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Galectin-3, a druggable vulnerability for KRAS-addicted cancers

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Abstract

Identifying the molecular basis for cancer cell dependence on oncogenes such as KRAS can provide new opportunities to target these addictions. Here, we identify a novel role for the carbohydrate-binding protein Galectin-3 as a lynchpin for KRAS dependence. By directly binding to the cell surface receptor integrin $\alpha v\beta 3$, Galectin-3 gives rise to KRAS addiction by enabling multiple functions of KRAS in anchorage-independent cells, including formation of macropinosomes that facilitate nutrient uptake and ability to maintain redox balance. Disrupting $\alpha v\beta 3$ /Galectin-3 binding with a clinically active drug prevents their association with mutant KRAS, thereby suppressing macropinocytosis while increasing reactive oxygen species to eradicate $\alpha v\beta 3$ -expressing KRAS mutant lung and pancreatic cancer patient-derived xenografts and spontaneous tumors in mice. Our work reveals Galectin-3 as a druggable target for KRAS-addicted lung and pancreas cancer, and indicates integrin $\alpha v\beta 3$ as a biomarker to identify susceptible tumors.

Keywords

Lung cancer; Galectin-3; integrin $\alpha v\beta 3$; oncogenic KRAS; macropinocytosis; reactive oxygen species

INTRODUCTION

Oncogenic KRAS drives a diverse set of cellular mechanisms that support tumor progression, from activation of its effectors MEK and AKT that broadly support cell survival

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and proliferation, to cellular processes representing specific functional advantages, including macropinocytosis [1] and redox balance [2]. However, targeting KRAS has yet to achieve clinical success. Not only does the frequency of KRAS mutation vary between cancer types (95% for pancreatic cancer vs. 25–30% for non-small cell lung cancer), but individual KRAS mutant tumors may develop KRAS indifference over the course of cancer progression [3, 4]. Successfully targeting KRAS will not only require strategies to identify which tumors remain dependent on KRAS expression, but also new approaches to undercut the ability of mutant KRAS to trigger its diverse array of effectors that drive survival and tumor progression.

KRAS nanoclustering has emerged as a critical determinant of KRAS function [5]. Unless oncogenic KRAS can be brought into proximity with its effectors at a relevant microdomain on the cell membrane, an activating KRAS mutation alone may not necessarily trigger specific downstream signaling events that generate KRAS addiction. Furthermore, KRAS signaling specificity is highly influenced by its association with distinct subsets of phospholipids that assemble at the plasma membrane [6], providing an explanation for the ability of KRAS to provoke diverse signaling outputs.

In matrix-adherent cells, KRAS is recruited into membrane nanoclusters by a variety of cell surface receptors that serve as interaction platforms to drive cell survival and proliferation. The redundancy in receptors capable of mediating KRAS clustering may explain why matrix-adherent cells can easily switch dependence between pathways when one is inhibited by a given targeted therapeutic. However, in the absence of matrix adhesion, epithelial cell surface receptors poorly cluster, as has been demonstrated for EGFR [7]. Because the ability of a cell to exhibit anchorage independent growth is a hallmark of tumor progression and metastasis [8, 9], we propose KRAS-containing signaling complexes that form in non-adherent cells as therapeutic targets to reverse anchorage-independence, thereby containing the spread of malignant cells.

Integrin $\alpha v \beta 3$ is unique among integrins for its ability to cluster on the surface of non-adherent tumor cells where it contributes to anchorage-independent growth [10]. This may account for the association of $\alpha v \beta 3$ expression with tumor progression, metastasis, and poor survival for a wide range of cancers [11]. For example, expression of $\alpha v \beta 3$ is increased from 10% in the primary tumor to 24% in metastases for lung cancer [12]. In the unligated state, integrin $\alpha v \beta 3$ is able to cluster and recruit tyrosine kinase signaling molecules to provide cancer cells with growth and survival signals across diverse microenvironments [13]. Anchorage-independent clustering of integrin $\alpha v \beta 3$ is influenced by Galectin-3, a carbohydrate binding protein that binds N-glycans on the integrin's extracellular domain [14], and we recently reported this $\alpha v \beta 3$ /Galectin-3 interaction as an important modulator of EGFR inhibitor resistance by virtue of its ability to induce KRAS clustering in non-adherent cells [15].

We therefore asked whether integrin $\alpha v \beta 3$ /Galectin-3 might represent an Achilles' heel for KRAS mutant lung and pancreatic cancers, and if this could explain why only certain KRAS mutant tumors remain addicted to KRAS for survival after tumor initiation. Not only do we

demonstrate the potential of integrin $\alpha v\beta 3$ as a biomarker to identify KRAS addiction, but we also provide a molecular explanation for this dependency.

RESULTS

Galectin-3 and integrin $\alpha v\beta 3$ drive lung cancer addiction to oncogenic KRAS

25–30% of non-small cell lung cancers express mutant KRAS [16], yet only about half of these are addicted or depend on KRAS for their survival [17], thus representing an interesting model to investigate the molecular basis for KRAS addiction. Since we previously reported that the association between integrin $\alpha v\beta 3$, Galectin-3, and KRAS on the surface of lung cancer cells led to EGFR resistance [15], we considered whether this interaction might be a contributing factor to KRAS oncogene addiction. To do this, we selected three KRAS mutant lung adenocarcinoma cell lines with detectable $\alpha v\beta 3$ expression and three lacking $\alpha v\beta 3$ expression to evaluate KRAS dependency on anchorage-independent growth (Figure 1A; Supplementary Fig. S1A, Supplementary Table S1). Notably, expression of other integrin subunits ($\beta 1$, $\beta 5$, and αv) or KRAS-GTP activity were not linked to $\beta 3$ expression. Strikingly, the $\alpha v\beta 3+$ cell lines were addicted to both KRAS and Galectin-3, while the $\alpha v\beta 3-$ cell lines showed no such dependence (Fig. 1B; Supplementary Fig. S1B). Not only was integrin $\alpha v\beta 3$ necessary for 3D growth (Fig. 1C; Supplementary Fig. S1C–D), but its ectopic expression was sufficient to render cells dependent on both KRAS and Galectin-3 (Fig. 1D; Supplementary Fig. S1C and E). Whereas 2D anchorage-dependent growth of $\alpha v\beta 3$ -positive cells did not require expression of KRAS, Galectin-3, or integrin $\beta 3$, $\alpha v\beta 3$ -negative cells did require KRAS for 2D growth (Fig. 1E). Together, these findings indicate that KRAS addiction arises from tumor cell reliance on an integrin $\alpha v\beta 3$ /Galectin-3 pathway that supports anchorage-independent growth in 3D, but which is dispensable in matrix-adherent cells.

