Cell

KRAS and YAP1 Converge to Regulate EMT and Tumor Survival

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 http://dx.doi.org/10.1016/j.cell.2014.06.004

SUMMARY

Cancer cells that express oncogenic alleles of *RAS* typically require sustained expression of the mutant allele for survival, but the molecular basis of this oncogene dependency remains incompletely understood. To identify genes that can functionally substitute for oncogenic RAS, we systematically expressed 15,294 open reading frames in a human KRAS-dependent colon cancer cell line engineered to express an inducible *KRAS*-specific shRNA. We found 147 genes that promoted survival upon KRAS suppression. In particular, the transcriptional coactivator YAP1 rescued cell viability in KRAS-dependent cells upon suppression of KRAS and was required for KRAS-induced cell transformation. Acquired resistance to Kras suppression in a *Kras*-driven murine lung cancer model also involved increased YAP1 signaling. KRAS and YAP1 converge on the transcription factor FOS and activate a transcriptional program involved in regulating the epithelial-mesenchymal transition (EMT). Together, these findings implicate transcriptional regulation of EMT by YAP1 as a significant component of oncogenic RAS signaling.

INTRODUCTION

Mutation of proto-oncogenes, such as *KRAS*, *BRAF*, and *EGFR*, induces a state in which cancers are dependent on signaling from the oncogene for survival (Sharma and Settleman, 2007). Although the mechanisms that lead to this oncogene addiction remain poorly understood, pharmacologic inhibition of such oncogenes results in clinical responses. Furthermore, tumors that are resistant to these therapeutic interventions often exhibit reactivation of the signaling pathways regulated by these onco-

genes. For example, EGFR-addicted cancers that relapse have been found to harbor *MET* copy-number amplification or *KRAS* mutation, which mediate resistance by activating the same downstream effector pathways independent of EGFR (Jänne et al., 2009).

Activating mutations of the *KRAS* proto-oncogene occur in a substantial fraction of pancreatic, lung, and colon cancers (Lau and Haigis, 2009). Oncogenic KRAS activates pleiotropic signaling pathways that contribute to tumor initiation and maintenance, including the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and Ral guanine nucleotide exchange factor (RalGEF) signaling pathways (Pylayeva-Gupta et al., 2011). Suppression or inhibition of these pathways prevents tumor initiation and slows the growth of established tumors (Ehrenreiter et al., 2009; González-García et al., 2005; Gupta et al., 2007). One consequence of mutant KRAS signaling is aberrant activation of the AP-1 family transcription factors, which promote responses to mitogenic signaling (Karin, 1995). Specifically, KRAS increases FOS and JUN activation through MAPK-dependent and -independent mechanisms (Deng and Karin, 1994).

YAP1 is a transcriptional coactivator that participates in several context-dependent transcriptional programs that regulate organ size and promote cell proliferation (Wang et al., 2009). Recurrent *YAP1* amplifications are observed in hepatocellular cancers, in which it is an essential oncogene (Zender et al., 2006). In addition, YAP1 is also implicated in the epithelial-to-mesenchymal transition (EMT) and the metastatic potential of mammary epithelial cells (Lamar et al., 2012; Overholtzer et al., 2006). YAP1 serves as an effector of the Hippo (Hpo) kinase cascade and regulates the transcriptional enhancer activator domain (TEAD) transcription factors (Pan, 2010). Serine phosphorylation of YAP1 by both Hpo-dependent and -independent factors inhibits YAP1 entry into the nucleus, preventing subsequent activation of not only TEAD but of other YAP1 transcriptional partners such as SMAD, RUNX, TBX5, and the ERBB4 internal cytoplasmic fragment (Wang et al., 2009). One Hpo-independent mechanism implicated in cancer involves phosphorylation of YAP1 at tyrosine-357 by YES1 to

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promote YAP1 interaction with β-catenin and modulation of Wnt signaling (Rosenbluh et al., 2012). These observations suggest that YAP1 interacts with specific transcription factors in particular contexts to promote cell proliferation, organ growth, or survival.

Identification of genes that promote resistance to targeted therapies can provide insight into signaling mechanisms activated by particular oncogenes. Here, we applied a similar concept to systematically probe pathways required for cancer cell lines that harbor and are dependent on oncogenic KRAS. Specifically, we performed a genetic screen to identify open reading frames (ORFs) that are able to sustain the survival of KRAS-dependent cancer cell lines in the setting of KRAS suppression.

RESULTS

Systematic Identification of Genes that Rescue the Loss of Oncogenic KRAS Expression

We performed a genome-scale genetic rescue screen to identify genes that support the survival of KRAS-dependent cancer cells upon suppression of KRAS. We generated a cell line for screening by stably introducing a doxycycline-inducible shRNA targeting the KRAS 3′ untranslated region (UTR) into the HCT116 KRAS mutant colon cancer cell line (HCTtetK) and introduced 15,294 ORFs from the Center for Cancer Systems Biology (CCSB)/Broad Institute ORF library (Yang et al., 2011) into these cells in an arrayed format in triplicate under optimized conditions in which each well was transduced at high efficiency (98%, Table S1 available online). We induced suppression of KRAS by doxycycline treatment and assessed cell proliferation/survival (Figure 1A). As a control, we expressed a mutant $KRAS^{G13D}$ ORF, which lacks the KRAS 3′UTR and thus cannot be suppressed (Figure 1B). We considered an ORF a "hit" if it obtained a KRAS rescue score greater than 3, i.e., the viability in that well was at least three standard deviations above the mean of negative controls. All of the 150 $KRAS^{G13D}$-expressing wells scored above this threshold, and only 1 of the 1,119 negative control wells (0.05%) scored.

We identified 147 genes that met this criterion (Table S1). The highest-scoring candidates included sterile α motif (SAM) proteins that function as posttranscriptional regulators (Baez and Boccaccio, 2005), the WW-domain-binding proteins YAP1 and WWTR1, and members of the FGF family (Figure 1C). In a separate screen focused on 597 kinases (CCSB/Broad Kinase ORF Collection), we also identified FGFR1 as a kinase that was able to rescue KRAS suppression (Figure S1A).

We then assessed the ability of each ORF to activate MAPK or PI3K signaling. Specifically, we expressed the 147 ORFs in HCTtetK cells in an arrayed format and quantified the activity of the MAPK and PI3K pathways by measuring the ratio of phospho-ERK to total-ERK levels and the ratio of phospho-S6 ribosomal protein to total S6 ribosomal protein levels, respectively (Figure 1D and Table S2). We found that 55.1% of the candidates activated at least one of the two pathways (16.1% MAPK only, 13.4% PI3K only, and 25.6% both pathways). A number of candidate genes failed to activate either pathway, suggesting that MEK- and PI3K-independent mechanisms may also play a

role in KRAS-dependent tumors. The observation that a large proportion of these candidates indeed activated KRAS effector pathways increased our confidence in the biological relevance of the rescue screen.

YAP1 Substitutes for Oncogenic KRAS in KRAS-Dependent Cancer Cell Lines

We focused on understanding YAP1, the highest-scoring gene in the screen. Expression of YAP1-2γ (hereafter referred to as YAP1) (Sudol, 2012) in HCTtetK cells prevented the morphological changes observed after suppression of KRAS (Figure 2A) and rescued the loss of viability induced by suppressing KRAS (Figure 2B). To ensure that the effects were not HCT116 specific, we tested the ability of YAP1 to complement loss of KRAS function in four additional KRAS mutant colon and pancreatic cancer cell lines (SW480, LS513, SU86.86, AsPC-1). We found that wild-type YAP1 rescued LS513, SU86.86, and AsPC-1 cell lines from KRAS suppression and that a constitutively active version of YAP1, which lacks five serine phosphorylation sites ($YAP1^{5SA}$) (Zhao et al., 2007), rescued loss of viability in SW480 cells (Figure 2C). We concluded that YAP1 signaling functionally replaces KRAS in KRAS-dependent cancer cells, although YAP1 itself may be differentially regulated in specific cell lines. In consonance with these findings, tumors that escape suppression of Kras in Kras-driven murine pancreatic ductal adenocarcinomas exhibit Yap1 amplifications (Kapoor et al., 2014 [this issue of Cell]).

To assess the specificity of the YAP1 phenotype to KRAS suppression, we tested whether YAP1 expression rescued suppression of additional oncogenes, c-MYC and PIK3CA, which are also activated in HCT116 cells. Suppression of c-MYC using two c-MYC-specific shRNAs reduced cell viability. Expression of c-MYC, but not YAP1, rescued this phenotype (Figure 2D). Furthermore, exposure to the PI3K inhibitor GDC-0941 arrested proliferation, and this effect was not rescued by YAP1 expression (Figure S2A). Together, these observations support the conclusion that rescue of KRAS suppression by YAP1 was not due to a general effect on survival by YAP1.

