar to the lung cancer mouse models described above, in these EGFR mutated lung cancers behave in an oncogene-addicted fashion following treatment with EGFR tyrosine kinase inhibitors [33,63].

Our observations illustrate that the combined inactivation of multiple oncogenes is more likely to be effective to treat some cancers [30,38,51,52]. The potential of targeting multiple oncogenic pathways in the treatment of human cancer has recently been illustrated in brain tumor lines in vitro [38]. The identification of the best gene products to therapeutically target in cancers is very likely to be much more complicated than simply identifying the genes mutated in a given tumor, as has recently been illustrated in human lung cancer patients who become resistant to tyrosine kinase inhibitors [37,51]. In this work, we illustrate that even the knowledge of the oncogene that initiated tumorigenesis is not necessarily sufficient to identify a gene

Figure 7. Combined inactivation of MYC and K-rasG12D in lung tumor cells results in a shutdown of Stat3 signaling. (A) Representative phospho-Stat5 and (B) phospho-Stat3 IHC analysis demonstrates little to no levels of nuclear staining in cells following dual inactivation of MYC/K-rasG12D (n = 3 “On” & 6 “Off”) similar to conditional K-rasG12D-induced tumors. MYC/K-ras #2-3 represent independent inactivated tumors with no to highest amount of staining observed, respectively. IHC was performed similar to Figure 1D with stated antibodies with CMR-induced lung tumors that were activated or inactivated (2–11 weeks). Adjacent bar graph panels represent scoring of individual tumors for IHC staining: negative, low (<50% positive cells) or high (≥50% positive cells) for phospho-Stat5 and phospho-Stat3 positive tumors.

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Figure 8. The inactivation of MYC and/or K-rasG12D in lymphoma is associated with the dephosphorylation of Stat3. Oncogene inactivation in lymphoma demonstrates decrease in Stat3 signaling. (A) LM lymphoma cells show decreased phospho-Stat 5 staining by flow cytometry analysis upon oncogene inactivation, while LR and LMR lymphoma cells do not. (B) LM, LR and LMR lymphoma cells show decreased phospho-Stat 3 staining by flow cytometry analysis following oncogene inactivation.

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product whose inactivation will result in tumor regression. The generation of transgenic mice with multiple conditional oncogenes is a tractable preclinical platform to define the combination of oncogenic targets most likely to be effective in the treatment of cancer.

Materials and Methods

Transgenic mice

The TetO-c-MYC and CCSP-rtTa transgenic lines generated for these experiments was described previously [15]. The Egr-1, and K-ras4bG12D transgenic lines were kindly provided by H. Bujard (University of Heidelberg, Germany), and H. Varmus (Memorial Sloan-Kettering Cancer Center, New York), respectively. Mice were mated and screened by PCR as described previously [23]. MYC and/or K-rasG12D expression was activated in the CM, CR, and CMR lung lines by administering doxycycline (Sigma) to the drinking water weekly [100 mg/mL] starting at the age of 3-4 weeks. All procedures were performed in accordance with APLAC protocols and animals were housed in a pathogen-free environment.

Oncogene Inactivation

Lung mice were followed by micro-computed tomography (microCT) scans for a total of 16 weeks. Serial microCT scans were performed at −10, −6, −2, 0, 2 and 6 weeks relative to oncogene inactivation occurring at time point “0”. Oncogenes were inactivated in the CM, CR and CMR cohorts in week 10 by removing doxycycline from the animals' drinking water. Oncogenes were inactivated in the LM, LR and LMR cohorts by injecting mice with 100 μg of doxycycline in PBS IP and adding doxycycline [100 μg/mL] to the drinking water weekly.

PCR genotyping

DNA was isolated from mouse tails using the Qiagen DNeasy kit (Qiagen) in accordance with the manufacturer’s directions. The CCSP-rtTA segment was detected using the following primers: CCSP-F 5′-ACTGGCCATTGCCAAACAC-3′ and CCSP-R 5′-AAAATCTTGGCAGCTTTCCC-3′ (yielding a 440 bp product). The TetO-Myc construct was detected with the following primers: Myc-F 5′-TAGTGAACCGTCAGATCGCCTG-3′ and Myc-R 5′-CTGTGCTTCTGCTTTTCG-3′ (yielding a 450 bp product). TetO-K-rasG12D and Egr-Sr-rtTA were screened as described previously [23]. DNA was amplified using the following PCR protocol: 94°C denaturation for 2 minutes followed by 35 cycles of 94°C for 15 seconds, 59°C annealing for 30 seconds, and 72°C for 30 seconds, followed by a 5 minute extension at 72°C. PCR products were resolved on a 1.5% gel.