We therefore considered the prevalence of the $\alpha v\beta 3$ -positive/KRAS mutant subpopulation in human lung cancer. For 86 KRAS mutant lung cancer patient-derived xenograft (PDX) models in the Crown Biosciences HuPrime database, 21% show significant integrin $\beta 3$ mRNA expression. Because the function of the integrin $\alpha v\beta 3$ heterodimer requires the $\beta 3$ subunit to couple with the αv subunit on the cell surface, we also screened 13 of these PDX models for tumor cell $\alpha v\beta 3$ protein expression by immunostaining tumor sections (Supplementary Table S2). We found a wide range of $\alpha v\beta 3$ expression on tumor cells, measured as the percent positive cells per field (Fig. 1F). As for our panel of lung cancer cell lines, expression of $\alpha v\beta 3$ was not linked to any particular KRAS mutation (Supplementary Table S1–S2). As expected, we also observed $\alpha v\beta 3$ expression on tumor-associated stromal cells and blood vessels [11]. For two PDX models with heterogeneous tumor cell expression of $\alpha v\beta 3$ (PDX-8 and PDX-9), we confirmed that knockdown of either KRAS, Galectin-3, or $\beta 3$ significantly decreased anchorage-independent growth (Fig. 1G; Supplementary Fig. S1F). Using flow cytometry to sort the heterogeneous tumors for $\beta 3$ -hi and $\beta 3$ -lo populations, we show that only the $\beta 3$ -hi population is sensitive to knockdown of either KRAS or Galectin-3 (Fig. 1G; Supplementary Fig. S1F). This work reveals an $\alpha v\beta 3$ -positive subset of KRAS-mutant lung cancer that shows KRAS addiction in the context of anchorage-independent growth.

While KRAS signals through multiple effector pathways, we find that $\beta 3$ expression is linked to the activation of phospho-Akt, but not phospho-Erk (Fig. 1H). While knockdown of KRAS decreases both p-Akt and p-Erk, knockdown of either $\beta 3$ or GAL3 primarily suppresses the ability of mutant KRAS to activate Akt only (Fig. 1I). These findings are in accordance with recent studies that highlight “context-dependent” functions of KRAS [18].

To examine these relationships in the absence of other oncogenic drivers, we generated mouse embryonic fibroblasts (MEFs) from animals expressing wildtype KRAS, mutant KRAS G12D, Galectin-3 knockout, or the combination. Whereas KRAS activity driven by the G12D mutation did not require Galectin-3 expression (Fig. 1J), the transforming potential of the KRAS G12D mutation requires Galectin-3 as does phosphorylation of Akt (Fig. 1K and Supplementary Fig. 1H). Together, these findings suggest that $\alpha v\beta 3$ -positive cells are uniquely addicted to mutant KRAS for anchorage-independent growth, and point to Galectin-3 as a critical mediator of this activity.

Only KRAS-addicted lung cancer cells expressing $\alpha v\beta 3$ depend on macropinocytosis

One recently described feature of KRAS mutant cancer is an enhanced ability for macropinocytosis [1], a process that provides tumor cells with the ability to consume protein from their in vivo microenvironment as a unique source of amino acids [19, 20]. We therefore asked if this functional benefit was common to all KRAS mutant cells, or if $\alpha v\beta 3$ /Galectin-3 might influence whether a given cell developed an addiction to this advantage. To do this, we measured cellular uptake of high molecular weight TMR-dextran and quantified the fluorescent signal emitted by degradation of a self-quenched albumin. As observed for the addiction to both KRAS and Galectin-3, $\alpha v\beta 3$ + cells showed markedly enhanced EIPA-sensitive nutrient uptake compared with $\alpha v\beta 3$ - cells growing in suspension (Fig. 2A–B) but not in 2D adherent culture conditions (Supplementary Fig. S2A). We selected H727 and A549 cells to represent $\beta 3$ - and $\beta 3$ + groups, respectively, based on their intermediate levels of TMR-dextran uptake. Importantly, macropinocytosis was inhibited by knockdown of KRAS, $\beta 3$, or Galectin-3 in A549 cells, and enhanced by ectopic $\beta 3$ expression in H727 cells (Fig. 2C; Supplementary Fig. S2B). For the PDX models with heterogeneous $\alpha v\beta 3$ expression, individual cells with high $\alpha v\beta 3$ expression showed the highest levels of macropinocytosis by flow cytometry analysis (Fig. 2D).

Consistent with these findings, treatment with ethylisopropyl amiloride (EIPA), an inhibitor of macropinocytosis that does not affect other endocytic pathways [21], suppressed anchorage-independent growth of $\alpha v\beta 3$ +, but not $\alpha v\beta 3$ -, cells (Fig. 2E). In contrast, an inhibitor of clathrin-mediated endocytosis had an equivalent effect on all cells (Fig. 2E). As further evidence that $\alpha v\beta 3$ is a critical determinant of this behavior, ectopic $\beta 3$ expression was sufficient to enhance H727 cell sensitivity to EIPA (Fig. 2F), and the $\beta 3$ -positive population of PDX cells were significantly more sensitive to EIPA than the $\beta 3$ -negative population (Fig. 2G; Supplementary Fig. S2C). Together, these findings suggest that macropinocytosis does not contribute to the anchorage independence of KRAS mutant lung cancer cells unless both integrin $\alpha v\beta 3$ and Galectin-3 are expressed.

Considering that the KRAS G12D mutation was required for anchorage-independent growth of mouse embryonic fibroblasts with endogenous $\alpha v\beta 3$ expression (Fig. 1J–K), we asked

whether sensitivity to EIPA may also require KRAS mutation. Whereas fibroblasts with wildtype KRAS were largely insensitive to EIPA, expression of the G12D KRAS mutation was sufficient to induce an addiction to macropinocytosis that, as for anchorage-independent growth, required expression of Galectin-3 (Fig. 2H). Together, these findings indicate that the enhanced capacity for anchorage-independent growth observed for $\alpha v\beta 3$ + KRAS mutant lung cancer cells can be attributed in part to their ability to use macropinocytosis for nutrient uptake. Because this process provides an additional energy route to support growth within nutrient-poor environments, blocking this pathway using EIPA or by targeting Galectin-3 provide new options to target the unique vulnerabilities of $\alpha v\beta 3$ -expressing KRAS mutant lung cancers.

