Oncogenic KRAS Regulates Tumor Cell Signaling via Stromal Reciprocation

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Graphical Abstract

Highlights
- KRAS\textsuperscript{G12D} establishes a reciprocal signaling axis via heterotypic stromal cells
- Reciprocal signaling further regulates tumor cell signaling downstream of KRAS\textsuperscript{G12D}
- Reciprocal signaling regulates tumor cell behavior via AXL/IGF1R-AKT
- Heterocellularity expands tumor cell signaling beyond cell-autonomous pathways

In Brief
Cell-specific proteome labeling reveals that oncogenic KRAS stimulates stromal cells to initiate reciprocal signaling back to pancreatic tumor cells, thereby enabling signaling capacity beyond the traditionally studied cell-autonomous pathways.

Accession Numbers
PXD003223
Oncogenic KRAS Regulates Tumor Cell Signaling via Stromal Reciprocation

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http://dx.doi.org/10.1016/j.cell.2016.03.029

SUMMARY

Oncogenic mutations regulate signaling within both tumor cells and adjacent stromal cells. Here, we show that oncogenic KRAS (KRASG12D) also regulates tumor cell signaling via stromal cells. By combining cell-specific proteome labeling with multivariate phosphoproteomics, we analyzed heterocellular KRASG12D signaling in pancreatic ductal adenocarcinoma (PDA) cells. Tumor cell KRASG12D engages heterotypic fibroblasts, which subsequently instigate reciprocal signaling in the tumor cells. Reciprocal signaling employs additional kinases and doubles the number of regulated signaling nodes from cell-autonomous KRASG12D. Consequently, reciprocal KRASG12D produces a tumor cell phosphoproteome and total proteome that is distinct from cell-autonomous KRASG12D alone. Reciprocal signaling regulates tumor cell proliferation and apoptosis and increases mitochondrial capacity via an IGF1R/AXL-AKT axis. These results demonstrate that oncogene signaling should be viewed as a heterocellular process and that our existing cell-autonomous perspective underrepresents the extent of oncogene signaling in cancer.

INTRODUCTION

Solid cancers are heterocellular systems containing both tumor cells and stromal cells. Coercion of stromal cells by tumor cell oncogenes profoundly impacts cancer biology (Friedl and Alexander, 2011; Quail and Joyce, 2013) and aberrant tumor-stroma signaling regulates many hallmarks of cancer (Hanahan and Weinberg, 2011). While individual oncogene-driven regulators of tumor-stroma signaling have been identified, the propagation of oncogene-dependent signals throughout a heterocellular system is poorly understood. Consequently, our perspective of oncogenic signaling is biased toward how oncogenes regulate tumor cells in isolation (Kolch et al., 2015).

In a heterocellular cancer, tumor cell oncogenes drive aberrant signaling both within tumor cells (cell-autonomous signaling) and adjacent stromal cells (non-cell-autonomous signaling) (Croce, 2008; Egeblad et al., 2010). As different cell types process signals via distinct pathways (Miller-Jensen et al., 2007), heterocellular systems (containing different cell types) theoretically provide increased signal processing capacity over homocellular systems (containing a single cell type). By extension, oncogene-dependent signaling can theoretically engage additional signaling pathways in a heterocellular system when compared to a homocellular system. However, to what extent activated stromal cells reciprocally regulate tumor cells beyond cell-autonomous signaling is not well understood.

We hypothesized that the expanded signaling capacity provided by stromal heterocellularity allows oncogenes to establish a differential reciprocal signaling state in tumor cells. To test this hypothesis, we studied oncogenic KRAS (KRASG12D) signaling in pancreatic ductal adenocarcinoma (PDA). KRAS is one of the most frequently activated oncogenic drivers in cancer (Pylayeva-Gupta et al., 2011) and is mutated in >90% of PDA tumor cells (Almoguera et al., 1988). PDA is an extremely heterocellular malignancy—composed of mutated tumor cells, stromal fibroblasts, endothelial cells, and immune cells (Neesse et al., 2011). Crucially, the gross stromal pancreatic stellate cell (PSC) expansion observed in the PDA microenvironment is non-cell-autonomously controlled by tumor cell KRASG12D in vivo (Collins et al., 2012; Ying et al., 2012). As a result, understanding the heterocellular signaling consequences of KRASG12D is essential to comprehend PDA tumor biology.