To interrogate the functional relationship between KRAS and YAP1 further, we assessed whether YAP1 is required for KRAS-induced cell transformation. We expressed $KRAS^{G13D}$ or YAP1 cDNAs in immortalized HA1E cells (Hahn et al., 1999) and confirmed that either $KRAS^{G13D}$ or YAP1 induced anchorage-independent colony formation when a control shRNA was expressed. We found that expression of two YAP1-specific shRNAs abrogated KRAS-driven anchorage-independent colony formation (Figures 2E and S2B). Expression of shYAP1-2 targets the YAP1 3′UTR and, as expected, failed to suppress YAP1-driven anchorage-independent colony formation.

We next examined the effect of manipulation of KRAS on the regulation of YAP1. Manipulating KRAS expression did not affect phosphorylation of YAP1 serine-127, a site implicated in regulation of YAP1 by Hpo signaling (Zhao et al., 2007), nor phosphorylation of components of the Hpo cascade such as LATS-1/2 and MST2 (Figure S2C). We concluded that KRAS does not alter YAP1 phosphorylation or activation.

In HCTtetK cells expressing the negative control LacZ, treatment with doxycycline for 2 days to induce KRAS suppression
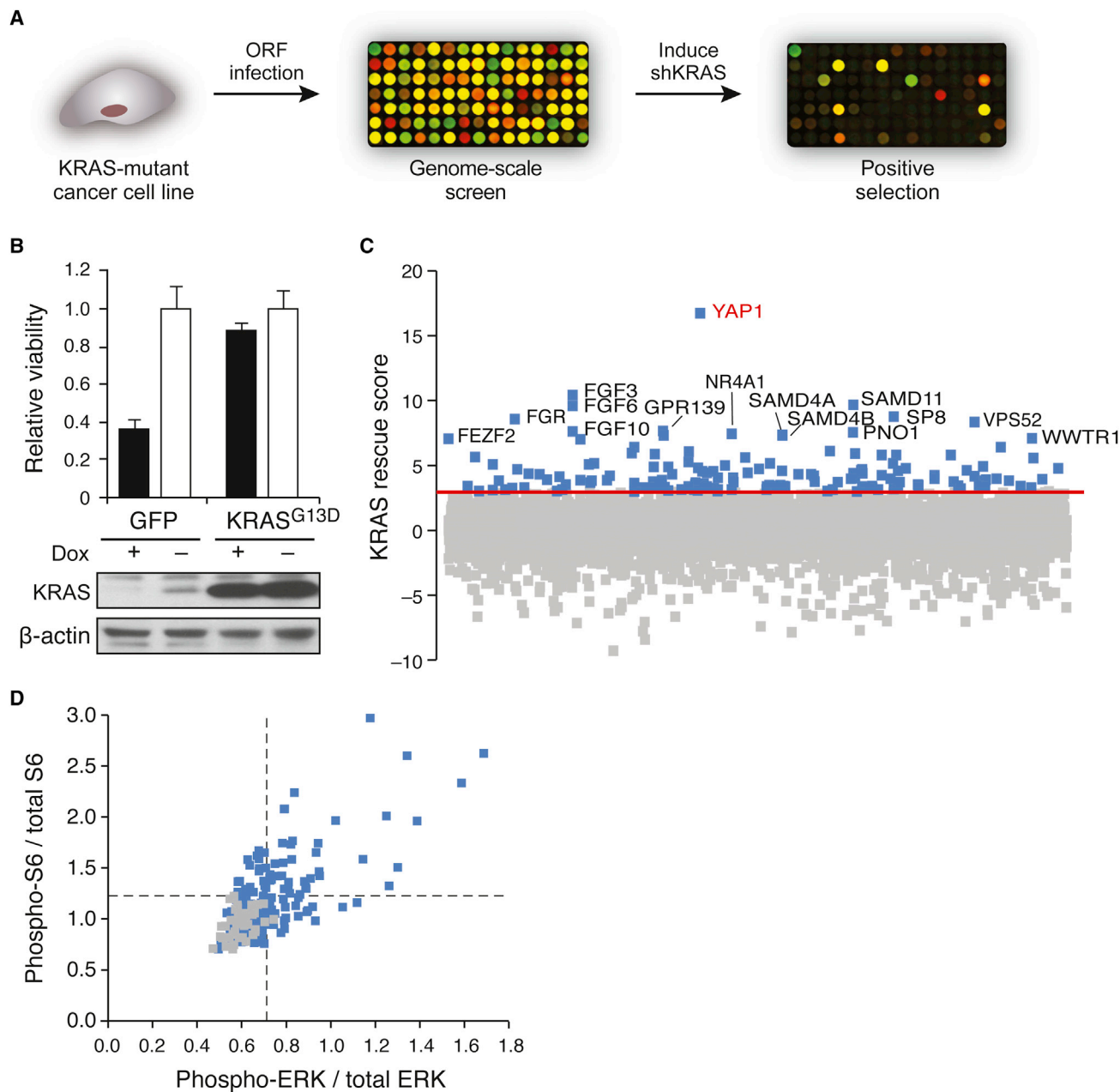
Figure 1. Systematic Identification of Genes that Rescue Loss of Viability Induced by KRAS Suppression

(A) Schematic diagram of an arrayed format screen to identify ORFs that rescue loss of cell viability induced by suppression of KRAS in KRAS-dependent cells.

(B) Suppression of KRAS in HCTtetK cells and rescue by KRAS ORF. Data represent mean ± SD normalized to cell viability in untreated conditions.

(C) Distribution of scores for all screened genes averaged across three replicates. KRAS rescue score indicates SD from mean of negative control wells. Red line, 3 SD. Blue, gene "hits."

(D) Characterization of 147 hits by in-cell western of ERK and S6 phosphorylation. Each point represents the average of duplicate wells. Lines indicate 2 SD above mean of negative controls. Gray, negative controls.

See also Figure S1 and Tables S1 and S2.

led to decreased levels of phosphorylated ERK, AKT, and S6. We found that YAP1 expression restored AKT and S6 phosphorylation to baseline levels and increased ERK phosphorylation (Figure 2F). These observations are in consonance with prior reports (Zhang et al., 2009; Overholtzer et al., 2006) that showed that

YAP1-regulated expression of the EGFR ligand amphiregulin led to activation of ERK and AKT. However, expression of ERK, MEK, or AKT failed to rescue HCTtetK cells upon suppression of KRAS in our original screen (Table S1), and YAP1 expression had no measureable effect on the activation of KRAS, as
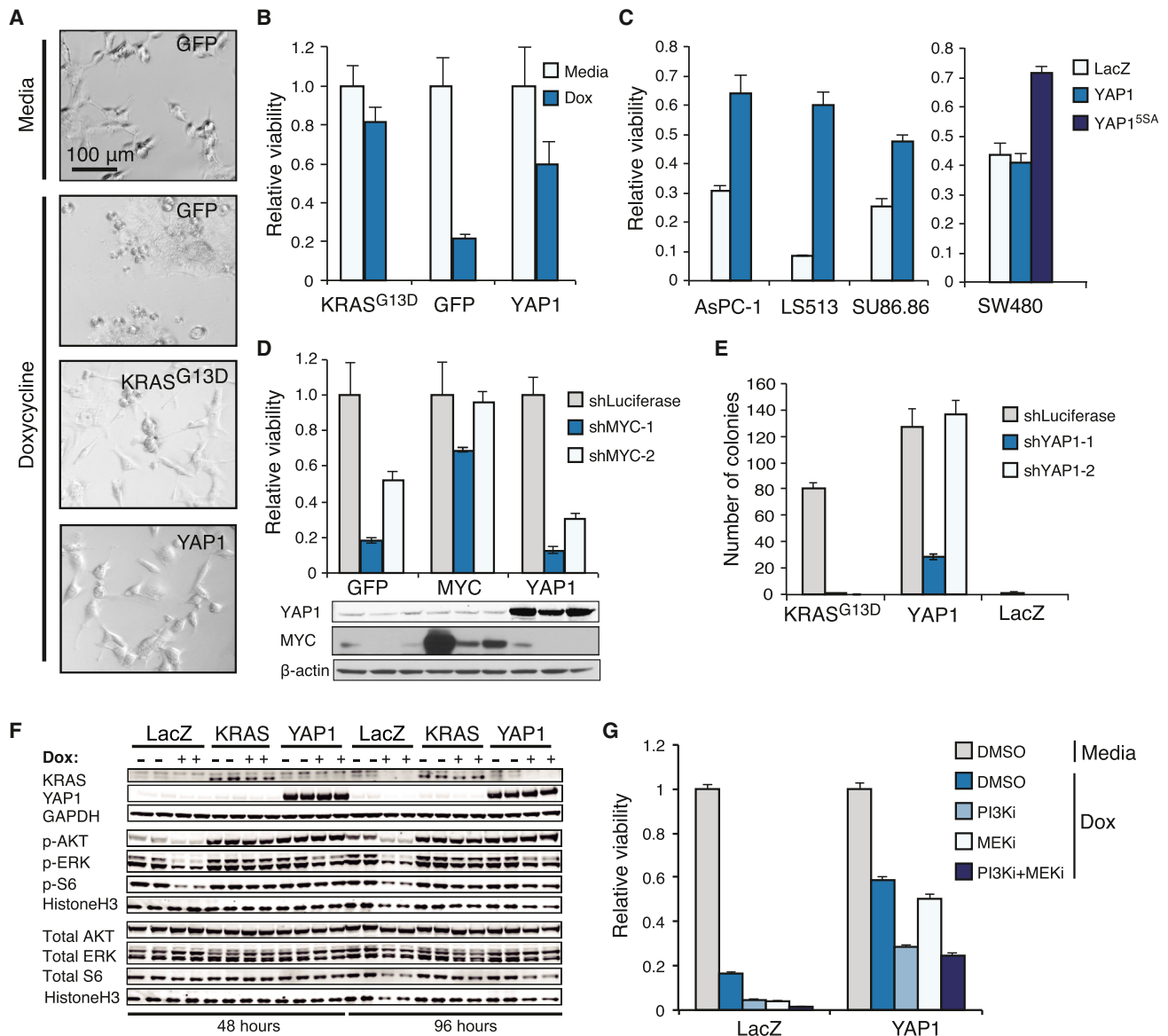
Figure 2. YAP1 Rescues *KRAS* Mutant Cancer Cells In Vitro