Only $\alpha v\beta 3$ + KRAS mutant tumor cells maintain low mitochondrial ROS levels

In addition to macropinocytosis, oncogenic KRAS has recently been linked to oxidative stress detoxification during tumorigenesis [2]. Because redox homeostasis has also been implicated in the adaptation to anchorage independence [22], we considered cellular detoxification as an additional hallmark vulnerability of KRAS-addicted lung cancer cells. Consistent with this notion, KRAS knockdown in $\alpha v\beta 3$ + cells strongly induced accumulation of cellular ROS, visualized by an increase in 8-oxo-dG or MitoSOX™ Red probes by immunofluorescence staining (Fig. 3A). In fact, only $\alpha v\beta 3$ + cells could manage the oxidative stress induced by hydrogen peroxide during anchorage-independent growth (Fig. 3B), suggesting a potential link between stress tolerance and the combination of mutant KRAS and $\alpha v\beta 3$ expression. Indeed, expressing $\beta 3$ in H727 cells increased their tolerance to this form of oxidative stress (Fig. 3C). As further evidence, the $\beta 3$ -positive population of the PDX-9 tumor shows lower ROS levels than the unsorted or $\beta 3$ -negative cells (Fig. 3D), and integrin $\alpha v\beta 3$ and Galectin-3 were both necessary and sufficient to maintain low ROS levels in KRAS mutant cells (Fig. 3E). These findings demonstrate that KRAS-addicted lung cancer cells require both Galectin-3 and integrin $\alpha v\beta 3$ to counteract the effects of oxidative stress, an advantage which may link KRAS addiction, tumor progression, and therapy resistance.

A clinically active Galectin-3 inhibitor disrupts Galectin-3/ $\alpha v\beta 3$ binding

Our findings reveal that the impact of mutant KRAS in lung cancer cells is dependent on both integrin $\alpha v\beta 3$ and Galectin-3, and we demonstrated the functional implications of this for both macropinocytosis and redox balance. Considering that this pathway leads to various biological functions and may involve multiple effectors that could vary between individual tumors, we reasoned that disrupting signaling at the highest upstream point should provide the most robust effect. While we previously reported a biochemical association between integrin $\alpha v\beta 3$, KRAS, and Galectin-3 [15], it was not clear whether there was a direct binding between these factors, and how such an interaction might be prevented. Using a cell-free binding assay, we report here that Galectin-3 directly binds to $\alpha v\beta 3$ in a dose-dependent and saturable manner, and this can be blocked by competitive binding with lactose, a sugar that mimics galactose binding to Galectin-3 (Fig. 4A–B). Furthermore, we demonstrate that this $\alpha v\beta 3$ /Galectin-3 interaction can be blocked with the anti-Galectin-3 drug GCS-100 (Fig. 4C), a Galectin-3-binding polysaccharide derived from citrus pectin that has shown a good safety profile and activity in early clinical phase trials for cancer [23–25]. For $\alpha v\beta 3$ -

expressing lung cancer cells growing in suspension, GCS-100 decreases the cell surface expression of GAL3 (Fig. 4D). This indicates that soluble extracellular GAL3 cannot bind to any of its cell surface receptors (including $\alpha\text{v}\beta 3$) in the presence of the GCS-100 drug. Thus, this inhibitor provides a novel opportunity to perturb mutant KRAS/GAL3/ $\alpha\text{v}\beta 3$ complex using an upstream approach.

Both $\alpha\text{v}\beta 3$ expression and KRAS mutation are required for sensitivity to the Galectin-3 inhibitor, GCS-100

Sensitivity to GCS-100 is dependent on integrin $\alpha\text{v}\beta 3$, since cells positive for $\beta 3$ expression were sensitive to the drug, while $\beta 3$ -negative cells were resistant (Fig. 4E). Furthermore, the $\beta 3$ -positive population of cells sorted from a heterogeneous PDX-9 lung tumor were more sensitive to the drug relative to the $\beta 3$ -negative or unsorted populations (Fig. 4F). We next asked whether the effects of GCS-100 were restricted to cells expressing mutant as opposed to wildtype KRAS. Interestingly, KRAS G12D MEFs (which express endogenous $\alpha\text{v}\beta 3$) are more sensitive to GCS-100 than MEFs isolated from KRAS wildtype mice, and we confirm that expression of Galectin-3 is required for this effect (Fig. 4G). Treating established $\alpha\text{v}\beta 3$ -positive lung cancer patient-derived xenograft (PDX) tumors with GCS-100 significantly decreased tumor growth (Fig. 5A), and this was associated with increased apoptosis evaluated by TUNEL staining (Fig. 5B).

We therefore considered whether GCS-100 could slow tumor progression for a spontaneous mouse model of KRAS-driven lung cancer. Three months after intratracheal adeno-Cre delivery to LSL-KRAS^{G12D} mice, we confirmed the presence of lung tumors with integrin $\alpha\text{v}\beta 3$ expression on tumor cells and pathological evidence of regions containing adenoma, dysplasia, and adenocarcinoma (Fig. 5C–D). Mice treated for 1 month with GCS-100 showed not only a significantly smaller tumor burden, but this agent prevented tumor progression as there was no evidence of adenocarcinoma in the mice (Fig. 5E). Importantly, treated tumors showed more apoptosis than controls (Fig. 5F). This finding demonstrates that Galectin-3 inhibition has the potential to alter the phenotype of KRAS-driven tumors to have a marked impact on tumor progression in immune-competent mice. In concordance with our hypothesis that tumor cell addiction to mutant KRAS is modulated by $\alpha\text{v}\beta 3$ /Galectin-3, the ability of GCS-100 to disrupt this interaction suggests its potential application as a therapeutic agent for KRAS mutant cancers.

We therefore asked whether $\alpha\text{v}\beta 3$ expression might predict GCS-100 sensitivity for pancreatic ductal adenocarcinoma, a cancer characterized by more than 95% frequency of KRAS mutation [26]. As for lung cancer, pancreatic carcinoma cell lines were varied in their expression of integrin $\beta 3$, and $\beta 3$ expression was associated with phosphorylation of Akt for cells in 3D, but not 2D (Fig. 6A). GCS-100 shows $\alpha\text{v}\beta 3$ -dependent activity in vitro (Fig. 6B) and in vivo for pancreatic cancer xenografts (Fig. 6C) and PDX models (Fig. 6D) that are driven by oncogenic KRAS mutations. As for the lung cancer models, the anti-tumor activity of GCS-100 appears to involve an induction of cell death (TUNEL staining) without altering the extent of cell proliferation (Ki-67 staining) (Fig. 6E). Thus, in both immune-compromised and immune-competent mice, inhibiting Galectin-3 with GCS-100 selectively inhibits the growth of KRAS mutant tumors expressing integrin $\alpha\text{v}\beta 3$.

