Endogenous Myc maintains the tumor microenvironment

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The ubiquitous deregulation of Myc in human cancers makes it an intriguing therapeutic target, a notion supported by recent studies in Ras-driven lung tumors showing that inhibiting endogenous Myc triggers ubiquitous tumor regression. However, neither the therapeutic mechanism nor the applicability of Myc inhibition to other tumor types driven by other oncogenic mechanisms is established. Here, we show that inhibition of endogenous Myc also triggers ubiquitous regression of tumors in a simian virus 40 (SV40)-driven pancreatic islet tumor model. Such regression is presaged by collapse of the tumor microenvironment and involution of tumor vasculature. Hence, in addition to its diverse intracellular roles, endogenous Myc serves an essential and nonredundant role in coupling diverse intracellular oncogenic pathways to the tumor microenvironment, further bolstering its credentials as a pharmacological target.

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tumor microenvironment plays a critical part in tumor progression and maintenance. We used this model to establish the role that endogenous Myc within tumor cells plays in maintenance of β-cell tumors and their peculiar microenvironment.

Results

Endogenous Myc is required for progression and maintenance of RIP1-Tag2 islet tumors

Expression of the dominant inhibitory Myc dimerization domain mutant Omomyc in TRE-Omomyc;CMVrTA mice is driven from a tetracycline-responsive promoter element [TRE] whose activity is reliant on a reverse tetracycline-dependent transactivator, rTA [Tet-On system], expressed off the CMV promoter (Soucek et al. 2008). The CMV promoter is highly active in most adult mouse tissue types (Furth et al. 1991; Kothary et al. 1991; Baskar et al. 1996; Soucek et al. 2008), and administration of doxycycline to TRE-Omomyc;CMVrTA mice elicits widespread inhibition of endogenous Myc trans-activation, including exocrine and endocrine pancreas (Soucek et al. 2008). TRE-Omomyc;CMVrTA mice were crossed into the RIP1-Tag2 mouse model of pancreatic β-cell cancer (Hanahan 1985). In the absence of doxycycline, TRE-Omomyc;CMVrTA;RIP1-Tag2 triple-transgenic mice developed tumors with incidence, multiplicity, and kinetics of tumor progression indistinguishable from their RIP1-Tag2 littermates (Supplemental Fig. 1). All of the tumors examined were well or moderately differentiated neuroendocrine tumors, based on clinical and pathological criteria, and appeared to be circumscribed, nodular masses with noninfiltrative borders [Fig. 1].

To ascertain the role of endogenous Myc function in the pathogenesis of SV40 T/t-driven islet cancer in RIP1-Tag2 mice, we first asked whether inhibition of Myc prevents expansion and progression of RIP1-Tag2-driven tumors. Systemic Omomyc expression was induced in 7-wk-old TRE-Omomyc;CMVrTA;RIP1-Tag2 mice by addition of doxycycline to their drinking water for seven subsequent weeks, then the animals were sacrificed and their pancreata were harvested and assessed immunohistochemically. As expected, at 14 wk of age, RIP1-Tag2 mice exhibited the predicted distribution of islet tumor sizes and grades as described in Christofori and Hanahan (1994). In contrast, no overt islet tumors were present in Omomyc-expressing animals [TRE-Omomyc;CMVrTA;RIP1-Tag2 mice treated with doxycycline]. Indeed, both islet size and distribution remained similar to that of control littermates not expressing RIP1-Tag2 [Fig. 1A; lower-power-magnification images are also provided as Supplemental Fig. 2]. Hence, Myc inhibition prevents formation of RIP1-Tag2 β-cell tumors.