(A) Morphology of HCTtetK cells expressing the indicated vectors at 20× magnification. The indicated ORFs were expressed, and cells were treated with doxycycline (KRAS suppressed).

(B) Viability of HCTtetK cells upon KRAS suppression in cells expressing the indicated genes, normalized to cell viability in media condition.

(C) Consequences of expressing YAP1 in KRAS mutant cell lines after KRAS suppression. Viability of shKRAS normalized to shLuciferase in the presence of each indicated ORF.

(D) Response of HCT116 cells to MYC suppression in cells that express the indicated ORFs.

(E) Effect of YAP1 suppression on anchorage-independent growth of HA1E transformed with KRAS[G13D] or YAP1 ORF.

(F) Effect of doxycycline-induced KRAS suppression on activation of ERK, AKT, and S6 in HCTtetK cells expressing LacZ, KRAS, or YAP1.

(G) Effect of a PI3K inhibitor (PI3Ki; GDC-0941) or a MEK inhibitor (MEKi; AZD-6244) on the ability of YAP1 to rescue KRAS suppression. Cells were treated with 1 uM of GDC-0941, 1 uM of AZD-6244, both, or DMSO. Data were normalized to viability of cells without KRAS suppression (media) with DMSO treatment.

(B–E and G) Mean ± SD of at least three replicates in a representative experiment shown. See also Figure S2.

assessed by GTP-bound KRAS levels after pull-down with the Ras-binding domain of RAF1 (Figure S2D).

To determine whether reactivation of MAPK and PI3K signaling was necessary for the ability of YAP1 to rescue KRAS suppression, we treated HCTtetK cells expressing YAP1 with the PI3K inhibitor GDC-0941 or the MEK inhibitor AZD-6244 (Figures 2G and S2E). We found that YAP1 rescued HCTtetK cells from KRAS suppression to the level that we observed previously when treated with the vehicle (DMSO, Figure 2B). Treatment with either MEK or PI3K inhibitor decreased but failed to fully
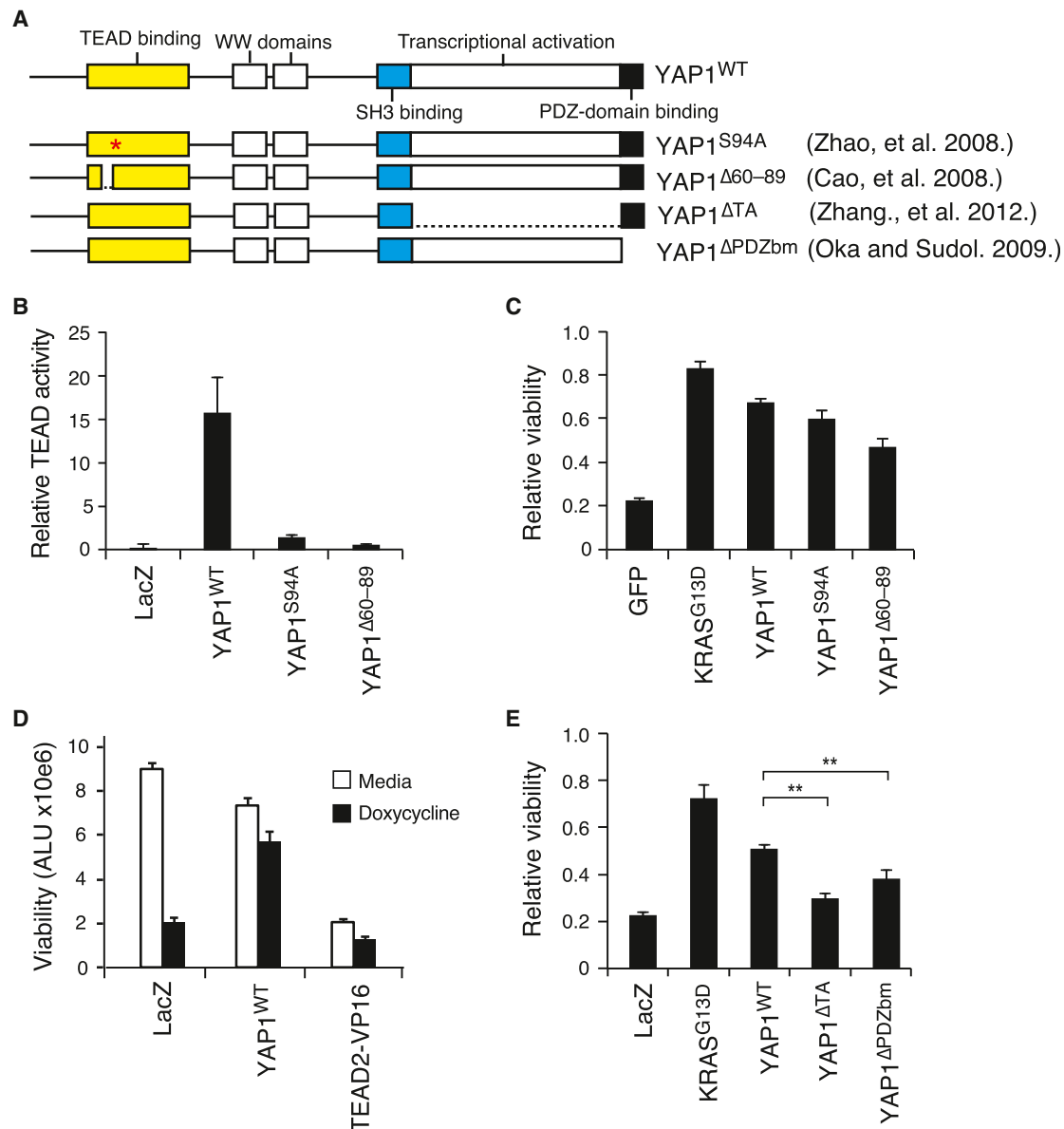
Figure 3. Structure-Function Analysis of YAP1

(A) YAP1 domain structure and YAP1 mutants.

(B) Effects of expressing YAP1 TEAD-defective mutants on the activity of a TEAD reporter in 293T cells.

(C) Viability after KRAS suppression in HCTtetK cells expressing YAP1 mutants defective in TEAD activation.

(D) Viability, in arbitrary luminescence units (ALU), after KRAS suppression in HCTtetK cells expressing a constitutively active TEAD2-VP16 fusion.

(E) Effects of expressing the YAP1 mutants defective in transcriptional activation or nuclear localization in HCTtetK cells after KRAS suppression. **p < 0.01.

(B–E) Mean ± SD of six replicates of a representative experiment shown. (C–E) Viability of doxycycline treated relative to untreated samples displayed. See also Figure S3.

suppress the ability of YAP1 to rescue KRAS inhibition as compared to cells expressing LacZ (Figure 2G). Furthermore, combined treatment with both inhibitors did not further decrease viability. Thus although YAP1 partially restores the activity of PI3K and MEK pathways after KRAS suppression, activation of these pathways fails to fully account for the ability of YAP1 to promote survival in KRAS-dependent cells after KRAS suppression.