Galectin-3 blockade disrupts KRAS clustering in anchorage-independent cells, to reduce nutrient uptake and increase mitochondrial ROS levels

Previous studies established a role for Galectin-3 in the clustering of integrin $\alpha v\beta 3$ on endothelial cells [14], and we reported that Galectin-3 knockdown prevented the clustering of $\alpha v\beta 3$, as well as the recruitment of KRAS, in cancer cells [15]. Here, we demonstrate how the biochemical association between $\beta 3$ /KRAS can be disrupted pharmacologically by using the Galectin-3 inhibitor GCS-100 (Fig. 7A–B). A consequence of this is a marked decrease in phospho-Akt, while the drug does not impact phospho-Erk or KRAS-GTP activity (Fig. 7A–B). In fact, phospho-Akt represents a very clear readout for activity of this drug in $\alpha v\beta 3$ -expressing tumors (Fig. 7C–E). By preventing the ability of integrin $\alpha v\beta 3$ to associate with KRAS, GCS-100 blocks the mutant KRAS-mediated survival advantages to which $\alpha v\beta 3$ -expressing cells have become addicted. Accordingly, treatment with GCS-100 not only prevents the cells from engulfing nutrients via macropinocytosis (Fig. 7F–G), but increases the ROS levels for KRAS mutant cells in 3D culture (Fig. 7H), tumor xenografts in vivo (Fig. 7I), and PDX tumors ex vivo (Fig. 7I).

DISCUSSION

Our findings indicate that lung adenocarcinoma addiction to oncogenic KRAS depends on Galectin-3 to assemble an $\alpha v\beta 3$ /KRAS complex (Fig. 7J). Direct binding between $\alpha v\beta 3$ and Galectin-3 can convert cells expressing mutant KRAS to a KRAS-addicted state, and disrupting this complex with a Galectin-3 inhibitor suppressed macropinocytosis, increased ROS, and selectively blocked the growth of KRAS-addicted lung and pancreatic cancer in mice. These findings provide a rationale for the design of clinical trials enabling the selective therapeutic targeting of KRAS-addicted cancers with the use of $\alpha v\beta 3$ as a biomarker for these tumors.

A new molecular definition of KRAS addiction

Despite intense efforts to target KRAS or its key effectors, compensatory pathways have limited the ability of such agents to achieve clinical impact [26, 27]. Although KRAS drives tumorigenesis of lung and pancreatic cancer, we and others [3] find that only certain KRAS mutant lung cancers continue to need KRAS during tumor progression. Our findings provide a molecular explanation for KRAS addiction driven by $\alpha v\beta 3$ /Galectin-3, and link this to the ability of mutant KRAS to initiate both macropinocytosis and ROS neutralization.

Alterations in nutrient uptake and metabolic reprogramming provide tumor cells with an enhanced ability to survive within an adverse environment. In particular, active RAS has been linked to a hyper-macropinocytotic phenotype that allows cells to engulf nutrients and alter their metabolic dependencies [1, 28]. In contrast, we find that only a subset of KRAS mutant lung cancer cells show enhanced macropinocytosis, and that expression of $\alpha v\beta 3$ defines this subset. In fact, cells that rely on KRAS-mediated macropinocytosis also depend on the ability of $\alpha v\beta 3$ to cluster on the surface of cells with Galectin-3, forming a complex that recruits active KRAS to the membrane. This is supported by the finding that only tumors with mutant KRAS and $\alpha v\beta 3$ expression are sensitive to the macropinocytosis inhibitor, EIPA.

In addition to driving macropinocytosis dependency, the $\alpha v\beta 3$ /Galectin-3 complex is required to maintain low mitochondrial ROS levels in KRAS-addicted cells. It is well appreciated that a tumor cell with enhanced cellular detoxification abilities, including some that are driven by active RAS [2], can survive and proliferate despite diverse environmental stresses or exposure to cancer therapy. It is therefore interesting to consider how individual cancer cells may be able to maintain redox balance, e.g. by upregulating antioxidant expression.

Notably, we propose integrin $\alpha v\beta 3$ as a biomarker capable of identifying KRAS addiction (and GCS-100 sensitivity) independent from any other genetic alterations. While the $\beta 3$ subunit associates only with αv (ubiquitously expressed) and αIIb (predominantly in platelets) [11], αv combines with multiple other β subunits to form heterodimers with diverse functions. As such, $\beta 3$ expression is rate limiting for $\alpha v\beta 3$ expression on virtually all epithelial cells. Differences in protein or mRNA expression of the $\beta 3$ subunit are therefore sufficient to identify tumor cell addiction to this pathway, whereas measuring αv expression alone would not predict KRAS addiction. Similarly, Galectin-3 protein expression is detectable in all cell lines and PDX models examined, suggesting that Galectin-3 levels are not likely to predict sensitivity to a Galectin-3 inhibitor. Within the tumor microenvironment, soluble Galectin-3 released from stromal cells might interact with $\alpha v\beta 3$ on the surface of a tumor cell. Taken together, our results suggest that the ability of Galectin-3 to directly bind the $\beta 3$ integrin ectodomain creates addiction to both Galectin-3 (as a mediator of $\alpha v\beta 3$ clustering in anchorage-independent cells) and KRAS (as a critical downstream effector that drives multiple survival advantages).

It is also interesting to point out that, among the $\alpha v\beta 3$ -positive KRAS mutant cell lines we have analyzed in our study, A549 cells have mutant LKB1 with wildtype p53, while the H1792 cells express wildtype LKB1 and mutant p53. Despite these differences, both cell lines are addicted to KRAS and require integrin $\alpha v\beta 3$ /Galectin-3. As such, targeting Galectin-3 as a requisite for KRAS clustering in cells with $\alpha v\beta 3$ expression may provide a more universal approach to disable the downstream effects that could vary between tumors with distinct genetic drivers of cancer.

KRAS-mediated viability in 3D culture as a surrogate for KRAS addiction and tumor progression in vivo

Notably, our determination of KRAS dependency in 3D culture varies from the designations previously reported for some of the same cell lines tested under 2D culture [3, 29]. We believe the key difference is that $\alpha v\beta 3$ is the only integrin capable of clustering on the surface of non-adherent cells [11], whereas multiple receptors are able to recruit KRAS into focal-adhesion type signaling nodes that are supported by cell-matrix adhesion. As a result, we propose a unique role for mutant KRAS in promoting the survival of the $\alpha v\beta 3$ -positive subset of lung cancer cells in the setting of anchorage-independent growth, in vitro conditions that partially recapitulate the environmental stresses tumor cells must overcome during metastasis and tumor progression in vivo [10, 30]. In fact, we show that ectopic expression of $\beta 3$ is sufficient to drive KRAS addiction, suggesting $\alpha v\beta 3$ -negative cells are simply unable to engage this survival mechanism (i.e., KRAS cannot otherwise be clustered)

in cells growing under anchorage-independent conditions. Our findings suggest that cooperation between integrin $\alpha v\beta 3$ and Galectin-3 provides an important contextual signal that is required for the function of mutant KRAS, and that disruption of this pathway could represent a unique vulnerability that could be targeted therapeutically.