Comprehensive analysis of tumor-stroma signaling requires concurrent measurement of cell-specific phosphorylation events. Recent advances in proteome labeling now permit cell-specific phosphoproteome analysis in heterocellular systems (Gauthier et al., 2013; Tape et al., 2014a). Furthermore, advances in proteomic multiplexing enable deep multivariate phospho-signaling analysis (McAlister et al., 2012; Tape et al., 2014b). Here, we combine cell-specific proteome labeling, multivariate phosphoproteomics, and inducible oncogenic mutations to describe KRASG12D cell-autonomous, non-cell-autonomous, and reciprocal signaling across a heterocellular system. This study reveals KRASG12D uniquely regulates tumor cells via heterotypic stromal cells. By exploiting heterocellularity, reciprocal signaling enables KRASG12D to engage oncogenic signaling
pathways beyond those regulated in a cell-autonomous manner. Expansion of KRASG12D signaling via stromal reciprocation suggests oncogenic communication should be viewed as a hetero-
cellular process.

RESULTS

Tumor Cell KRASG12D Non-cell-autonomously Regulates Stromal Cells

To investigate how KRASG12D supports heterocellular communication, we first analyzed tumor cell-secreted signals (using PDA tumor cells containing an endogenous doxycycline inducible KRASG12D) (Collins et al., 2012; Ying et al., 2012). Measuring 144 growth factors, cytokines, and receptors across three unique PDA isolations, we observed that KRASG12D increased secretion of GM-CSF, GCSF cytokines, and the growth factor production from PSCs (e.g., IGF1 and GAS6), we hypothesized that KRASG12D-activated PSCs initiate a reciprocal signaling axis back in the tumor cells. However, given that tumor cells already undergo phosphoproteomic deregulation by KRASG12D, it was unclear whether additional reciprocal signals from PSCs can further regulate the tumor cell phosphoproteome. To investigate this, the phosphoproteome of KRASWT and KRASG12D PDA cells were directly compared to PDA cells treated with conditioned media from SHH-activated PSCs (Figure 3A). Despite the considerable regulation of cell-autonomous signaling by KRASG12D, PDA cells are further modulated by signals from SHH-activated PSCs (Figure 3B). In fact, PSC-signaling regulates (+/-1 log2, p < 0.01) comparable numbers of PDA tumor cell phosphosites (6.7% phosphoproteome) when compared to KRASG12D alone (7.2% phosphoproteome) (Figure 3C; Data S1). This implies stromal cells can substantially alter tumor cell signaling beyond cell-autonomous KRASG12D. Notably, while PDA KRASG12D expression does not activate AKT in a cell-autonomous manner (Figures 2 and S3), tumor cell AKT substrate phosphosites (e.g., AKTS1 [pT247] and GSK3β [pS21]) are exclusively regulated by stromal PSCs (Figures S4C–S4E).

Targeted temporal analysis revealed SHH-activated PSCs induce rapid phosphorylation of IGF1R (receptor for IGF1), AXL/TYRO3 (receptor for GAS6), and downstream IRS-1 and AKT (pT308/pS473) in KRASG12D PDA cells (Figure 3D). Tumor cells treated with conditioned media from SHH-activated PSCs and perturbed with either MEK and/or AKT (MK-2206) inhibitors and analyzed by quantitative phosphoproteomics. This analysis confirmed MEK-ERK1/2, not AKT, controls the differential phosphoproteome of KRASG12D (Figure 3E; Data S1).

Activated Stromal Cells Extend Tumor Cell Signaling beyond Cell-Autonomous KRASG12D

Given that KRASG12D non-cell-autonomously regulates growth factor production from PSCs (e.g., IGF1 and GAS6), we hypothesized that KRASG12D-activated PSCs initiate a reciprocal signaling axis back in the tumor cells. However, given that tumor cells already undergo phosphoproteomic deregulation by KRASG12D, it was unclear whether additional reciprocal signals from PSCs can further regulate the tumor cell phosphoproteome. To investigate this, the phosphoproteome of KRASWT and KRASG12D PDA cells were directly compared to PDA cells treated with conditioned media from SHH-activated PSCs (Figure 3A). Despite the considerable regulation of cell-autonomous signaling by KRASG12D, PDA cells are further modulated by signals from SHH-activated PSCs (Figure 3B). In fact, PSC-signaling regulates (+/-1 log2, p < 0.01) comparable numbers of PDA tumor cell phosphosites (6.7% phosphoproteome) when compared to KRASG12D alone (7.2% phosphoproteome) (Figures 3C, S4A, and S4B; Data S1). This implies stromal cells can substantially alter tumor cell signaling beyond cell-autonomous KRASG12D. Notably, while PDA KRASG12D expression does not activate AKT in a cell-autonomous manner (Figures 2 and S3), tumor cell AKT substrate phosphosites (e.g., AKTS1 [pT247] and GSK3β [pS21]) are exclusively regulated by stromal PSCs (Figures S4C–S4E).