To assess the impact of inhibiting endogenous Myc on established RIP1-Tag2 islet tumors, we induced Omomyc expression in 11-wk-old TRE-Omomyc;CMVrTA;RIP1-Tag2 mice—which at this age harbor hyperplastic lesions, angiogenic tumors, and carcinomas (Supplemental Fig. 3)—and maintained Omomyc expression for three
triggers rapid degeneration of the tumor microenvironment. Inactivation of ectopic Myc in Myc-driven islet tumors, Omomyc-regressed tumors was comparable with that in glucagon-positive peripheral glucagon expression, and, in general, the ratio of tumor cell proliferation and a significant increase in apoptosis (Fig. 2A), irrespective of tumor grade or stage. Nonetheless, whether endogenous Myc plays an analogous role in driving and maintaining the microenvironment in tumors driven by other oncogenes is unknown. Since the switch to an angiogenic phenotype is a prerequisite for expansion, invasion, and metastasis of RIP1-Tag2 tumors (Bergers et al. 2000), we next examined the impact of Myc inhibition on the microenvironment supporting established RIP1-Tag2 islet tumors. First, Omomyc was induced in 13-wk-old RIP1-Tag2 mice for 1 wk, and tumor hypoxia was assessed. Hypoxia was absent from all normal islets in control mice and was only occasionally evident in some regions of the many large but extensively vascularized β-cell tumors of RIP1-Tag2 mice (19% ± 3.8% of tumors). In contrast, hypoxia was widespread among islet tumors from Omomyc-expressing TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice (68.75% ± 9.5% of tumors) (Fig. 3A). Regions of hypoxia coincided with regions of significant endothelial cell death (27% ± 3.2% of all dying cells in angiogenic lesions and overt tumors of Omomyc-expressing TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice are endothelial), suggesting that hypoxia arises from attrition of tumor vasculature (Fig. 3B). Detailed kinetic analysis of such phenomenon by double staining for Meca32 and active Caspase 3 clearly showed that, at 3 d after doxycycline treatment, the great majority of apoptotic cells in the TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice are endothelial, not tumor (Fig. 4). Onset of tumor cell death follows rapidly thereafter, and, by 7 d of Myc inhibition, the majority of cells dying are tumor β cells. Hence, vasculature collapse precedes tumor death and is not a secondary consequence of tumor regression. Comcomitant analysis of pericytes by immunohistochemistry with NG2 antibody revealed only occasional cell deaths at either 3 or 7 d of Myc inhibition (Supplemental Fig. 6).

The principle proximal mediator of angiogenesis in RIP1-Tag2 tumors is VEGF, which is constitutively present in normal pancreatic islets but is sequestered in an inactive state by the extracellular matrix. The onset of islet tumor angiogenesis in RIP1-Tag2 mice is precipitated by proteolytic release of ECM-bound VEGF and further weeks. Pancreata were then harvested, and tumor status was assessed histologically. Strikingly, whereas the 14-wk-old RIP1-Tag2 Omomyc-negative control group exhibited multiple, large, angiogenic, and aggressive islet tumors, Omomyc-expressing animals (TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice treated with doxycycline) lacked any such lesions (Fig. 1B; low-power-magnification images are also provided as Supplemental Fig. 2). To exclude the formal possibility that Omomyc suppresses expression of SV40 T antigens driven from the insulin promoter in RIP1-Tag2 islets, we directly assayed expression of SV40 large T immunohistochemically. Omomyc induction had no impact on T-antigen expression in RIP1-Tag2 β cells (Supplemental Fig. 4).

To investigate the mechanism of islet tumor regression in RIP1-Tag2 mice following Myc inhibition, we induced Omomyc expression for 1 wk in 13-wk-old TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice and assayed both proliferation and apoptosis. Although overall tumor size is not significantly reduced after only 1 wk of Omomyc expression, Omomyc expression elicited both a profound suppression of tumor cell proliferation and a significant increase in apoptosis (Fig. 2A), irrespective of tumor grade or stage (Supplemental Fig. 5). Hence, Myc inhibition is both cytostatic and cytotoxic in RIP1-Tag2 tumors. Nonetheless, the β cells in the regressed islet tumors of Omomyc-expressing TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice retained the classic aberrant cytologic features of transformed β cells (Fig. 2B), indicating that Myc inhibition does not block SV40 T/t-oncogenic function per se, only the propagation and expansion of such transformed cells. Of note, the regressed islet tumors retained strong peripheral glucagon expression, and, in general, the ratio of glucagon-positive α cells to insulin-positive β cells in Omomyc-regressed tumors was comparable with that in normal islets (Fig. 2C).