In the context of 2D growth, Singh and colleagues classify the $\beta 3$ -positive A549, SKLU1, and PANC1 cells as KRAS independent, and the $\beta 3$ -negative H727 and H441 cells as KRAS-dependent [3], and they show that A549/SKLU1/PANC1 cells are able to activate Akt in the absence of KRAS during 2D growth, while we suggest that integrin $\alpha v\beta 3$ is the only integrin capable of recruiting and associating with KRAS to activate Akt in 3D. While $\alpha v\beta 3$ might drive KRAS independence in 2D by activating Akt in a manner that circumvents KRAS, we focus primarily on 3D growth conditions that mimic the in vivo tumor growth environment required for invasion and metastasis [31]. As such, we believe that the growth advantage imparted by $\alpha v\beta 3$ /KRAS in 3D can be explained by their ability to promote Akt activity that drives both macropinocytosis and redox balance, features that may be less critical for cells growing in a 2D monolayer with media containing full serum.

Galectin-3, a clinically relevant target to attack KRAS-addicted lung cancer

Previous studies have shown that Galectin-3, a metastasis-inducing protein expressed by tumor cells and inflammatory cells [32–34], not only binds in a multimeric manner to the oligosaccharides on the extracellular domain of integrin $\alpha v\beta 3$ [14], but it also associates directly or indirectly with KRAS [35], mediates KRAS clustering at the plasma membrane [36–38], and can augment KRAS activity [39, 40]. In fact, we previously reported that $\alpha v\beta 3$ and Galectin-3 form a membrane complex with KRAS to promote tumor stemness and drug resistance [15]. It is therefore remarkable that only certain KRAS mutant lung cancer cells are inherently “addicted” to Galectin-3 during anchorage-independent growth, a feature we attribute to its impact on $\alpha v\beta 3$ recruitment of KRAS.

One of the most interesting outcomes of our study is that a clinically active Galectin-3 inhibitor, GCS-100, has the capacity to undermine the survival of tumor cells that are $\alpha v\beta 3$ -positive and KRAS mutant in vitro and in vivo, including a spontaneous KRAS driven lung cancer model. In this model GSC-100 actually prevented tumor progression as measured by dysplasia and adenoma but a complete absence of adenocarcinoma. In fact, among KRAS mutant lung and pancreatic tumors, $\alpha v\beta 3$ expression was necessary and sufficient for the effects of this drug. This activity was not only related to epithelial cancers, as Galectin-3 was required to drive tumor formation among MEFs expressing mutant but not wildtype KRAS. This suggests that oncogenic activation of KRAS is not sufficient to drive KRAS addiction, but that the recruitment of KRAS by $\alpha v\beta 3$ /Galectin-3 is critically important.

The selective activation of Akt in $\alpha v\beta 3$ -expressing KRAS mutant carcinoma cells may also contribute to KRAS addiction, considering that PI3K/Akt drives survival signaling. Although Akt has many effectors, its phosphorylation in cells and tumors treated with GCS-100 is markedly suppressed, suggesting that $\alpha v\beta 3$ /KRAS/GAL3 is a driver of p-Akt during anchorage-independent growth and tumor progression of KRAS-addicted tumors. While p-Akt provides a readout for activity of the Galectin-3 inhibitor GCS-100, our work does not exclude the possibility that additional KRAS effectors may also contribute to the

survival advantages driven by KRAS in $\alpha v\beta 3$ -expressing cells. As such, targeting KRAS function by virtue of its association with integrin $\alpha v\beta 3$ represents a strategy to block multiple KRAS-driven functions including but not limited to macropinocytosis and redox balance.

Galectin-3 can modulate the function of multiple cell types important during for progression [33, 41], and the early development of Galectin-3 inhibitors for cancer therapy focused on blocking the function of tumor-associated endothelial cells [42]. While we cannot rule out that the tumor growth inhibition observed for GCS-100 is exclusively due to its disruption of $\alpha v\beta 3$ clustering on tumor cells, we do show a similar level of tumor growth inhibition in immune-compromised vs. immune-competent mice (Fig. 5C–F), and we show selective drug activity for tumors with ectopic $\beta 3$ expression but not the respective parental line (Fig. 6C). Together, these findings suggest that blockade of the $\alpha v\beta 3$ /Gal3 interaction on the surface of tumor cells represents a novel approach to target KRAS addicted cancers.

Concluding remarks

Although mutant KRAS is involved both in lung cancer tumorigenesis and progression, it becomes dispensable for the progression of a subset of tumors. Here, we identify lung cancers that remain addicted to KRAS by virtue of their dependence on key KRAS-mediated survival advantages, including nutrient uptake and redox balance. While previous studies link macropinocytosis to oncogenic KRAS in pancreas cancer [1], we find that only certain KRAS mutant lung cancers (those expressing both Galectin-3 and integrin $\alpha v\beta 3$) rely on this process for anchorage-independent growth. Although KRAS is implicated as a driver of redox gene expression [2, 43], we show that $\alpha v\beta 3$ -negative cells are more sensitive to oxidative stress and show higher basal ROS levels than $\alpha v\beta 3$ -positive cancer cells. In this respect, our findings provide a new mechanistic explanation to account for KRAS addiction by linking established KRAS functions to the ability of KRAS to form a complex with integrin $\alpha v\beta 3$ in anchorage-independent cells. Since tumors addicted to this pathway for survival are highly sensitive to Galectin-3 inhibition, this strategy represents an approach to treat the subset of KRAS mutant lung cancers for which there are currently few viable options (Fig. 7J). Interrupting this pathway at the level of $\alpha v\beta 3$ /KRAS association may prove more promising than strategies to block individual KRAS-driven functions, which are not likely limited to only macropinocytosis and redox balance.