Targeted temporal analysis revealed SHH-activated PSCs induce rapid phosphorylation of IGF1R (receptor for IGF1), AXL/TYRO3 (receptor for GAS6), and downstream IRS-1 and AKT (pT308/pS473) in KRASG12D PDA cells (Figure 3D). Tumor cells treated with conditioned media from control or SHH-activated PSCs and perturbed with either MEK and/or AKT inhibitors further confirmed PSCs drive a differential phosphoproteome in PDA cells. However, unlike cell-autonomous KRASG12D, stromal-driven signaling depends on both active MEK and AKT (Figure 3E; Data S1). As IGF1 and GAS6 are secreted by activated PSCs, we investigated the dependency of IGF1R and AXL activity on the
PSC-induced tumor cell phosphoproteome. Combined IGF1R and AXL inhibitors are required to block the PSC-induced tumor cell phosphoproteome—suggesting a Boolean “OR” axis between PSC IGF1/GAS6 and PDA pAKT (Figures 3F, 3G, and S4F; Data S1).

Collectively, these results reveal activated stromal cells can return a differential signal to tumor cells via an IGF1R/AXL-AKT axis. The stromal-driven tumor cell phosphoproteome is distinct from the KRASG12D regulated cell-autonomous phosphoproteome and responds differently to pharmacological perturbation.

**KRASG12D Regulates Tumor Cell Signaling via a Reciprocal Signaling Axis**

Our data suggests that oncogenic KRAS in tumor cells establishes a reciprocal signaling axis between stromal cells and tumor cells. Herein, we define an oncogenic reciprocal signaling axis as an oncogenic cue that signals via an adjacent heterotypic...
cell to produce a distinct response in the oncogene-expressing cell. For this heterocellular variation on the “cue-signal-response” systems biology paradigm (Janes et al., 2004, 2005; Miller-Jensen et al., 2007) to be valid, we hypothesized that oncogenic reciprocal signaling requires three essential features: (1) an oncogenic cue (e.g., KRAS\textsuperscript{G12D}), (2) a cue-driven non-cell-autonomous signal (e.g., KRAS\textsuperscript{G12D}-induced SHH), and (3) a heterotypic cell capable of transducing the signal response back to the instigating oncogenic cell (e.g., PSC). To test this multi-node reciprocal signaling hypothesis, we systematically perturbed each reciprocal feature in a native heterocellular tumor-stroma context.

To measure multivariate signaling in a heterocellular system, concurrent cell-specific and variable-specific phosphoproteomic data are required. We have previously shown that stable isotopic proteome labeling (Ong et al., 2002) can resolve between discrete cell types in direct culture of heterotypic cells (Jorgensen et al., 2009) and recently introduced cell type-specific labeling with amino acid precursors (CTAP) (Gauthier et al., 2013) Ly\textsuperscript{MYT-KDEL} and DDC\textsuperscript{MYT-KDEL} enzymes for cell-specific isotopic labeling (Tape et al., 2014a). To this end, we combined CTAP labeling (spatial resolution) with isotopic tandem mass tag (TMT) phosphoproteomics (variable resolution) (Tape et al., 2014b; Thompson et al., 2003) to enable heterocellular multivariate phosphoproteomic analysis of each reciprocal signaling component (Figure 4A). This technique allows simultaneous observation of cell-autonomous, non-cell-autonomous, and reciprocal oncogenic phosphoproteomes at cell-specific resolution.

Cell-specific phosphoproteomes were interrogated in PDA cells expressing either KRAS\textsuperscript{WT} or KRAS\textsuperscript{G12D}, either in homo- or heteroculture with isotopically “heavy”-labeled PSCs, and treated with either SHH inhibitor or vehicle. We monitored 3,695 lysine-containing (8,566 total) phosphopeptides across eight conditions, two heterotypic cell types, and three biological replicates with cell-specific resolution (Figures 4B and S5; Data S1). As expected, expression of KRAS\textsuperscript{G12D} in tumor cells alone regulates (+/−1 log\textsubscript{2}) 7.2% of the identified cell-autonomous phosphoproteome. In parallel, tumor cell KRAS\textsuperscript{G12D} non-cell-autonomously regulates 4.7% of the PSC phosphoproteome. Moreover, when KRAS\textsuperscript{G12D} is allowed to communicate with PSCs via SHH, a reciprocal axis is completed and the differentially regulated tumor cell phosphoproteome almost doubles to 13.8%. Importantly, perturbation by a SHH blocking antibody decreases the phosphoproteomic regulation on PSCs back down to 1.2% and PDA phosphoproteome to 8.1% (close to cell-autonomous at 7.2%).