Endogenous Myc is required for maintenance of the RIP1-Tag2 islet tumor microenvironment

Inactivation of ectopic Myc in Myc-driven β-cell tumors triggers rapid degeneration of the tumor microenvironment, marked by collapse of tumor vasculature [Pelengaris et al. 2002, Shchors et al. 2006, Soucek et al. 2007]. However, whether endogenous Myc plays an analogous role in driving and maintaining the microenvironment in tumors driven by other oncogenes is unknown. Since the switch to an angiogenic phenotype is a prerequisite for expansion, invasion, and metastasis of RIP1-Tag2 tumors (Bergers et al. 2000), we next examined the impact of Myc inhibition on the microenvironment supporting established RIP1-Tag2 islet tumors. First, Omomyc was induced in 13-wk-old RIP1-Tag2 mice for 1 wk, and tumor hypoxia was assessed. Hypoxia was absent from all normal islets in control mice and was only occasionally evident in some regions of the many large but extensively vascularized β-cell tumors of RIP1-Tag2 mice (19% ± 3.8% of tumors). In contrast, hypoxia was widespread among islet tumors from Omomyc-expressing TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice (68.75% ± 9.5% of tumors) (Fig. 3A). Regions of hypoxia coincided with regions of significant endothelial cell death (27% ± 3.2% of all dying cells in angiogenic lesions and overt tumors of Omomyc-expressing TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice are endothelial), suggesting that hypoxia arises from attrition of tumor vasculature (Fig. 3B). Detailed kinetic analysis of such phenomenon by double staining for Meca32 and active Caspase 3 clearly showed that, at 3 d after doxycycline treatment, the great majority of apoptotic cells in the TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice are endothelial, not tumor (Fig. 4). Onset of tumor cell death follows rapidly thereafter, and, by 7 d of Myc inhibition, the majority of cells dying are tumor β cells. Hence, vasculature collapse precedes tumor death and is not a secondary consequence of tumor regression. Comcomitant analysis of pericytes by immunohistochemistry with NG2 antibody revealed only occasional cell deaths at either 3 or 7 d of Myc inhibition (Supplemental Fig. 6).

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ligation to its principal receptor, VEGF-R2/Flk-1 [Bergers et al. 2000]. Since Myc inhibition triggers rapid vascular attrition, we ascertained whether Myc inhibition abrogates VEGF:VEGF-R2 ligation by staining pancreas sections from Omomyc-expressing TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice with the GVM39 monoclonal antibody that specifically recognizes VEGF:VEGFR2 (Flk1)-ligated conjugates [Brekken et al. 1998]. Omomyc expression blocked all detectable interaction between VEGF and its receptor [Fig. 5], offering a compelling proximal mechanism.

Figure 2. Inhibition of endogenous Myc function decreases proliferation and increases apoptosis in RIP1-Tag2 pancreatic β cells but does not suppress SV40 T/t-induced cellular transformation or disrupt α-cell disposition. (A) Immunohistochemical quantitation of Ki67-positive [top] and TUNEL-positive [bottom] cells in islets from 14-wk-old nontransgenic control [ctr], RIP1-Tag2, and TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice treated with doxycycline for 1 wk prior to sacrifice. Data are represented as mean ± SD. Representative images of islet/β-cell tumors used in this immunohistochemical quantitation are shown on the right [see also Supplemental Fig. 5]. (B) Higher-magnification images of H&E-stained islets isolated from 14-wk-old nontransgenic control [ctr], RIP1-Tag2, and TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice treated with doxycycline for 3 wk prior to sacrifice showing aberrant transformed morphology of β cells in RIP1-Tag2 and TRE-Omomyc;CMVrtTA;RIP1-Tag2 β-cell tumors. (C) Immunohistochemical staining of glucagon-expressing α cells [in red] and insulin-expressing β cells [in green] in islets from 14-wk-old nontransgenic control [ctr], RIP1-Tag2, and TRE-Omomyc;CMVrtTA;RIP1-Tag2 mice treated with doxycycline for 3 wk prior to sacrifice. Nuclear Hoechst counterstaining is shown in blue.
sustained expression of endogenous Myc is required for both recruitment and retention of inflammatory cells by established RIP1-Tag2 tumors.