METHODS

Cell culture

Cells were confirmed mycoplasma-free before experiments. Cells were purchased in 2013 (A549, H441 and PANC-1), 2014 (SKLU-1 and H1792) and 2015 (A427 and H727), from the American Type Culture Collection (ATCC) and grown in recommended medium supplemented with 10% fetal bovine serum and glutamine (RPMI for A549, H441, H1792 and H727; EMEM for SKLU-1 and A427, DMEM for PANC-1). FG and FG $\beta 3$ cells (DMEM) were obtained as described [10]. Identity of the A549 cell line was confirmed using DNA fingerprinting. In all experiments, each cell line was passaged less than 15 times. Unless stated otherwise, tissue culture plates were coated with a 6% poly-HEMA solution to

create a non-adherent condition wherein cells tend to grow in floating clumps rather than attach to the bottom of the wells.

Compounds and reagents

EIPA (5-(N-Ethyl-N-isopropyl)amiloride), hydrogen peroxide, chlorpromazine, lactose, and sucrose were purchased from Sigma-Aldrich. The Galectin-3 inhibitor GCS-100 was provided by La Jolla Pharmaceutical Company.

Genetic knockdown and ectopic expression

Cells were transfected with vector control or integrin $\beta 3$ using a lentiviral system as described [15]. For knockdown experiments, cells were transfected with shRNA (Open Biosystems) using a lentiviral system and the constructs listed in Supplementary Table 3. Lentiviruses were produced by co-transfection of 293T cells with lentiviral backbone constructs and packaging vectors (ps-PAX2 and VSVG) using Lipofectamine 3000 (Thermo Fisher). Supernatant was collected 48 and 72 h post-transfection and resuspended in an appropriate volume of OptiMEM (Gibco). Knockdowns were confirmed by immunoblot.

Immunoblotting

Immunoblotting was performed as described [15] for lysates generated using RIPA, Triton, or NP-40 buffers. 25 μ g protein was boiled in Laemmli buffer and resolved on an 8–15% gel. Primary antibodies: integrin $\beta 3$ (Cell Signaling Technology (CST) 13166S 1:1000), KRAS (Abgent AT2650a 1:1000), Galectin-3 (Santa Cruz sc-20157 1:500), pAKT (CST 2606S 1:1000), AKT (CST 2938S 1:1000), pERK (CST 9101S 1:2000), ERK1/2 (CST 4695S 1:1000), HSP70 (Santa Cruz sc-221731 1:1000), GAPDH (CST 2118S 1:5000) and HSP90 (Santa Cruz sc-7947 1:1000).

GST-pull down of activated RAS

Levels of activated RAS in cell lysates were determined using a RAS Activation Assay Kit (EMD Millipore # 17-218) following the manufacturer instructions with anti-KRAS mouse monoclonal antibody (Abgent AT2650a).

Immunoprecipitation

Lysates from cells grown in 3D were generated using MLB buffer (Millipore # 20-168). Immunoprecipitation experiments were performed using 500 μ g of protein and anti-KRAS (Abgent AT2650a).

Anchorage-independent growth and cell viability

Soft agar assays were performed as described [10] for 14–21 days with weekly media replacement. Cells were grown on poly-HEMA coated plates for 3–6 days in serum-free media, and CellTiterGLO viability assays (Promega) were performed according to manufacturer instructions [44]. To evaluate growth in a clonogenic assay, mouse embryonic fibroblasts were seeded in a 96-well plate in complete media. After 1 day, serum-free media containing test agents was added and refreshed twice per week, and crystal violet staining was performed after 2 weeks.

Evaluation of reactive oxygen species (ROS)

MitoSOX™ Red (Thermo Fisher) was used according to manufacturer instructions to assess mitochondrial oxidation by superoxide in live cells using confocal microscopy [45] or flow cytometry. Alternatively, cells were immunostained with mouse monoclonal 8 hydroxy 2' deoxyguanosine (Abcam #ab48508) as a measure of oxidative damage to DNA. Images were acquired using confocal or light microscopy.

Macropinosome visualization and quantification

As described [1, 46], cells were grown in 3D using poly-HEMA coated plates in serum-free media for 3 hours. Macropinosomes were marked using a high-molecular TMR-dextran and/or DQ-Green BSA (Life Technologies) at a final concentration of 1 mg/ml for 1 hour at 37°C. Cells were rinsed in cold PBS, fixed in 3.7% formaldehyde, and coverslips mounted using Hard Set (Vector Labs). Images were captured using a Nikon Eclipse C1 confocal microscope with 1.4 NA 60× oil-immersion lens and minimum pinhole (30 μm), and analyzed using the 'Analyze Particles' feature in ImageJ. Particle area per cell was determined from at least three randomly selected fields, each containing approximately 75–150 cells depending on cell size and seeding density. As described for the *ex vivo* assay [47], tumors were cut into 3-mm cubes, immersed in serum-free RPMI with 1 mg/ml of FITC-dextran at 37°C for 1 hour, rinsed in PBS, and frozen in optimal cutting temperature (OCT) compound.

Isolation of cells from patient-derived xenograft tumors

PDX tumors were harvested from mice, washed with cold PBS containing antibiotics, chopped with a sterile blade, and incubated in 0.001% DNase (Sigma–Aldrich), 1 mg/ml collagenase/dispase (Roche), 200 U/ml penicillin and 200 mg/ml streptomycin in DMEM/F12 medium in a 37°C water bath for 1 h with intermittent shaking. The suspensions were repeatedly triturated, passed through 70- and 40-mm cell strainers and centrifuged at 300g for 5 min at 4°C. Cells were re-suspended in red blood cell lysis buffer for 4 min at room temperature with intermittent shaking before re-suspension in serum-free medium. Viability was evaluated by exclusion of Trypan blue dye (Thermo Fisher). For some experiments, cells were sorted into β3+ and β3- populations using flow cytometry as described [15].

Immunofluorescence microscopy and flow cytometry

Single cell suspensions from patient-derived xenograft tumors were processed as described [15] using anti-αvβ3 (LM609, Millipore, 1:1000) followed by AlexaFluor-labeled secondary antibodies (Invitrogen) with DAPI (Thermo Fisher) as a nuclear marker. Samples were imaged on a Nikon Eclipse C1 confocal microscope with 1.4 NA 60× oil-immersion lens, using minimum pinhole (30 μm). For FACS analysis, cells were stained with Galectin-3 (BioLegend 125401, 0.5 mg/mL diluted 1:200), LM609 (1:1000), or isotype control.

Immunohistochemical analysis

Tumor sections were subjected to haematoxylin and eosin (H&E) staining following standard protocols. TUNEL staining for apoptosis was performed using the APO-BrdU-IHC

assay (Phoenix Flow Systems #AH1001). 5 μ m-thick sections of paraffin-embedded tumors were immunostained using the VECTASTAIN Elite ABC HRP kit (Vector Labs). Primary antibodies for human tumors included integrin β 3 (CST #13166) pAKT (CST # 3787) and 8-oxo-d-Guo (Abcam #ab48508). Mouse tumors were stained using integrin β 3 (Abcam #ab119992). H&E-stained lung tissues were imaged on a NanoZoomer Slide Scanning System (Hamamatsu). Tumor burden was measured with the ImageJ Threshold Color plugin.