Heterocellular multivariate phosphoproteomics demonstrates how tumor cell oncogenes exploit the differential signaling capacity of stromal cells to achieve a unique signaling state in the inceptive tumor cell. KRAS\textsuperscript{G12D} reciprocal signaling engages additional phospho-nodes to cell-autonomous KRAS\textsuperscript{G12D} alone, allowing KRAS\textsuperscript{G12D} to extend the oncogenic signaling capacity in the inceptive tumor cells. Crucially, these observations are the product of native tumor-stroma signaling and are independent of exogenous stimulation.

KRAS\textsuperscript{G12D}-Driven Reciprocal Signaling Regulates the Tumor Cell Phosphoproteome and Total Proteome

Comprehensive phosphoproteomic quantification of reciprocally engaged PDA cells (Figures 5A–5C and S6A; Data S1) revealed upregulation of several AKT substrates (e.g., BAD [pS136], PDCD4 [pS457], CHSP1 [pSS3], AKTS1 [T247], and GSK-3\textsuperscript{\alpha} [pS21]). Interestingly, cell-autonomous targets of KRAS\textsuperscript{G12D} were not regulated by reciprocal signaling—further implying reciprocal KRAS\textsuperscript{G12D} supplements cell-autonomous KRAS\textsuperscript{G12D} by engaging additional tumor cell kinases (Figure S6B).
Reciprocal signaling also activates several translational mediators (e.g., RPS6 [pS235/pS236], PDCD4 [pS457], and EIF4B [pS422]). Concordantly, RNA sequencing (RNA-seq) analysis of PDA cells revealed reciprocal signaling upregulates RNA associated with translational control (Figures S6C–S6F), further suggesting a de novo control of PDA protein abundance. To validate whether the SHH-driven reciprocal signaling axis regulates de novo tumor cell protein turnover, PSC+DDC_M.tub-KDEL and PDA+LyrM37-KDEL CTAP cells were differentially isotopically labeled, treated with a SHH inhibitor or vehicle, and cell-specific proteomes were quantified in heteroculture (Figure 5D). This experimental format permitted cell-specific quantification of changes to the KRASG12D tumor cell proteome following inhibition of the PSC targeting signal (SHH). Parallel perturbations with AKT and IGF1R/AXL inhibitors provided additional insight into the role of each reciprocal node.

Cell-specific proteomics confirmed KRASG12D reciprocally regulates the PDA proteome and is dependent on active SHH, IGF1R/AXL, and AKT signaling (Figure 5E; Data S1). As with the PDA phosphoproteome, reciprocal signaling regulates the PDA proteome differently to cell-autonomous KRASG12D. For example, while cell-autonomous KRASG12D rapidly depletes distinct mitochondrial components from PDA cells (Data S1) (Viale et al., 2014), reciprocally engaged KRASG12D restores mitochondrial proteins in an SHH-, IGF1R/AXL-, and AKT-dependent manner. Moreover, PDA proteins involved with DNA replication are also upregulated under reciprocal conditions. These results demonstrate reciprocal signaling uniquely regulates both the tumor cell phosphoproteome and global proteome when compared to cell-autonomous signaling. Reciprocal signaling states are unique to a heterocellular environment and are not observed in tumor cells alone.

KRASG12D-Driven Reciprocal Signaling Regulates Tumor Cell Phenotypes

Reciprocal signaling regulates proteins and phospho-sites known to control several important biological processes. For example, while cell-autonomous and reciprocal KRASG12D signaling both regulate mitochondrial proteins, many of these are asymmetrically regulated. As a result, we hypothesized PDA mitochondrial activity would be differentially regulated by cell-autonomous and reciprocal KRASG12D. Concordantly, cell-autonomous KRASG12D decreases PDA mitochondria polarization (Δψm) and mitochondrial superoxide production, whereas reciprocal signaling increases these processes (via SHH, IGF1R/AXL, and AKT) (Figures 6A and S7). Furthermore, reciprocal
signaling increases spare mitochondrial respiratory capacity in tumor cells (Figure 6B). These results demonstrate KRASG12D can differentially regulate mitochondrial performance via heterocellular communication.