Functions of YAP1 Required for the Survival of KRAS-Dependent Cells

YAP1 is regulated by multiple signaling pathways and regulates the function of several transcription factors. YAP1 is composed of a TEAD-binding domain, an SH3-binding motif, two WW domains, and a transcription activation domain (Figure 3A), and YAP1 nuclear localization is mediated by a C-terminal PDZ domain-binding motif (Oka and Sudol, 2009). To identify regions

of YAP1 necessary to promote survival after KRAS suppression, we expressed previously described YAP1 domain-specific mutants in HCTtetK cells to assess their effect on YAP1 function (Figure 3A).

First, we explored the role of TEAD transcription factors for the KRAS-related functions of YAP1. Prior work identified YAP1 TEAD-binding domain mutants that disrupt binding to TEAD transcription factors (YAP1^{S94A} and YAP1$^{\Delta60-89}$) (Zhao et al., 2008b; Cao et al., 2008). Expression of these mutants indeed abolished the ability of YAP1 to activate a TEAD-specific reporter (Figure 3B) (Ota and Sasaki, 2008). However, these YAP1 mutants rescued the proliferation effects of KRAS suppression similar to wild-type YAP1 (YAP^{WT}; Figure 3C), suggesting that TEAD-specific effects are dispensable for YAP1 rescue. These findings differ from those of Kapoor et al. (2014), who identified TEAD2 as one mediator of YAP1 function by showing that a constitutively active fusion of the VP16 domain to the DNA-binding region of TEAD2 (TEAD2-VP16) (Cao et al., 2008) was able to rescue KRAS suppression. We tested the ability of this construct to rescue in the HCTtetK model and found that TEAD2-VP16 activated a TEAD-specific reporter (Figure S3A) but failed to rescue the effect of KRAS suppression (Figures 3D and S3B). TEAD2-VP16 expression slowed the proliferation of HCTtetK cells (Figure 3D) and resulted in a change in cell morphology different than what we observed upon expression of YAP1 or expression of KRAS. Together, these results suggest that the TEAD family is not the primary mediator of the ability of YAP1 to complement loss of KRAS in this model system.

Because we recently reported a role for YES1 phosphorylation of YAP1 for β-catenin signaling (Rosenbluh et al., 2012), we investigated whether YES1 played a role in KRAS signaling. We expressed two YAP1 mutants: YAP1$^{\Delta SH3bm}$, which disrupts the interaction of YES1 with YAP1 (Vassilev et al., 2001; Sudol, 1994), and YAP^{Y357F}, which prevents YES1 phosphorylation of YAP1 (Rosenbluh et al., 2012). Expression of either YAP1$^{\Delta SH3bm}$ or YAP1^{Y357F} rescued the loss of cell viability observed after KRAS suppression to the level of YAP1^{WT} (Figures S3C and S3D). Thus, YES1 modulation of β-catenin signaling also does not contribute to YAP1 activity in the context of KRAS suppression.

We then tested whether YAP1-induced transcriptional activation was required to rescue KRAS suppression. Expression of YAP1 mutants that harbor a deletion of the transcriptional activation domain (YAP$^{\Delta TA}$; Zhang et al., 2012) disrupted the ability of YAP1 to rescue cells from KRAS suppression in HCTtetK cells, as did expression of YAP1 mutants that harbored a deletion of the 5 amino acid PDZ domain-binding motif (YAP$^{\Delta PDZbm}$), reported to disrupt YAP1 nuclear localization (Figures 3E and S3E). These two YAP1 mutants also prevented YAP1-induced anchorage-independent growth (Figure S3F). These observations show that YAP1 mediates survival after suppression of KRAS through interactions with transcription factors in the nucleus.

YAP1 Regulates AP-1 Family Transcription Factors and EMT

Because transcriptional activation was important for the ability of YAP1 to rescue KRAS suppression, we analyzed transcriptional profiles in HCTtetK cells in which we manipulated KRAS or YAP1 expression. We first identified genes that were downregulated after 30 hr of doxycycline (KRAS suppressed) treatment compared to untreated cells. Of those, we identified genes whose expression was rescued by the expression of both YAP1 and KRAS (Figure 4A and Table S3). Separately, we analyzed the transcriptional profiles of cells expressing the YAP1 TEAD-binding defective mutant YAP1^{S94A}, compared to KRAS^{G13D} and YAP1^{WT} as positive controls or to LACZ and YAP1$^{\Delta TA}$ as negative controls. We noted that the expression signature induced by YAP1^{S94A} overlapped but was distinct from that of YAP1^{WT}, suggesting differences in specific transcriptional programs regulated by these alleles. Yet we found that YAP1^{S94A} rescued expression of genes that decreased upon KRAS suppression (Figure S4A), as expected.

Using the 1,045 genes that were rescued by both YAP1^{WT} and KRAS (Figure 4A and Table S3), we searched for coregulated transcription factor motifs and coregulated gene sets. We used TransFind motif analysis (Kiełbasa et al., 2010) to identify transcription factor motifs enriched in promoter regions of genes rescued by both YAP1 and KRAS compared to motifs rescued by KRAS alone. Enriched motifs (p < 0.05) included transcriptional factors involved in the immediate early gene response, c-MYC, HIF, and E2F (Figures 4B and S4B). Kapoor et al. (2014) showed that E2F1 is required for Yap1 to permit Kras-independent tumor growth in a murine pancreatic ductal adenocarcinoma model. Depending on the context, multiple transcription factors may play roles in YAP1 function.

We focused on the largest category, transcription factors involved in the immediate early gene response, which are regulated by both growth factor stimulation and Ras signaling (O'Donnell et al., 2012). Specifically, this category includes gene families such as activating transcription factor (ATF), early growth response protein (EGR), and specificity protein (SP) (Figure S4B). To determine which of these genes plays a role in YAP1 function, we systematically suppressed members of these gene families using multiple independent shRNAs and assessed the effect on YAP1-driven anchorage independent growth (Figure S4C). Suppression of several SP family and ATF family transcription factors repressed YAP1-mediated cell transformation. Genes in the SP family are required for development and are ubiquitously involved in maintaining cell survival (Suske, 1999). We were particularly interested in the role of the ATF genes, as they are members of the AP-1 transcription factor family, previously shown to be regulated by KRAS-MAPK signaling (Mechta et al., 1997). Further supporting the role of AP-1, we found that expression of YAP1 or KRAS activated an AP-1 luciferase reporter driven by a consensus AP-1-binding element (Figure 4C).

From our genome-scale screening data, we noted that FOS was the only member of the AP-1 transcription factor family that scored (3.75 SD above controls; Table S1). We confirmed that expression of FOS rescued HCTtetK cells upon suppression of KRAS (Figure 4D). To test whether FOS expression was necessary for YAP1 function, we assessed whether suppression of FOS affected YAP1-induced transformation. Depletion of FOS with two FOS-specific shRNAs reduced YAP1-driven anchorage-independent colony formation in HA1E cells
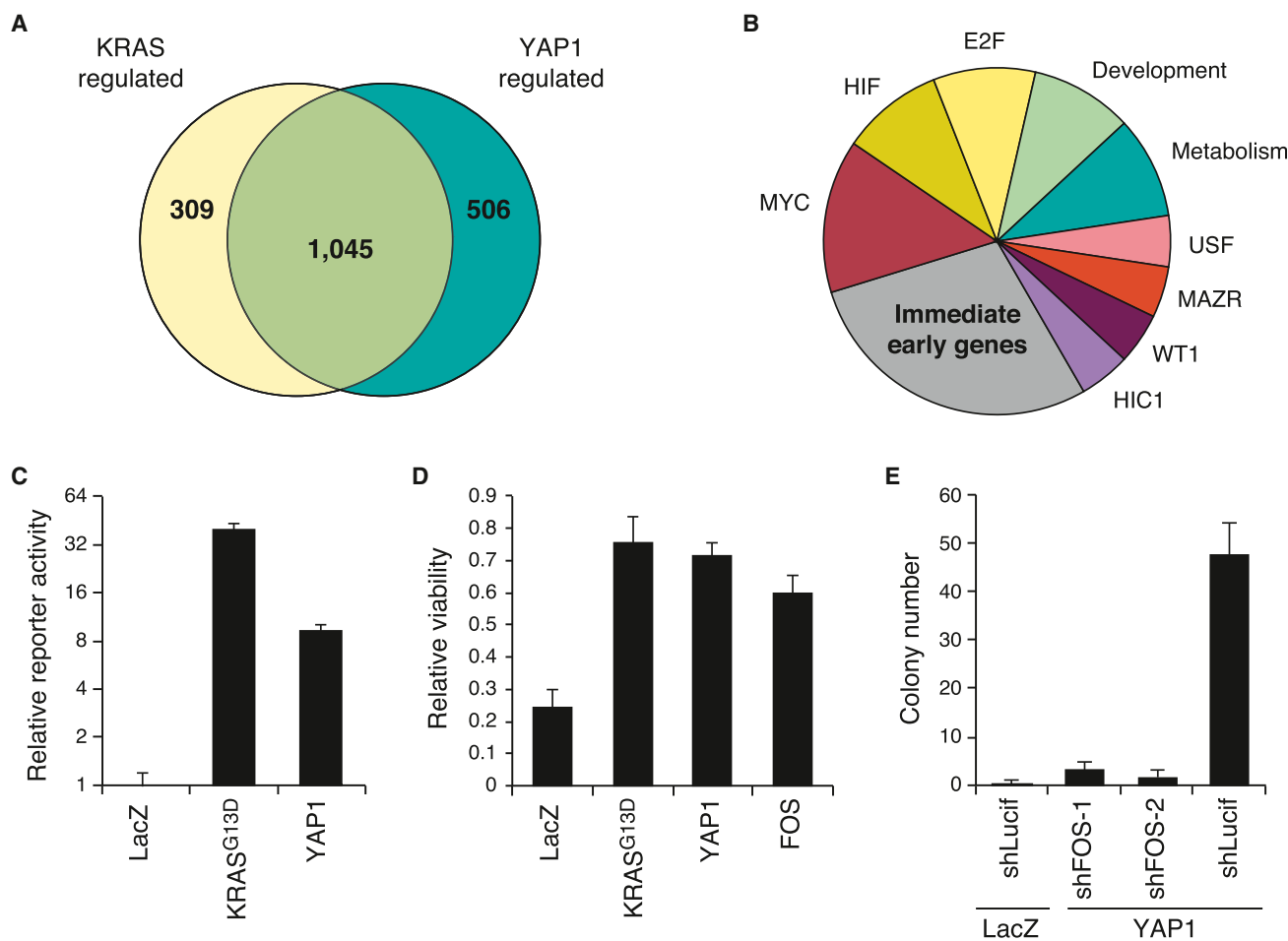
Figure 4. YAP1 and KRAS Converge to Regulate the AP-1 Transcription Factor FOS
(A) Genes rescued by YAP1, KRAS, or both in the context of KRAS suppression.
(B) Categories of transcription factor motifs enriched among genes rescued by both KRAS and YAP1.
(C) AP-1 reporter activity in 293T cells expressing YAP1. Arbitrary luminescence units normalized to LacZ condition.
(D) Expression of FOS rescues suppression of KRAS in HCTtetK cells. Viability of doxycycline treated relative to untreated samples is displayed.
(E) Effects of suppressing FOS on YAP1-mediated cell transformation of HA1E cells.
(C–E) Mean ± SD of at least three replicates in a representative experiment shown. See also Figure S4 and Table S3.