Integrin α v β 3-Galectin-3 cell-free binding assay

96-well plates were coated with purified human integrin α v β 3 (Millipore CC1021, 0.5 μ g in 100 μ l), incubated at 4°C overnight, and blocked with 50 mg/mL BSA for 90 minutes at 30°C. Recombinant human Galectin-3 (R&D Systems 1154-GA-050, 1 μ g/well) and test agents were added for a total volume of 50 μ l, then incubated for 4 hours at 30°C. Wells were washed, fixed with 2% PFA in PBS for 15 minutes at room temperature, washed, and incubated with rat monoclonal Galectin-3 antibody (BioLegend 125401, 0.5 mg/mL diluted 1:100) for 30 minutes on ice. After washing, wells were incubated with secondary antibody (Life Technologies A21210, AF488 rabbit anti-rat IgG diluted 1:200) for 30 minutes on ice. After washing, fluorescence was read using a Tecan Infinite M200 (excitation 485, emission 538) to quantify α v β 3/Galectin-3 binding.

Animals

All research was conducted under protocol S05018 and approved by the UCSD Institutional Animal Care and Use Committee. All studies are in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

LSL-KRAS-G12D mouse model

As previously published for KRAS^{LSLG12D} mice, intra-tracheal delivery of adeno-Cre induces oncogenic KRAS in lung airway cells, leading to multi-focal adenocarcinomas and a median survival of about 6 months [48]. Starting with tumors established for 3 months in adult B6.129 KRAS^{LSLG12D} (Jackson Labs, 008179) mice of either gender, systemic dosing with vehicle or 20 mg/kg GCS-100 three times a week by i.p. injection was performed for 1 additional month. Tumors were harvested, fixed, and stained for tumor burden analysis using H&E. Scanned slides were scored by a pathologist for the presence of adenomas with benign proliferation versus preneoplastic lesions (dysplasia) and adenocarcinomas, as defined [49, 50].

Patient-derived xenograft models

Subcutaneous PDX tumors were grown in 6–8 week old female SCID mice as reported [15]. Molecular Response LLC (now Crown Bioscience) provided slides containing tumor sections of the PDX models listed in Supplementary Table S2 to allow immunohistological screening for integrin α v β 3 expression, as well as cryovials of the 2001030397 PDX model for use in vitro and implantation in vivo. The UC San Diego Moores Cancer Center Biorepository and Jackson Labs provided the MCCT-009.4-LG1202F PDX model. The pancreatic PDX model was provided by Dr. Andrew Lowy at UCSD under UCSD

Institutional Review Board (IRB) protocol #090401. FG and FG β 3 human pancreatic carcinoma cells (5×10^6 tumor cells in 200 μ l PBS) were injected subcutaneously to the flank of 6–8 week old female nu/nu mice. Animals bearing 150–250 mm³ tumors were randomly allocated for treatment with vehicle or 20 mg/kg GCS-100 i.p. three times per week.

Statistical analyses

All analyses were carried out using Prism software (GraphPad). One-way ANOVA or Welch's t-tests were used to calculate significance using $P < 0.05$. 2+ independent experiments were carried out for all in vitro tests. All of the figures showing immunoblots or micrographs were independently repeated three times. The number of independent experiments versus technical replicates is specified in each figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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STATEMENT OF SIGNIFICANCE

There is a significant unmet need for therapies targeting KRAS mutant cancers. Here, we identify integrin $\alpha v \beta 3$ as a biomarker to identify mutant KRAS-addicted tumors that are highly sensitive to inhibition of Galectin-3, a glycoprotein that binds to integrin $\alpha v \beta 3$ to promote KRAS-mediated activation of Akt.

Figure 1. Integrin $\alpha v\beta 3$ and Galectin-3 drive KRAS addiction for a subset of KRAS mutant lung cancers

A) A subset of KRAS mutant lung cancer cells express integrin $\alpha v\beta 3$

B–D) 3D growth capacity was quantified by counting colony formation in soft agar for 14 days.

E) Effect of shRNA-mediated knockdowns on 2D or 3D viability was measured using CellTiter-Glo® for 72 hours.

F–G) Patient-derived xenograft (PDX) tumors were stained for $\beta 3$ expression (brown) using immunohistochemistry. Scale bar, 50 μ m. The effect of KRAS, Galectin-3, or $\beta 3$ knockdown on 3D growth was evaluated as colony formation in soft agar.

H–I) Western blots show phospho-Akt and phospho-Erk.

J–K) Embryonic fibroblasts from genetically-engineered mice (wildtype, KRAS G12D, Galectin-3 knockout, or the combination) were compared for KRAS activity using a GST-pull down assay and colony formation in 2D. Scale bar, 250 μ m.

All data represent mean $\pm$ standard deviation from at least 3 independent experiments.

Statistical significance was determined by Student's t-test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

See also Supplementary Fig. S1.

Figure 2. KRAS-addicted lung cancer cells achieve enhanced nutrient uptake via macropinocytosis

A) Macropinocytosis uptake assay using TMR-dextran as a marker of macropinosomes (red) in lung cancer cells after 4 hours in 3D under serum deprivation, including the macropinocytosis inhibitor, EIPA. Scale bar, 10 μ m.

B) Uptake and proteolytic cleavage de-quenches the DQ-BSA signal (green), indicating uptake of nutrients into functional macropinosomes. Scale bar, 10 μ m.

C) α v β 3-positive A549 cells require expression of KRAS, β 3, and Galectin-3 for uptake of TMR-dextran, while this is enhanced in α v β 3-negative H727 cells by ectopic β 3 expression.

D) For patient-derived xenograft tumors with heterogeneous expression of $\alpha v\beta 3$, the DQ-BSA signal indicating macropinocytotic uptake is enhanced in cells positive for $\beta 3$ expression. Scale bar, 10 μ m.

E) Inhibitors of macropinocytosis (EIPA) vs. clathrin-mediated endocytosis (Chlorpromazine) were tested for their effect on cell viability in 3D.

F) Ectopic expression of $\beta 3$ integrin sensitized H727 cells to the effects of the macropinocytosis inhibitor EIPA.

G) Patient-derived xenograft tumors with heterogeneous expression of $\alpha v\beta 3$ were sorted by $\beta 3$ expression and the effects of EIPA on cell viability in 3D was tested using the CellTiter-Glo assay.