To identify possible mediators of the Myc-dependent cross-talk between tumor and microenvironment, we used an unbiased approach to identify inflammatory and vascular modulators that are directly and rapidly influenced by Myc inhibition. Laser-captured tumor islets were isolated from the pancreata of 13-wk-old TRE-Omomyc; CMVrtTA;RIP1-Tag2 mice treated with either doxycycline or sucrose for 2 d, and gene expression was profiled and compared. Our analysis focused on cytokine, chemokine, and inflammatory response genes described in the PCR arrays from SA Biosciences (http://www.sabiosciences.com/Cytokines_Inflammation.php). We then identified only those genes that showed a fold change of at least 0.2 between Omomyc-treated and untreated states. We found that multiple genes implicated in inflammation and inflammatory cell recruitment were rapidly modulated by Myc inhibition (Fig. 7). Among the genes down-regulated upon Myc inhibition—such as IL8rb, Il19, Il3, LTB4r2, Il11, Ccl7, Ccl8, and Ccl12—have well-characterized roles in chemotaxis, proliferation, and maturation of various inflammatory cells; others—such as Il1r and Ccr4—are known to influence tumor angiogenesis and invasion. Hif1a was also significantly up-regulated, consistent with the rapid onset of tissue hypoxia observed upon Myc inhibition. Among the genes up-regulated following Myc inhibition were Il12b, ccl5, and Th2, all known to stimulate macrophage and NK activation.

Systemic expression of Omomyc in TRE-Omomyc; CMVrtTA;RIP1-Tag2 mice models a Myc inhibitory drug by blocking Myc function in both SV40 T/t-antigen-transformed β cells themselves and all other cell types of the β-cell compartment of TRE-Omomyc;RIP1-Tag2 tumors. Hence, it is not possible to determine the extent to which the tumoricidal effects of Myc inhibition are due to its effect on the tumor cells themselves versus the tumor microenvironment. To ascertain whether the dramatic collapse of the RIP1-Tag2 tumor microenvironment triggered by Myc inhibition is mediated through the tumor or the stromal compartments, we crossed our TRE-Omomyc;RIP1-Tag2 model
function is absolutely required for RIP1-Tag2 tumor maintenance.