Reciprocal signaling also regulates proteins known to control cell proliferation and survival. In agreement, cell-specific analysis of PDA proliferation in homo and heterocellular cultures revealed increased tumor cell proliferation under heterocellular conditions (via SHH, IGF1R/AXL, and AKT activity) (Figure 6C). Upregulation of AKT substrates (e.g., inhibition of BAD [pS136]) also suggested reciprocal signaling might protect tumor cells from apoptosis. Concordantly, TUNEL and caspase 3/7 profiling revealed activated PSCs protect tumor cells from apoptosis and sensitize tumor cells to reciprocal node inhibitors (IGF1R/AXL and AKT) (Figures 6D–6E).

Increased mitochondrial performance, proliferative capacity, and resistance to apoptosis collectively implied reciprocal signaling supports tumor cell phenotypes beyond cell-autonomous KRASG12D. In accordance, reciprocal signaling increases semi-solid colony growth relative to cell-autonomous KRASG12D alone (Figure 6F). Reciprocal colony growth is dependent on SHH activation of PSCs and IGF1R/AXL-AKT activity in tumor cells. Collectively, these results demonstrate the unique signals produced by reciprocal KRASG12D control distinct metabolic, proliferative, anti-apoptotic, and anchorage-independent growth phenotypes in tumor cells.

**DISCUSSION**

Whether oncogenes regulate tumor cell signaling via stromal cells is a fundamental question in tumor biology. Using heterocellular multivariate phosphoproteomics, we demonstrate how oncogenic KRAS signals through local non-tumor cells to achieve a differential reciprocal signaling state in the inceptive tumor cells. In PDA, this reciprocal axis supplements oncogenic cell-autonomous signaling to control protein abundance, transcription, mitochondrial activity, proliferation, apoptosis, and colony formation. Reciprocal signaling is the exclusive product of heterocellularity and cannot be achieved by tumor cells alone. These observations imply oncogenes expand their capacity to deregulate cellular signaling via stromal heterocellularity (Figure 7).

Despite the well-established heterocellularity of cancer, our understanding of oncogenic signaling within tumor cells has largely excluded non-tumor cells. We observe that stromal cells approximately double the number of tumor cell signaling nodes regulated by oncogenic KRAS, suggesting both cell-autonomous (internal) and reciprocal (external) stimuli should be considered when defining aberrant oncogenic signaling states. For example, although KRAS is thought to cell-autonomously regulate AKT in PDA (Eser et al., 2014), we show that KRASG12D activates AKT, not cell-autonomously, but reciprocally. As PI3K signaling is essential for PDA formation in vivo (Baer et al., 2014; Eser et al., 2013; Wu et al., 2014) reciprocal signaling may control oncogene-dependent tumorigenesis. Our findings suggest future genetic studies should consider the heterocellular signaling consequences of oncogene/tumor-suppressor deregulation.

The observation that many oncogene-dependent tumor cell signaling nodes require reciprocal activation has important implications for identifying pharmacological inhibitors of oncogene signaling. For example, if PDA tumor cells were screened alone, one would expect MEK, MAPK, and CDK inhibitors to perturb KRASG12D signaling. However, when screened in conjunction with heterotypic stromal cells, our study additionally identified SHH, AKT, and IGF1R/AXL inhibitors as KRASG12D-dependent...
targets in tumor cells. Inhibitors of signaling specific to reciprocally engaged tumor cells, such as or AKT or IGF1R/AXL, block heterocellular phenotypes (e.g., protein expression, proliferation, mitochondrial performance, and anti-apoptosis), but have little effect on KRASG12D tumor cells alone. An appreciation of reciprocal nodes increases our molecular understanding of drug targets downstream of oncogenic drivers and highlights focal points where reciprocal signals converge (e.g., AKT). These trans-cellular observations reinforce the importance of understanding cancer as a heterocellular disease.

Previous work in PDA tumor cells under homocellular conditions demonstrated cell-autonomous KRASG12D shifts metabolism...
toward the non-oxidative pentose phosphate pathway (Ying et al., 2012), whereas KRASG12D-ablated cells depend on mitochondrial activity (Viale et al., 2014). Here, we show that heterocellular reciprocal signaling can restore the expression of mitochondrial proteins and subsequently re-establish both mitochondrial polarity and superoxide levels. This suggests KRASG12D regulates non-oxidative flux through cell-autonomous signaling and mitochondrial oxidative phosphorylation through reciprocal signaling. These results provide a unique example of context-dependent metabolic control by oncogenes and reinforce the emerging role of tumor-stroma communication in regulating cancer metabolism (Ghesquiere et al., 2014).