H) In mouse fibroblasts, expression of oncogenic KRAS and Galectin-3 is required for sensitivity to EIPA.

All data represent mean $\pm$ standard deviation from at least 3 independent experiments.

Statistical significance was determined by Student's t-test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

See also Supplementary Fig. S2.

Figure 3. KRAS-addicted lung cancer cells need $\alpha v\beta 3$ /Galectin-3 to maintain low ROS levels

A) The effect of KRAS knockdown on mitochondrial ROS levels was visualized as fluorescence staining using MitoSOX Red or 8-oxo-dG. MitoSOX Red staining was quantified by flow cytometry (4 hours). Scale bar, 10μm.

B) The effect of oxidative stress (hydrogen peroxide) on viability was compared for cells growing in suspension.

C) Ectopic $\beta 3$ expression protects H727 cells from the effects of oxidative stress.

D) By flow cytometry, the $\beta 3$ -high population sorted from patient-derived xenograft PDX-8 show lower levels of mitochondrial stress (MitoSOX) compared with the $\beta 3$ -low population.

E) The effect of $\beta 3$ or Galectin-3 knockdown on ROS levels was evaluated using flow cytometry (MitoSOX).

All data represent mean $\pm$ standard deviation from at least 3 independent experiments.

Statistical significance was determined by Student's t-test *P<0.05, **P<0.01, ***P<0.001.

Figure 4. Blocking $\alpha v \beta 3$ /Galectin-3 binding with GCS-100 selectively kills KRAS-addicted lung cancer cells

A–B) A cell-free binding assay shows direct binding between integrin $\alpha v \beta 3$ and Galectin-3, and its competitive inhibition by lactose.

C) A Galectin-3 inhibitor, GCS-100, disrupts $\alpha v \beta 3$ /Galectin-3 binding in the cell free assay.

D) Flow cytometer analysis for cell surface Galectin-3 expression.

E–F) The effect of GCS-100 on 3D viability of KRAS mutant lung cancer cell lines and PDX models is enhanced by $\alpha v \beta 3$ expression.

G) Embryonic fibroblasts from genetically-engineered mice (wildtype, KRAS G12D, Galectin-3 knockout, or the combination) were compared for sensitivity to GCS-100. All data represent mean $\pm$ standard deviation from at least 3 independent experiments. Statistical significance was determined by Student's t-test *P<0.05, **P<0.01, ***P<0.001.

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Figure 5. GCS-100 selectively kills KRAS-addicted lung tumors

A) Mice with established subcutaneous KRAS mutant lung PDX-8 tumors were treated with vehicle (n=9) or GCS-100 (n=10) (20 mg/kg i.p. 3 times per week) for 15 days. Change in tumor volume vs. time (left) and after 15 days of treatment (right).

B) Sections from PDX-8 tumors were stained for TUNEL as an indicator of apoptosis, and the percent of TUNEL+ cells quantified using ImageJ. Scale bar, 50 μ m.

C–D) KRAS^{LSLG12D} mice were infected with Adeno-Cre via intratracheal injection. After 3 months, sections of lung tissue reveal β 3-expressing tumors with areas of adenoma, dysplasia, and adenocarcinoma. Scale bar, 50 μ m.

E) Mice were randomized and treated with either vehicle control or GCS-100 for 1 additional month. Representative histological images show lung tumor burden. Scale bar, 2 mm and 500 μ m for inset. Tumor burden (tumor area as a percentage of total lung area) in KRAS^{LSLG12D} mice treated with vehicle (n=7) or GCS-100 (n=7) at a dose of 20 mg/kg i.p. 3 times per week. Table shows effect of the drug on tumor histopathology.

F) Effect of GCS-100 on apoptosis, evaluated using TUNEL staining by IHC. Scale bar, 50 μ m.

All data represent mean $\pm$ standard deviation from at least 3 independent experiments.

Statistical significance was determined by Student's t-test *P<0.05, **P<0.01, ***P<0.001.

Figure 6. GCS-100 shows efficacy only for KRAS mutant $\alpha\beta3$ + cancer cells

A) Protein expression of integrin $\beta3$ is shown for a panel of KRAS mutant pancreatic cancer cell lines.

B) Expression of $\alpha\beta3$ enhances sensitivity to GCS-100 for pancreatic cancer cells in vitro.

C–D) Mice with established subcutaneous FG, FG+ $\beta3$, or PDX pancreatic tumors were treated with vehicle or GCS-100 (20 mg/kg i.p. 3 times per week). Change in tumor volume vs. time (left) and at the endpoint (right). Scale bar, 5mm.

E) PDX tumor sections were stained for TUNEL as an indicator of apoptosis and Ki-67 as an indicator of cell proliferation, and the percent of positive cells quantified using ImageJ. Scale bar, 100 μ m.

All data represent mean $\pm$ standard deviation from at least 3 independent experiments.

Statistical significance was determined by Student's t-test *P<0.05, **P<0.01, ***P<0.001.

Figure 7. Galectin-3 blockade prevents $\alpha v\beta 3$ /KRAS complex, reduces nutrient uptake, and enhances ROS levels

A–B) Disrupting Galectin-3 using GCS-100 prevents biochemical association between $\alpha v\beta 3$ and KRAS, and blocks phosphorylation of Akt.

C–E) p-Akt immunostaining is significantly suppressed in tumors from mice treated systemically with GCS-100. Scale bar, 5 μ m.

F) GCS-100 treatment reduces macropinocytosis in $\alpha v\beta 3$ -positive lung cancer cells, measured as TMR-dextran uptake and DQ-BSA de-quenching. Scale bar, 10 μ m.

G) Representative images from vehicle or GCS-100-treated PDX tumors incubated ex vivo with DQ-BSA (green). Tumor cells are marked by anti-cytokeratin staining (red) Scale bar, 50 μ m. FITC-BSA uptake was quantified using ImageJ.

H) Treating α v β 3-positive A549 or H1792 cells with GCS-100 (to inhibit Galectin-3) increases mitochondrial ROS levels (MitoSox signal analyzed by flow cytometry).

I) Immunohistochemical analysis of oxidative stress (8-oxo-dG expression) in FG, FG+ β 3, and PDX-8 tumors treated with vehicle or GCS-100. Scale bars, 20 μ m (in E) and 100 μ m (in F).

J) Schematic summarizes the molecular basis for KRAS addiction of KRAS-dependent lung cancer and potential for clinical development of GCS-100.

All data represent mean $\pm$ standard deviation from at least 3 independent experiments.

Statistical significance was determined by Student's t-test *P<0.05, **P<0.01, ***P<0.001.