SYBR-green quantitative RT-PCR and RT-PCR

Total RNA was isolated from tissue using the Stratagene RNase-Free kit (Promega) and RT-PCR was performed using Superscript One-Step RT-PCR (Life Technologies) for 35 cycles with an annealing temperature of 57°C with 0.25 μg of total RNA per sample. Control reactions were run using Taq polymerase without RT enzyme (Perkin Elmer). cDNA was generated from 1 μg of total RNA using the Superscript II kit (Invitrogen Technologies). 50 μg of cDNA equivalents were amplified for the transcript described below in an ABI-prism 7700 (Perkin Elmer Applied Biosystems) for 40 cycles using SYBR green PCR Master mix (Perkin Elmer Applied Biosystems) according to manufacturer’s directions. PCR reactions were performed in at least triplicate in a final volume of 20 μL. Thermal cycling conditions were: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 57°C for 30 seconds, 72°C for 30 seconds, and a dissociation stage of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. Following amplification, the data was processed with the analysis program Gene Amp System 7700 Software (Applied Biosystems). For each sample, the level of RNA for the genes of interest was standardized to the level of ubiquitin within that sample; subsequently, the level of a transcript of interest was normalized to the expression of that transcript in wildtype lungs. Primers for qRT-PCR were the following: transgenic K-ras exon 4b (K-ras4b-fw 5′-CAAGGCAAGGTTGACAGTTTGACTG-3′ and downstream primer mp-1 pA (mp-1-real time-rev 5′-GGCATCTGCTCTGCTCAG-3′; and endogenous K-ras4b 3UTR (K-ras4b-UTR-fw 5′-GCAGGGTGTTGGGCTCTTACAT-3′ and K-ras4b-UTR rev 5′-ATGCGTCCGCACATTGAAT-3′); MYC (forward 5′-ACCAGATCAGGATGTGGA-3′ and (MYC reverse 5′-CGTGCTTTCCGCCAACAAGTGC-3′); ornithine decarboxylase (ODC) (ODC forward 5′-CTGTGCTCTGCTAGGATCAATGT-3′) and (ODC reverse 5′-GGCTTAAACAAGCTAAACTTGGCA-3′); and (MYC reverse 5′-GGAGGCAATGGAGATGAGG-3′) and (nucleolin forward 5′-GGAGGCAATGGAGATGAGG-3′) and (nucleolin reverse 5′-CACCCTCCTGCCGAACACT-3′) and (ubiquitin (ubiquitin forward 5′-AGCCCCAGTGTACCACCAGA-3′) and ubiquitin reverse 5′-ACCCAAAGACAGGCAACAGG-3′).

Histology and Immunohistochemistry

Tissues were fixed in 10% buffered formalin for 24 h and then transferred to 70% ethanol until embedding in paraffin. Tissue sections 5 μm thick were cut from paraffin embedded blocks, placed on glass slides and hematoxylin and eosin (H&E) staining was performed using standard procedures (Stanford Histology Core). Antibodies used in our study: c-Myc (C19) (Santa Cruz Biotech.), phospho-AKT-S497 (Cell Signaling Tech.), phospho-EGFR-Y1173 (Cell Signaling Tech.), phospho-Erk-1/2-T202/Y204 (Cell Signaling Tech.), phospho-Stat3-Y705 (Cell Signaling Tech.) and phospho-Stat5-Y694 (Cell Signaling Tech.). Samples were dewaxed in xylene and rehydrated in a graded series of ethanol. Antigen retrieval for c-Myc, phospho-AKT and phospho-EGFR were performed by 14 min microwave irradiation in citrate-based Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA, USA). Antigen retrieval for phospho-Stat3 and -Stat5 were performed by 14 min microwave irradiation in EDTA, pH 8.0, and antigen retrieval for phospho-Erk1/2 was performed by 10 min incubation in Pronase (Roche, Basel, Switzerland). Endogenous peroxidases were blocked in either 3% hydrogen peroxide in deionized water (phospho-AKT, -pErk, -EGFR and -pStat3/5) or 0.3% hydrogen peroxide in methanol (c-Myc) for 10–20 minutes. Non-specific binding was blocked with 5–10% goat serum for 60 minutes. Primary antibodies were used at appropriate dilutions (c-Myc, phospho-AKT, and -pErk at 1:100; phospho-Stat3 at 1:200; and phospho-Stat3 and -EGFR at 1:50) and sections incubated overnight at 4 degrees Celsius. Detection was conducted using the Vector Elite ABC detection kit (Vector Laboratories) with 3,3′-diaminobenzidine tetrahydrochloride as the chromogen. Sections were counterstained with Gill’s hematoxylin (Vector Laboratories).