In PDA, the stroma has dichotomous pro-tumor (Kraman et al., 2010; Sherman et al., 2014) and anti-tumor (Lee et al., 2014; Rhim et al., 2014) properties. It is becoming increasingly evident that non-cell-autonomously activated stromal cells vary within a tumor and can influence tumors in a non-obvious manner. For example, while vitamin D receptor normalization of stromal fibroblasts improves PDA therapeutic response (Sherman et al., 2014), total stromal ablation increases malignant behavior (Lee et al., 2014; Rhim et al., 2014). Thus, while stromal purging is unlikely to provide therapeutic benefit in PDA, “stromal reprogramming” toward an anti-tumor stroma is now desirable (Brock et al., 2015). Although we describe a largely pro-tumor reciprocal axis, both pro- and anti-tumor stromal phenotypes likely transduce across reciprocal signaling networks. Our work suggests future efforts to therapeutically reprogram the PDA stroma toward anti-tumor phenotypes will require an understanding of reciprocal signaling. In describing the first oncogenic reciprocal axis, this study provides a foundation to measure the cell-cell communication required for anti-tumor stromal reprogramming.

Figure 6. Reciprocal Signaling Regulates Tumor Cell Phenotypes
(A) High-content live-cell TMRE analysis of PDA mitochondrial polarity. As predicted by heterocellular proteomics, reciprocal signaling restores mitochondrial polarity via SHH, IGF1R/AXL, and AKT (Δd∫m) (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001.
(B) PDA mitochondrial flux analysis. As predicted by heterocellular proteomics, reciprocal signaling increases spare mitochondrial capacity when compared to cell-autonomous KRASG12D alone (two-way ANOVA). OCR, oxygen consumption rate. *p < 0.05, **p < 0.01, ***p < 0.001.
(C) Cell-autonomous and reciprocal proliferation of luciferase-labeled tumor cells. Reciprocal KRASG12D (heterocellular, red) increases PDA proliferation relative to cell-autonomous KRASG12D (homocellular, orange). Inhibitors of reciprocal nodes only perturb heterocellular tumor cells (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
(D) High-content TUNEL imaging of PDA apoptosis. Reciprocal signaling protects tumor cells from apoptosis beyond cell-autonomous KRASG12D. Inhibiting IGF1R/AXL or AKT increases apoptosis when reciprocal signaling is active (n = 9). *p < 0.05, **p < 0.01, ***p < 0.001.
(E) Caspase 3/7 activity in (D) (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
(F) Semi-solid PDA colony formation. Reciprocal signals increase colony formation (via SHH, IGF1R/AXL, and AKT) relative to cell-autonomous KRASG12D alone (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.
See also Figure S7.
We demonstrate heterocellular multivariate phosphoproteomics can be used to observe reciprocal signaling in vitro. Unfortunately, cell-specific isotopic phosphoproteomics is not currently possible in vivo. To delineate reciprocal signaling in vivo, experimental systems must support manipulation of multiple cell-specific variables and provide cell-specific signaling readouts. Simple pharmacological perturbation of reciprocal nodes (e.g., IGF1R, AXL, AKT, etc.) in existing PDA GEMMs will in principle affect all cell types (e.g., tumor cells, PSCs, immune cells) and cannot provide axis-specific information in vivo. Future in vivo studies of reciprocal signaling will require parallel inductive genetic manipulation (e.g., oncogene activation in cancer cell and/or inhibition of reciprocal node in stromal cell), combined with cell-specific signaling data (e.g., using epithelial tissue mass-cytometry) (Simmons et al., 2015).

We describe KRAS<sup>G12D</sup> reciprocal signaling between PDA tumor cells and PSCs. However, it is likely oncogenic reciprocal signaling occurs across multiple different cell types in the tumor microenvironment. For example, in PDA, FAP<sup>+</sup> stromal fibroblasts secrete SDF1 that binds tumor cells to suppress T cells (Feig et al., 2013). Our model predicts oncogene signaling expands across several cell types in the tumor microenvironment—including immune cells. Moreover, as oncogenes non-cell-autonomously regulate the stroma in many other tumor types (Croce, 2008), our model predicts oncogenic reciprocal signaling to be a broad phenomenon across all heterocellular cancers. The presented heterocellular multivariate phosphoproteomic workflow now enables future characterization of oncogenic reciprocal signaling in alternative cancer types.