Discussion

We previously used a systemic, switchable dominant-negative approach to show that lung tumors driven by oncogenic KRas in vivo are continuously dependent on endogenous Myc function for their maintenance at all stages of their evolution (Soucek et al. 2008). However, neither the mechanism by which Myc inhibition triggers tumor regression nor the general applicability of Myc inhibition to other tumor types driven by other oncogenic mechanisms was known. Here, we show that inhibition of endogenous Myc also triggers regression of pancreatic islet tumors driven by SV40 large T and small t antigens in RIP1-Tag2 mice. Just as with KRas<sup>G12D</sup>-driven lung tumors, Myc inhibition elicits rapid and widespread onset of tumor cell arrest and apoptosis, culminating in universal regression of all tumors, irrespective of tumor grade or stage. Hence, endogenous Myc function is an obligate requirement for RIP1-Tag2 tumor maintenance. Extensive studies have delineated the mechanisms by which SV40-encoded large T antigen and small t antigen drive tumorigenesis. Large T antigen, SV40's principal oncoprotein, simultaneously incapacitates Rb and p53, the two principal tumor suppressors that restrain promiscuous proliferation. In parallel, small t modulates the pleiotropic impact of the PP2A Ser/Thr phosphatase that modulates multiple kinases downstream from Ras (Ahuja et al. 2005; Sablina and Hahn 2008). Any role played by endogenous Myc in SV40 transformation and tumorigenesis remains undefined. At no stage of RIP1-Tag2 islet tumor evolution do we observe any overt up-regulation of c-Myc protein (Supplemental Fig. 7), suggesting that oncogenically activated Myc is never the oncogenic “driver” of these tumors, but, instead, is a downstream effector of the SV40 T/t oncoproteins. Indeed, t antigen has been shown to enhance...
c-Myc activity by suppressing c-Myc dephosphorylation at Ser 62 and stabilizing the protein. Moreover, a stabilized c-Myc mutant can substitute for t-antigen expression in cell culture transformation assays (Sablina and Hahn 2008), again suggesting that endogenous Myc is a bona fide downstream effector of small t. Large T’s association with p300 is also thought to promote c-Myc activity and potentiate large T-mediated cell cycle entry and cell transformation (Singhal et al. 2008). Of note, the β cells within the regressed islet tumors in Omomyc-expressing TRE-Omomyc; CMVrtTA;RIP1-Tag2 mice remain clearly transformed even when Myc is inhibited. Hence, while growth and maintenance of macroscopic β-cell tumors driven by SV40 T/t require endogenous Myc, at least some cell-intrinsic aspects of SV40 T/t cellular transformation do not. It is also intriguing that, despite their transformed cytological features, the regressed islet tumors in Omomyc-expressing RIP1-Tag2 mice exhibit size, distribution, and α-cell to β-cell ratios similar to islets in normal littermates. Since the α-cell compartment does not expand alongside that of β cells during RIP1-Tag2 tumorigenesis, it would appear that the tumors regress back to both the size and site of their origins. Tumor cells, like their normal counterparts, are completely dependent on their local microenvironment for provision of oxygen, nutrients, survival factors, and supporting stroma. Moreover, tumor growth, just like regeneration of normal tissues, requires a complex interplay between the expanding tissue and various accessory cells—most notably inflammatory cells, endothelial cells, and fibroblasts. However, the reciprocal interaction between tumor cells and stroma is complicated because it evolves over time, making it difficult to address the most important therapeutic question: Which driving processes within a tumor are causally responsible for maintaining the tumor microenvironment, and do those processes originate in the tumor cells themselves or within the multifarious stromal compartment? While multiple studies indicate that ectopic Myc drives and maintains multiple aspects of the tumor microenvironment in transgenic Myc-driven tumors (Pelengaris et al. 1999, 2002; Shchors et al. 2006; Soucek et al. 2007), the role played by endogenous Myc in tumors driven by other oncogenic mechanisms in other tissue types has remained unclear. Our data unequivocally show that short-term systemic Myc inhibition in TRE-Omomyc;CMVrtTA;RIP1-Tag2 β-cell tumors is sufficient to trigger collapse of the tumor microenvironment, with concomitant death of endothelial cells, attenuation of inflammatory cells, vascular collapse, and hypoxia. Importantly, collapse of the tumor microenvironment temporally precedes detectable regression of tumors, implicating it as a potential cause, and certainly not just a consequence, of tumor regression. Moreover, since tumor regression occurs with identical kinetics and gross pathology when endogenous Myc is inhibited solely in the β-cell compartment, this confirms that it is endogenous
Myc within the tumor cells themselves that is responsible for instating and sustaining the signal cross-talk between tumor and microenvironment. Of course, we cannot exclude the possibility that endogenous Myc may also play a tumor maintenance role in cells within the stromal compartment, a role that could further augment the therapeutic impact of any systemic Myc inhibitory therapy. Of note, our data support the notion that targeting Myc, a nonredundant obligate effector lying upstream of the plethora of signals that maintain the tumor microenvironment, may sidestep the adaptive compensation that bedevils existing anti-angiogenic and anti-inflammatory cancer therapies. These same obligate and nonredundant attributes also have the potential to minimize the other principal reason for failure of targeted therapies in cancer treatment; namely, the evolution within tumors of de novo mechanisms that circumvent the tumor cells’ initial requirement for that target. In this regard, it is interesting that, out of several hundred individual β-cell tumors, each comprised of many thousands of tumor cells in which both Rb and p53 had been concomitantly incapacitated, the only occasional tumors in TRE-Omomyc; CMVrtTA; RIP1-Tag2 mice that were refractory to doxycycline had lost expression of the Omomyc transgene, consistent with the idea that Myc inhibition per se cannot be circumvented by compensatory or evolutionary mechanisms. This, together with the remarkable mildness of side effects that systemic Myc inhibition elicits (Soucek et al. 2008), reinforces the notion that inhibiting Myc is an exceptionally promising and generic strategy for treating diverse cancers.

Materials and methods

Generation and maintenance of genetically engineered mice

TRE-Omomyc and RIP1-Tag2 mice have been described previously [Hanahan 1985; Soucek et al. 2008]. CMVrtTA [Tg(rtTAhCMV)/4Bjd/J] mice were purchased from the Jackson Laboratory. RIP-rtTA [Tg(Ins2-rtTA)2Efr/J] mice were obtained from Dr. Shimon Efrat. Omomyc expression was systemically induced in TRE-Omomyc;CMVrtTA; RIP1-Tag2 mice by addition of doxycycline (2 mg/mL, plus 5% sucrose) to their drinking water.
Sucrose only was administered to control mice. Animals were maintained and treated in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California at San Francisco (UCSF). For each experiment, cohorts of at least five mice were used for each data point.