Western blots analysis

Western analysis was performed using conventional techniques [66]. Tissues were disrupted and protein was isolated using a pestle and tube homogenizer in NP-40 lysis buffer. Equal protein was loaded in each lane, as quantitated by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, Illinois, United States). Proteins
were electrophoresed on 10% Tris-HCl polyacrylamide gels at 100 V for 60 min and transferred on PVDF membranes at 100 V for 60 min. Blotting was then performed as directed by the antibody manufacturer. MYC protein expression was detected using the 9E10 antibody that recognizes human MYC (Santa Cruz Biotech).

**Computed Tomography**

Micro-computed tomography (μCT) scans were performed on a custom GEHC (London, Ontario) RS150 cone-beam scanner, which uses a fixed anode with tungsten target source. Animals were anesthetized with 2% isoflurane in a nitrogen/oxygen mixture. Scans were performed at 97 μm resolution, using a 70 kV (40 mA) beam to acquire images at 286 radial views over 200 degrees around the subject. Four frames were exposed and averaged in each position. Data were corrected using the GEHC reconstruction utility and volumes generated using the same application, which were viewed using the GEHC Microview software.

**Tumor Volume Measurements**

We used the open source application, ITK-Snap, for segmentation of the lung nodules in three-dimensions [67]. The post-processing of the segmented data provides the voxel counts and the volume (cubic millimeters) and displays the shape of the segmented structure. We calculated the volume of individual lung tumor nodules right before and 6 weeks following oncogene-inactivation. Volumes at 6 weeks were normalized relative to the volume before oncogene-inactivation and all values for a given genotype were averaged: CR, n = 11; CM, n = 8; and CMR, n = 10.

**Intracellular phospho-protein detection using flow cytometry**

LM, LR, and LMR- lymphoma derived cell lines were treated with doxycyline \( \alpha \text{rito} \). 1 million cells from each condition were fixed for 10 minutes in 1.6% paraformaldehyde at 37°C, permeabilized for 10 minutes in 100% methanol at room temperature, washed twice with PBS 1% BSA, then stained with 10 ul anti-Phospho-Stat 5: Alexa-488 (BD Biosciences Pharmingen) or 10ul anti-Phospho-Stat 3: Alexa-488 (BD Biosciences Pharmingen) in 100 ul PBS 1% BSA, incubated for 30 minutes in the dark at room temperature. Finally, samples were washed once with PBS 1% BSA and then analyzed using a benchtop FACSCAN (Becton-Dickinson) flow cytometer. 10,000 un gated events were collected per primary tumor and one derived cell line transplanted into SCID mice were assayed for mutations in exon one hotspots, codon 12 and 13. (C) Similar samples assayed for exon 2 hotspot codon 61.

**K-Ras mutation analysis**

Total genomic DNA was harvested using DNeasy Blood & Tissue Kit (Qiagen) as directed by the manufacturer and PCR amplified using primers specific for K-Ras. PCR products were purified with a QIAquick column (Qiagen) as directed by the manufacturer and sequenced to detect point mutations at codons 12, 13, and 61.

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**Author Contributions**

Conceived and designed the experiments: DF PB PT AF SG. Performed the experiments: PB PT AF SK KK JC GH SG CW. Analyzed the data: DF PB PT AF SK KK JC GH SG CW. Wrote the paper: DF PB PT AF.

**Statistics**

Survival graphs were generated by the product limit method of Kaplan and Meier and log-rank analysis was utilized for differences between proportions. Pair-wise and multiple comparisons were made using Mann-Whitney and Kruskal-Wallis nonparametric tests, respectively. Analysis was facilitated using Prism v5.0 by GraphPad.
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