As differentiated cells process signals in unique ways, heterocellularity provides increased signal processing space over homocellularity. We provide evidence that KRAS<sup>G12D</sup> exploits heterocellularity via reciprocal signaling to expand tumor cell signaling space beyond cell-autonomous pathways. Given the frequent heterocellularity of solid tumors, we suspect reciprocal signaling to be a common—albeit under-studied—axis in oncogene-dependent signal transduction.

**EXPERIMENTAL PROCEDURES**

**KRAS<sup>G12D</sup>-Induced Soluble Signaling Molecules**

KRAS<sup>WT</sup> PDA cells (1 x 10<sup>6</sup>) were plated in a 6-well dish and cultured in DMEM + 0.5% FBS and conditioned media was collected after 48 hr. Conditioned media was analyzed for relative changes in KRAS<sup>G12D</sup>-driven cytokines and growth factors using the RayBio Mouse Cytokine Antibody Array G2000 (RayBiotech AAM-CYT-G2000-8) (144 proteins quantified in duplicate per sample). SHH-N expression after 24 hr was further validated by sandwich ELISA (R&D Systems DY461).

**KRAS<sup>G12D</sup> Cell-Autonomous Signaling**

For comprehensive phosphoproteomic quantification of KRAS<sup>G12D</sup>-dependent cell-autonomous signaling, 1 x 10<sup>6</sup> KRAS<sup>WT</sup> PDA cells were plated in a 6-well dish (DMEM + 0.5% FBS) and cultured ± 1 μg/ml doxycycline for 24 hr (biological replicates n = 5). Cells were lysed in 6 M urea, 10 mM NaPPi, 20 mM HEPES, pH 8.0, sonicated, centrifuged to clear cell debris, and protein concentration was determined by BCA (Pierce 23225). One hundred micrograms of each condition were individually digested by FASP (Wisniewski et al., 2009), amine-TMT-10-plex-labeled (Pierce 90111) on membrane (IFASP) (McDowell et al., 2014), eluted, pooled, lyophilized, and subjected to automated phosphopeptide enrichment (APE) (Tape et al., 2014b). Phosphopeptides were desalted using OLIGO R3 resin (Life Technologies 1-1339-03) and lyophilized prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (see the Supplemental Experimental Procedures).

**Automated Phosphopeptide Enrichment**

For TMT-labeled samples, phosphopeptides were enriched from each fraction using the automated phosphopeptide enrichment (APE) method described by Tape et al. (2014b). Phosphopeptide fractions were individually desalted using OLIGO R3 resin (Life Technologies 1-1339-03) and resuspended in 0.1% formic acid prior to Q-Exactive Plus HCD FT/FT LC-MS/MS (see the Supplemental Experimental Procedures). For reciprocal phosphoproteomics PSC-PDA co-cultures, 15 mg protein was digested with 150 μg Lys-C (Wako 125-05061) (24 hr) and 150 μg Tryptsin (Worthington) (24 hr) using 2 ml FASP. Lyophilized tryptic peptides were re-suspended in 60% MeCN and resolved using a Ultimate 3000 (Dionex) high-performance liquid chromatography fitted with a 10 μm particle size, 7.8 mm ID, and 30 cm TSKgel Amide-80 hydrophilic interaction liquid chromatography (HILIC) column (Tosoh 14459) (McNulty and Annan, 2008) into 24 fractions. Phosphopeptides were enriched from each fraction by APE. Phosphopeptide fractions (n = 192) were individually desalted using OLIGO R3 resin (Life Technologies 1-1339-03) and re-suspended in 0.1% formic acid prior to LTQ Velos HCD FT/FT LC-MS/MS (see the Supplemental Experimental Procedures).

**Multi-axis Phosphoproteomics**

For concurrent PDA cell-autonomous and reciprocal phosphoproteomics, 1 x 10<sup>6</sup> PSCs were plated in a 6-well dish, stimulated with 5 mM SHH-N (C25i) (R&D Systems 464-SH-025/CF) in DMEM + 0.5% FBS, and conditioned media was collected after 48 hr. PDA cells (1 x 10<sup>6</sup>) were cultured without doxycycline (KRAS<sup>WT</sup>), with 1 μg/ml doxycycline (KRAS<sup>G12D</sup>), or with 1 μg/ml doxycycline (KRAS<sup>G12D</sup>) + PSC+SHH conditioned media (biological n = 3) (all in +0.5% dialyzed FBS). One hundred micrograms of each condition was then processed for TMT and APE analysis as described above.