(Figure 4E). RAS-induced transformation in vivo requires FOS (Ledwith et al., 1990; Saez et al., 1995), and as expected, FOS suppression reduced colony formation by HCT116 cells or HA1E cells expressing KRAS^{G13D} (Figures S4D and S4E). These observations support the role of AP-1 family transcription factors as effectors of YAP1 and KRAS.

We then looked for transcriptional programs regulated by both KRAS and YAP1 in our microarray data (Figure 4A; Table S3). Using the Molecular Signatures Database (MSigDB) (Subramanian et al., 2005), the top gene sets, enriched to p < 10^{-3}, included several related to differentiation and development (Figure 5A). In many cancers, normal cell differentiation is perturbed by the interruption of differentiation steps or by the aberrant activation of EMT programs. Furthermore, EMT has been implicated in resistance to therapies targeting receptor tyrosine kinases (Witta et al., 2006), and FOS has been shown to regulate EMT directly (Reichmann et al., 1992). When we analyzed tran-

scriptional profiles of the YAP1 mutants (Figure S4A), we found that YAP transcriptional signature (YAP_DUPONT) and EMT signatures (SARRIO_EPITHELIAL_MESENCHYMAL_TRANSITION_UP) were enriched among genes rescued by YAP1^{WT} (Fisher exact, p = 0.005 and < 10^{-16}, respectively; threshold log(−0.5) compared to LacZ), and neither signature was enriched among the genes rescued by functionally deficient YAP1^{ΔTA} mutant (Fisher's exact test, p = 0.48 and 0.53, respectively). Thus, we hypothesized that EMT induced by YAP1 contributed to the survival of cells after suppression of KRAS.

We found that both KRAS and YAP1 expression strongly induced expression of mesenchymal genes such as Vimentin (*VIM*), Fibronectin (*FN1*), Slug (*SNAI2*), and Zinc-finger E-box-binding homeobox 1 (*ZEB1*) and reduced the expression of epithelial genes such as E-cadherin (*CDH1*) and Occludin (*OCLN*) (Figure 5B). Although only a subset of genes implicated in EMT were regulated by KRAS or YAP1 (Figure S5A), we found
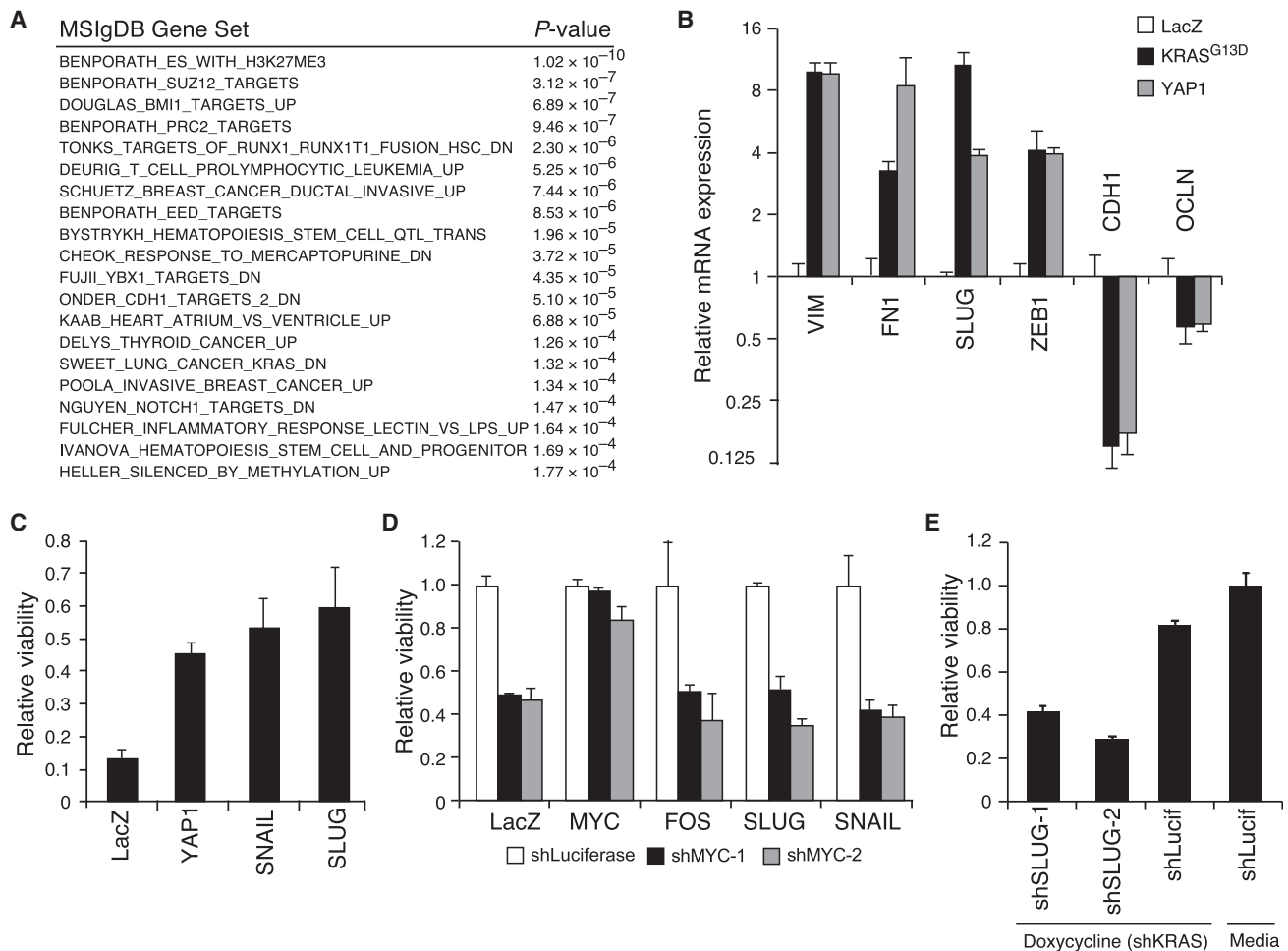
Figure 5. YAP1 Regulates the Epithelial-Mesenchymal Transition

(A) Enriched gene sets rescued by both YAP1 and KRAS.

(B) qRT-PCR validation of epithelial-mesenchymal transition (EMT) regulation by KRAS and YAP1 in HCT116 cells. Data represent mean ± SD of four replicates relative to LacZ control.

(C) Viability of doxycycline-treated relative to untreated HCTtetK cells expressing Slug and Snail.