**Tissue preparation and histology**

Mice were euthanized and cardiac-perfused with PBS followed by zinc-buffered formalin. Pancreata were removed, fixed overnight in zinc-buffered formalin, and processed for paraffin embedding. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) using standard reagents and protocols. For frozen sections, pancreata were embedded in OCT, frozen on dry ice, and stored at −80°C.

**Immunohistochemistry**

For immunohistochemical analysis, sections were deparaffinized, rehydrated, and subjected to high-temperature antigen retrieval in 10 mM citrate buffer (pH 6.0). Primary antibodies were as follows: rabbit monoclonal anti-Ki67 (clone SP6; Lab Vision), rabbit polyclonal anti-OMomyc (prepared in-house, Evan laboratory) [Soucek et al. 2004], rat monoclonal anti-MECA32 (BD PharMingen), rabbit polyclonal anti-cleaved Caspase-3 (R&D Systems), mouse monoclonal anti-VEGF/VEGFR complex (clone Gv39M, EastCoast Biol), guinea pig polyclonal anti-insulin (Millipore), rabbit anti-Tag (prepared in the Hanahan laboratory) [Casanovas et al. 2005], rat monoclonal anti-macrophage F4/80 (AbD Serotec), rat monoclonal anti-neutrophils (Clone 7/4, Cedarlane), and rabbit polyclonal anti-NG2 (Millipore). Primary antibodies were applied for 2 h in blocking buffer (2.5% BSA, 5% goat serum, 0.3% Triton X-100 in PBS), sections were washed, and species-appropriate secondary Alexa Fluor 488 dye-conjugated antibodies [Amersham] or Vectastain ABC kit and DAB reagents [Vector Laboratories] were applied. Fluorescence antibody-labeled slides were mounted in DAKO fluorescent mounting medium containing 1 μg/mL Hoechst counterstain. HRP-conjugated secondary antibodies were visualized by DAB staining [Vector Laboratories]. Apoptotic cells were detected by TUNEL [ApopTag Detection kit, Chemicon International] or by expression of activated caspase 3. To stain tissues systemically for hypoxia, 60 mg/kg hypoxypoxy-1 [1-[[2-hydroxy-3-piperidinyl] propyl]-2-nitroimidazole hydrochloride] was administered intraperitoneally in saline 15 min prior to euthanasia. Protein adducts of reductively activated pimonidazole were identified immunohistochemically in fixed tissues with a monoclonal antibody against hypoxypoxy-1. Images were collected with an Axiosvert 5100 TV inverted fluorescence microscope [Zeiss] and Open Lab 3.5.1 software, or with an Axiovert 100 inverted microscope [Zeiss] equipped with a Hamamatsu Orca digital camera.

**Microarray analysis**

For expression profiling, 13-wk-old TRE-OMomyc:CMVrtTA, RIP1-Tag2 mice were treated with either doxycycline (2 mg/mL, plus 5% sucrose; referred to as “treated”) or 5% sucrose (referred to as “untreated”) in their drinking water for 2 d. Pancreata were isolated and embedded immediately with OCT. Fresh-frozen pancreatic sections were fixed in ice-cold 70% ethanol before laser-capture microdissection. A modified H&E staining protocol was used for microscopic visualization of islets while preserving RNA integrity [Goldsworthy et al. 1999, Luzzi et al. 2001]. Tumor islets were isolated with a Zeiss PALM MicroBeam Laser Microdissection system. For each independent sample (n = 6: three treated and three untreated), three to six tumors were captured from four to six adjacent tissue sections and pooled for RNA isolation. Total RNA was isolated using an Arcturus PicoPure RNA isolation kit, and RNA was amplified with a NuGEN FFPE kit [NuGEN Technologies, Inc.] and processed for hybridization on Affymetrix mouse set ST1.0. Robust multivariate (RMA) analysis using Affymetrix Power Tool “APT” software was used to normalize and summarize probe level intensities, and any transcript clusters that, after normalization, had a log-base-2 score <3.0 were discarded. The fold change in expression for each gene was calculated from the ratio of median expression of treated samples versus untreated.

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