(D) Effects of suppressing MYC after expressing FOS, Slug, and Snail in HCT116 cells. Viability of shMYC normalized to shLuciferase control in the presence of each indicated ORF.

(E) Effect of Slug on the ability of YAP1 to rescue KRAS suppression. Viability of doxycycline-treated HCTtetK cells expressing YAP1 after expression of each indicated shRNA, normalized to media-treated shLuciferase control.

(B–E) Mean ± SD of at least three replicates in a representative experiment is shown. See also Figure S5.

that KRAS and YAP1 regulated a similar set of EMT markers in both HCTtetK cells (Figures 5B and S5A) and SU86.86 (Figure S5B).

We then tested whether key transcriptional regulators of EMT such as Slug and Snail were necessary and sufficient to rescue KRAS dependence. Expression of either Slug or Snail in HCTtetK cells rescued the loss of viability induced by suppressing KRAS (Figure 5C). To assess the specificity of this effect to KRAS, we suppressed MYC and expressed FOS, Slug, or Snail (Figure S5C). Neither expression of EMT transcriptional regulators nor FOS rescued loss of viability upon MYC suppression (Figure 5D), suggesting that induction of EMT did not broadly rescue oncogenic suppression. Moreover, we tested whether Slug was

required for YAP1 rescue of KRAS suppression. Expression of two Slug-specific shRNAs reduced Slug expression and decreased the ability of YAP1 to rescue suppression of KRAS (Figures 5E and S5D).

FOS and YAP1 Coordinately Regulate Downstream Targets

Because FOS expression was required for YAP1-induced cell transformation, we examined whether FOS and YAP directly interact. We expressed a V5-epitope-tagged FOS in HCT116 cells and isolated YAP1 or control (anti-GFP) immune complexes. In YAP1 complexes, but not control immune complexes, we detected FOS (Figure 6A), and in V5 immune complexes
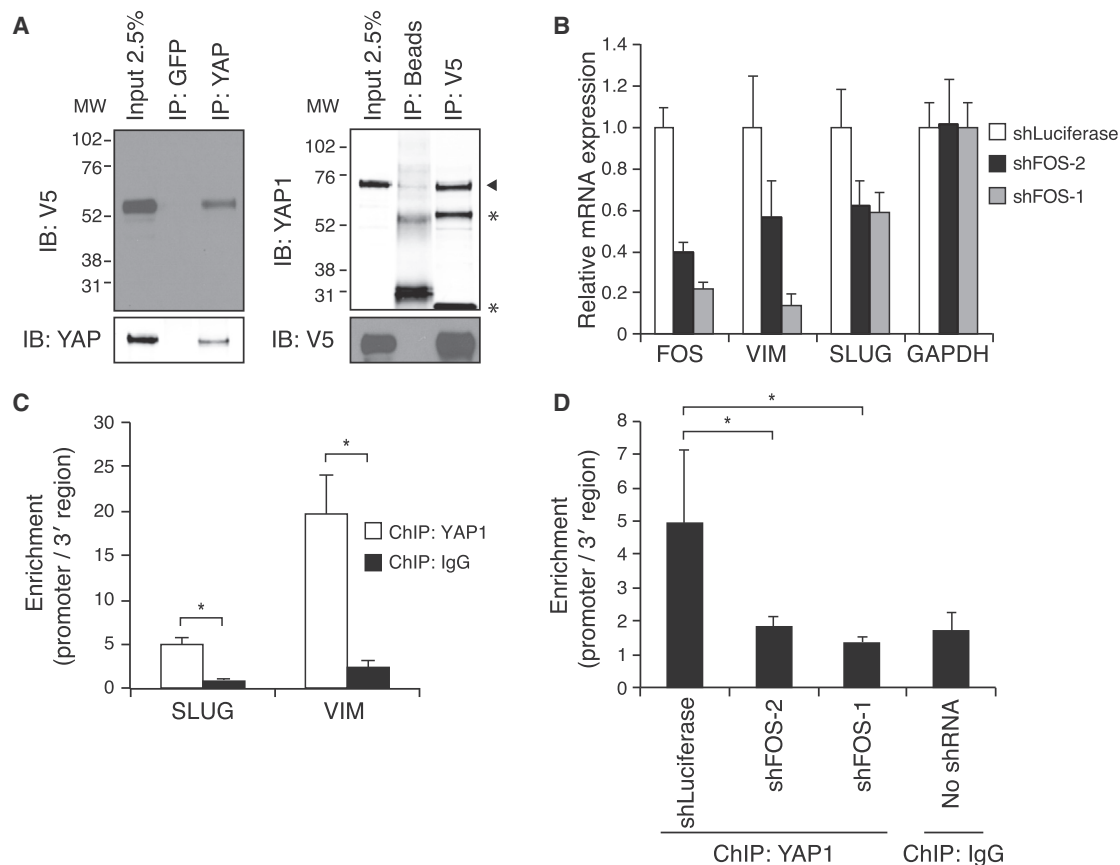
Figure 6. YAP1 and FOS Interact at Promoter Regions to Regulate EMT

(A) Coimmunoprecipitation using control antibody or target-specific antibody for YAP1 and FOS in lysates from HCT116 cells expressing YAP1 and V5-tagged FOS. Binding of the reciprocal protein was assessed by immunoblotting. YAP1 is indicated by arrowhead. *, IgG heavy and light chains; MW, molecular weight in kDa.

(B) mRNA expression of Vimentin (VIM) and Slug after FOS suppression. Mean ± SD of four replicates relative to shLuciferase control in HCT116 cells is shown.

(C) Chromatin immunoprecipitation in HCT116 to assess YAP1 DNA binding at promoter regions of *SLUG* (*SNAI2*) and Vimentin (*VIM*).

(D) Chromatin immunoprecipitation in HCT116 to assess YAP1 binding at *SLUG promoter* after FOS suppression.

(C and D) Bars represent enrichment of promoter compared to 3′ region of each gene. Mean ± SD of 3 replicates is shown. *$p < 0.05$. See also Figure S6 and Table S4.

(FOS), we found endogenous YAP1 (Figure 6A). In contrast, coimmunoprecipitation experiments with a V5-epitope tagged version of JUN, another AP-1 transcription factor, failed to show an interaction between YAP1 and JUN (Figure S6A).

We hypothesized that FOS and YAP1 regulate common downstream target genes that are important for KRAS dependence. Using transcription factor ChIP-seq data from the Encyclopedia of DNA Elements (ENCODE) (Bernstein et al., 2012), we found that YAP1-regulated genes such as *VIM* and *Slug* (*SNAI2*) harbor FOS-binding sites in their promoter regions. We confirmed that FOS suppression using FOS-specific shRNA decreased *VIM* and *Slug* expression levels (Figure 6B). To determine whether FOS and YAP1 bind at the same loci, we performed chromatin immunoprecipitation using antibodies specific to YAP1, FOS, or control IgG in HCT116 cells. As reported by ENCODE, FOS was enriched at the promoter regions of *VIM* and *Slug* (compared to a region 3′ of each gene) (Figure S6B). We found that YAP1 binding was also enriched at the same loci, whereas

binding of IgG was not (Figure 6C). We confirmed the specificity of the YAP1 antibody by showing decreased YAP1 binding at those loci when YAP1-specific shRNAs were expressed (Figure S6C). Notably, suppression of FOS also decreased YAP binding at the *VIM* promoter (Figure 6D), suggesting that YAP may function through FOS as a transcriptional coactivator.

We then interrogated ChIP-on-chip and ChIP-seq data to identify FOS- and YAP1-binding sites across the genome. Specifically, we looked for genes co-occupied by YAP1 and FOS by using ingenuity pathway analysis on a list of YAP1-occupied genes in MCF10A cells (Zhao et al., 2008b) and corresponding FOS-occupied genes (1 kb upstream, 0.3 kb downstream of transcription start site in MCF10A-ER-Src cells; Bernstein et al., 2012). As a control, we used a list of androgen receptor (AR)-occupied genes (Zhao et al., 2008b). We found that the EMT canonical pathway was enriched in the YAP1-FOS overlap set ($p < 0.05$) but was not enriched in the AR-FOS overlap set (Table S4). This observation provides additional support that

YAP1 and FOS regulate EMT to rescue viability upon KRAS suppression.

Yap1 Mediates Resistance to Kras Suppression in a Kras[G12D]-Driven Lung Cancer Mouse Model

We then examined the consequences of Kras inhibition in a well-studied mouse model of lung adenocarcinoma (Tuveson et al., 2004; DuPage et al., 2009). We first used primary lung adenocarcinoma cells derived from the Kras[Lox-STOP-Lox-G12D];p53[flox/flox] (KP) mouse lung cancer model into which we introduced a doxycycline-inducible shRNA-targeting Kras expressed from the 3′UTR of GFP (KP-KrasA cells). In this system, doxycycline treatment activates the GFP reporter as well as shKras, resulting in suppression of endogenous wild-type Kras and mutant Kras[G12D]. After intravenous injection of tumor cells, tumor burden in the lung was monitored weekly by a constitutively expressed luciferase construct in the tumor cells (Figure 7A). At 7 days posttransplantation, the mice were fed a doxycycline-containing diet, which resulted in rapid lung tumor regression within 7 days (Figure 7B), confirming that these lung tumors depend on ongoing oncogenic Kras signaling. However, tumors recurred over the course of the next 2 weeks even though Kras remained suppressed in tumor tissue, as confirmed by imaging of GFP reporter and by qRT-PCR of Kras mRNA from microdissected tumors (Figures 7C and S7A). Thus, in this model, some Kras-driven tumor cells can continue to proliferate in a Kras-independent manner after prolonged suppression of oncogenic Kras.

To assess the molecular basis of this Kras-independent process, KP-KrasA cells were cultured in the presence of doxycycline, resulting in cells that continued to proliferate despite suppression of Kras. RNA sequence profiling (RNA-seq) of these cells after 21 days on doxycycline compared to cells without exposure to doxycycline showed significant upregulation of a published Yap1 gene signature (Figure 7D and Table S5) (Dupont et al., 2011). Because we showed that EMT played a critical role in mediating resistance to KRAS suppression in human cells (Figure 5), we also assessed whether EMT was involved in the Kras resistance observed in the murine cancer cell system. Using a published EMT gene signature (Taube et al., 2010), we noted a significant enrichment of the signature in Kras-independent cells compared to parental cells (Figure 7E). In addition, EMT-associated markers, including Fn1, Cdh2, Snai1, Snai2, Zeb1, and Ocln, were altered in RNA samples from Kras-independent cells (Figure S7B). We confirmed by qRT-PCR that expression of several of these markers was upregulated in tumor cells that had escaped Kras suppression in vivo as compared to tumors in which Kras expression was maintained (Figure S7C). Notably, we found that Yap1 expression was not significantly different in these two groups. Thus, we examined Yap1 localization by immunohistochemistry from tumor tissue. We found that Yap1 showed increased nuclear localization in tumors that escaped Kras suppression (Figure 7F), which may explain the observed upregulation of genes involved in EMT. Together, these observations suggest that Kras-independent mouse lung cancer cells exhibit signatures similar to those that we observed when we expressed YAP1 or KRAS in human cancer cell lines.

To explore whether Yap1 signaling was involved in Kras oncogenic addiction in vivo, we performed both gain-of-function and loss-of-function experiments in this mouse model. We observed that forced expression of YAP1 partially prevented the tumor regression observed upon initial suppression of Kras (Figures S7D and S7E). To test whether relapse from Kras suppression requires Yap1 signaling, we generated KP cells in which two shRNAs are expressed simultaneously. Specifically, in addition to the Kras-specific shRNA, we expressed a doxycycline-inducible construct that drives the expression of red fluorescent protein (RFP) carrying shRNAs targeting either Yap1 or Renilla luciferase within the 3′UTR (Zuber et al., 2011). Whereas tumors with Kras suppression alone relapsed over time, concurrent Yap1 suppression delayed tumor relapse while the expression of the Renilla luciferase shRNA did not (Figure 7G). We found that the residual tumors that formed in the presence of the Yap1-specific shRNA after 28 days no longer suppressed Yap1, as assessed by qRT-PCR (Figure S7F), suggesting that Kras-independent proliferation in vivo occurs in cells with higher Yap1 activity. Together, the observations from this murine model confirm that the upregulation of Yap1 signaling correlates with the expression of an EMT-like transcriptional program and plays a compensatory role in vivo upon loss of Kras signaling.

DISCUSSION

Using a systematic functional approach, we identified YAP1 as a gene whose expression rescued cell death induced by suppression of KRAS in KRAS-dependent cancer cells. In a murine model of Kras-driven lung cancer, we found that tumors that escaped Kras suppression in vivo also exhibited increased YAP1 activity. Through transcriptional profiling, we described a YAP1-driven transcriptional program that recapitulates the oncogenic signals in KRAS-driven cancers through involvement of AP-1 family transcription factors—specifically FOS—and the regulation of EMT.

Complementation Screening in Cancer Cells to Elucidate KRAS Signaling

We found that a substantial proportion of genes identified through our rescue screen activated known KRAS downstream MAPK and PI3K pathways, in line with previous observations that MAPK and PI3K signaling provide a general mechanism to substitute for Ras signaling (Lim and Counter, 2005). Although we focused on YAP1 and FOS herein, these observations suggest that other genes that scored in this screen may represent novel components of KRAS-regulated signaling pathways.

Models of Resistance to RAS Oncogenic Addiction

Withdrawal of oncogenic Ras results in rapid tumor regression in mouse models that used tetracycline-inducible overexpression of oncogenic Ras (Fisher et al., 2001; Chin et al., 1999; Jechlinger et al., 2009). Relapse was often associated with mutations in the tetracycline transactivator (Podsypanina et al., 2008), resulting in reactivation of the oncogene. Here, we used inducible in vivo RNAi to model Kras inhibition in mouse lung adenocarcinoma cells driven by Kras[G12D] expressed from its endogenous promoter and observed tumor relapse through Kras-independent
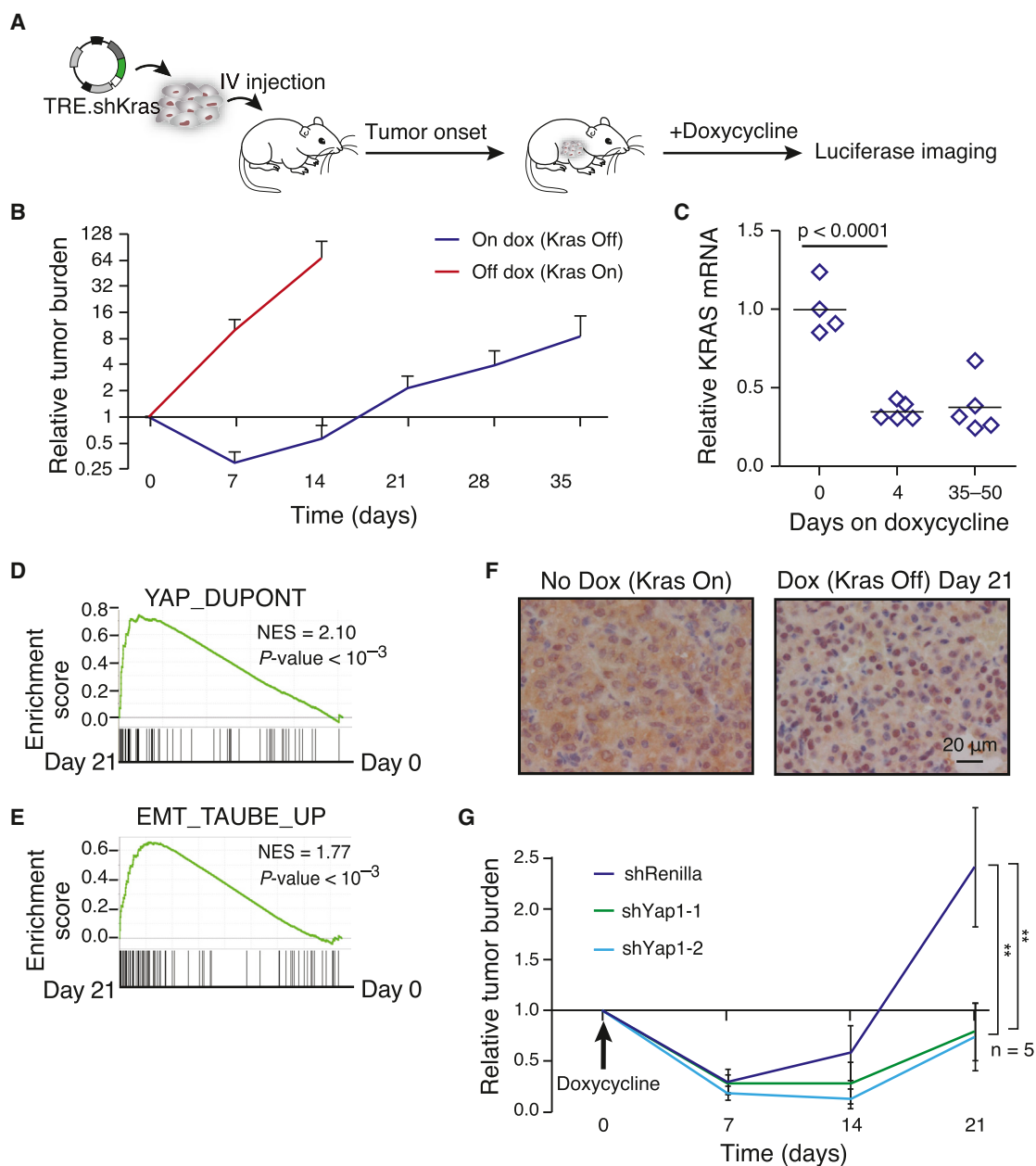
Figure 7. Yap1 Activity Is Required for Endogenously Acquired KRAS Resistance In Vivo

(A) Schematic of mouse transplant model of KRAS-driven lung cancer. $Kras^{G12D};p53^{fl/fl}$ lung adenocarcinoma cells were infected with retroviral vectors expressing rtTA3, luciferase, and a tet-on shKras. Cells were transplanted into recipient mice by tail-vein injection. At 7 days later, mice were fed a doxycycline diet to induce shKras in tumor cells (D0).

(B) Time course of tumor regression and relapse after Kras suppression. Mean ± SD is shown. n = 3 off dox and n = 10 on dox.

(C) Suppression of Kras in tumor tissue. Kras mRNA was measured by qRT-PCR in microdissected lung tumors after the indicated days of doxycycline treatment.

(D) Enrichment of a published YAP1 signature (Dupont et al., 2011) after 21 days doxycycline treatment versus untreated cells.

(E) Enrichment of a published EMT signature (Taube et al., 2010) after 21 days of doxycycline treatment versus untreated cells.

(F) Yap1 localization in tumors that escape Kras suppression. Immunohistochemistry was performed with Yap1 antibody on frozen tissue sections from tumors that developed after Kras suppression (dox on) for 21 days and tumors that formed with continued Kras expression (dox off).

(G) Tumor response to suppression of Kras in combination with Yap1 or control suppression. Mean ± SD is shown. **p < 0.01.

See also Figure S7 and Table S5.

mechanisms. This model of relapse thus provided strong evidence that increased activity of YAP1 is a physiologically relevant mechanism to bypass loss of KRAS signaling.

We found that YAP1 replaces oncogenic KRAS signaling, at least in part, by regulating an EMT-like transcriptional program. Singh et al. (2009) previously showed a correlation between

KRAS dependency and epithelial morphology among *KRAS* mutant cell lines and argued that induction of EMT makes cells insensitive to KRAS suppression. However, RAS itself activates EMT (Wong et al., 2013), and genes involved in EMT, such as *Slug,* are essential in *KRAS* mutant cells (Wang et al., 2010). Furthermore, our observation that YAP1 and FOS cooperate to regulate portions of the EMT program is in consonance with a prior report that FOS itself drives an EMT phenotype (Eger et al., 2000). We report here that sustaining the established EMT phenotype is necessary to rescue KRAS suppression. Based on the available evidence, it is clear that the correlation between KRAS dependency, morphology, and EMT is complex and that the interplay of YAP1, KRAS, and EMT regulators will be context dependent.

In consonance with our observations, Kapoor et al. (2014) used a model of murine pancreatic adenocarcinoma in which Kras is expressed from an inducible promoter. After Kras suppression, some tumor cells spontaneously relapsed through a mechanism involving amplifications of a genomic locus containing *Yap1* and a gene signature associated with EMT. They showed that TEAD2 and E2F1 cooperate with YAP1 in these Kras-independent tumors. We also observed that E2F motifs were enriched in genes regulated by both KRAS and YAP1 (Figure 4B) but did not find a role for TEAD2. Because YAP1 engages different transcription factors in a context-dependent manner, TEAD2 involvement may be related to contextual differences of species or cell lineage, to the method of gene suppression, or to functional differences between suppression of both mutant and wild-type KRAS (as occurs with shRNA) compared to suppression of mutant KRAS alone. We also observed that YAP1 reactivated MAPK and PI3K signaling in our experimental model, whereas Kapoor et al. observed baseline activation of these pathways by Yap1 in their system. This may be attributed to different baseline levels of MAPK/PI3K signaling in the two systems or to the influence of wild-type KRAS (To et al., 2013). Despite these differences, the observation that YAP1 can rescue KRAS suppression in three independent experimental models underscores the ability of YAP1 to promote survival in KRAS-dependent cancers.

KRAS and YAP1 Converge at the Transcriptional Level

RAS signaling has been linked to YAP1 in *D. melanogaster* imaginal wing discs, in which YAP1 was required for EGFR and RAS activity (Reddy and Irvine, 2013). We showed that YAP1 is required for KRAS-driven transformation but did not observe biochemical modulation of YAP1 activity by KRAS signaling. Although downstream effectors of KRAS such as RASSF1 and AKT have been shown to regulate YAP1 phosphorylation in specific contexts (Zhao et al., 2008a), their influence on YAP1 may be context specific.

Recurrent amplifications of *YAP1* have been observed in liver, breast, and esophageal cancer (Overholtzer et al., 2006; Muramatsu et al., 2011; Zender et al., 2006). As an oncogene, YAP1 has been shown to drive development of hepatocellular cancer and to induce colonic adenomas in mouse models (Zender et al., 2006; Camargo et al., 2007), though the gene programs regulated by YAP1 to induce transformation may differ from that engaged by YAP1 to mediate survival in the setting of KRAS suppression. In the context of KRAS suppression, we found that YAP1 regulation of FOS was required for rescue and that YAP1 and FOS interact at promoter sites of regulated genes to activate an EMT-like program in order to mediate survival.

EMT phenotypes are upregulated in cancer cells resistant to chemotherapy (Fuchs et al., 2002; Kajiyama et al., 2007; Cheng et al., 2007), and modulation of EMT influences cellular dependency on receptor tyrosine kinases (Witta et al., 2006). We found that YAP1-induced regulation of EMT was specific for oncogenic KRAS, but not other oncogenes. The specificity of this mechanism to KRAS suggests that resistance associated with EMT might be attributed to bypass of specific oncogenic pathways. Understanding the role of YAP1 and FOS in regulating EMT will not only provide further insights into the transcriptional programs regulated by oncogenic KRAS, but as therapeutic strategies to target KRAS are developed, will also elucidate potential mechanisms by which KRAS-driven cancers escape these interventions.

EXPERIMENTAL PROCEDURES

For additional details, see the Extended Experimental Procedures.

KRAS Rescue Screen

HCTtetK cells were seeded at 300 cells per well in 50 ul in 384-well plates. The next day, cells were infected using 1 ul of virus (1.4×10^8 infectious particles/ml using the RNAi Consortium virus tittering protocol; http://www.broadinstitute.org/rnai/public/resources/protocols) in 25 ul media supplemented with 8 ug/ml polybrene. The media was changed the following day. At 2 days after infection, 500 ng/ml doxycycline was added. For 10% of plates, additional replicates received treatment with blasticidin or no treatment to confirm overall infection efficiency. At 7 days after infection, viability of each well was determined by CellTiterGlo (Promega). B score adjustment was performed for each plate (Brideau et al., 2003), and final score for each ORF was normalized to ~40 negative control values on each plate (uninfected wells and wells infected with HcRed, eGFP, BFP, LacZ, or Luciferase).

Rescue Experiments In Vitro

ORFs were introduced by lentiviruses followed by selection with 10ug/ml Blasticidin for ≥4 days. For cell lines harboring a tet-inducible shRNA, cells expressing each ORF were seeded in 96-well plates and were treated with media supplemented with doxycycline or media alone for 5 days. Viability was quantified by CellTiterGlo (Promega). For cell lines that did not harbor a tet-inducible shRNA, the desired shRNA was introduced by lentiviral delivery and selected with puromycin for 48 hr before replating at 10,000 cells/well in 12-well plates. Results were quantified by Vi-Cell Cell Viability Analyzer (Beckman Coulter).

Generation of KP-KrasA Cells and Derivative Lines

Kras^{G12D};p53$^{fl/fl}$ lung adenocarcinoma cells were infected with retroviral vectors TRE-GFP-miR30 shKras-PGK-Puro (Zuber et al., 2011), rtTA3-PGK-Hygro, and MSCV-luciferase-IRES-GFP. GFP$^+$ cells were sorted into single-cell clones to screen for cells showing efficient doxycycline-inducible Kras knockdown, resulting in KP-KrasA, KP-KrasB, and KP-KrasC lines from independent clones. KP-KrasA were stably infected with TRE-dsRed-miR30 shYap1-PGK-Venus-IRES-NeoR (Zuber et al., 2011) to simultaneously express shKras and shYap1 from both TRE promoters upon doxycycline treatment.

Mouse Lung Transplant Model

5×10^4 cells were transplanted into NCr-nu/nu recipient mice (Taconic) by tail-vein injection, and mice were treated with Doxycycline diet *(*Harlan Laboratories). Bioluminescence imaging was performed as described (Xue et al., 2011). Luciferase signal in the lung was quantified using Xenogen software and was normalized to tumors on day 0, before doxycycline treatment.

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