**Cell-Type Labeling with Amino Acid Precursors**

Mycobacterium tuberculosis (DDC<sup><mbox>am</mbox></sup>/h<sup><mbox>am</mbox></sup>) (PDA5M4) diaminopimelate decarboxylase (DDC) and Proteus mirabilis lysine racemase (Lyr<sup><mbox>am</mbox></sup>/h<sup><mbox>am</mbox></sup>) (M4GGR9) were synthesized by GenArt. Full details can be found in Tape et al. (2014a). DDC cells were grown in DMEM (-K/-R) (Caisson DMP49) supplemented with 10% (v/v) dialyzed FBS (GibCO), 0.3 mM L-arginine (Sigma A8094) and 5 mM meso-2,6-diaminopimelate (DAP) (Sigma 07038). Lyr cells were grown in DMEM (-K/-R) supplemented with 10% (v/v) dialyzed FBS,
0.3 mM L-arginine and either 2.5 mM "Medium" D-lysine-4,4,5,5-d4 HCl (C/D/N D-7334) (Delta mass: 0.025104) or 2.5 mM "Heavy" D-lysine-3,3,4,4,5,5,6,6-d8 2HCl (C/D/N D-6367) (Delta mass: 0.0502136, Delta average mass: 0.04928).

**Heterocellular Multivariate Phosphoproteomics**

PDA cells were transfected with DDCOM.Lys3-3,3,4,4,5,5,6,6-d8 and grown on 5 mM DAP ("Light"). PDA+DDC cells (3 x 10⁶) were cultured in a 10 cm dish ± 1 mg/ml doxycycline, ±10 μg/ml SHH-neutralizing monoclonal antibody (mAb) (R&D Systems MAB4641) and ±3 x 10⁻⁵M "Heavy" PSC+Lyr cells (biological triplicates). All cells were grown in DMEM (-K/-R) supplemented with 0.5% (v/v) dialyzed FBS, 0.3 mM L-arginine, 5 mM DAP, and 2.5 mM "Heavy" D-lysine. After 5 days, each condition was lysed in 6 M urea, sonicated, centrifuged to clear cell debris, and protein concentration was determined by BCA. One hundred micrograms of each variable was then processed for TMT and APE analysis as described above.

**Heterocellular Reciprocal Proteomics**

To investigate reciprocal regulation of protein abundance, "Heavy" PDA-Lyr3-3,3,4,4,5,5,6,6-d8 and "Medium" PDA-DCO cells were co-cultured with "Light" PSC-ΔNat-ΔKDEL in the presence of 2.5 mM "Heavy" D-lysine-3,3,4,4,5,5,6,6-d8 and 5 mM "Light" DAP (biological n = 3). For each experiment, a control co-culture of "Medium" PDA+Lyr3-3,3,4,4,5,5,6,6-d8 and "Light" PSC-ΔNat-ΔKDEL was performed in the presence of either PDA pre-treatment with IGF1R inhibitor (250 nM micropodophyllin (MPP)), AXL inhibitor (500 nM R428), or 20 μg/ml SHH-neutralizing antibody (R&D Systems MAB4641). All co-cultures were performed in +0.5% dialyzed FBS for 72 hr. Co-cultures were lysed in 100 mM Na₂CO₃ (pH 11.0), pooled, snap-frozen in liquid nitrogen, treated with Benzonase (Novagen 7074B), centrifuged at 40,000 rpm (to resolve membrane-bound proteins from cytosolic proteins), and denatured in 6 M urea 2 M thiourea. Differential changes in cytoplasmic and membrane protein levels were determined using "In-gel digestion" (see the Supplemental Experimental Procedures). To investigate the comparative KRASG12D cell-autonomous proteome, KRASWT "Medium" and "Heavy" PDA+Lyr3-3,3,4,4,5,5,6,6-d8 cells were seeded into 10-cm dishes (biological n = 3) (5 x 10⁶ PDA cells/plate). Doxycycline (1 μg/ml) was then added to the "Heavy" PDA cells (i.e., KRASG12D) and the "Medium" cells were left untreated (i.e., KRASWT) in +0.5% dialyzed FBS. After 72 hr, cells were lysed as above.

**ACCESSION NUMBERS**

The accession number for all 617 mass spectrometry proteomic files have been deposited to the ProteomeXchange Consortium (http://www.proteomexchange.org) via the PRIDE partner repository (Vizcarra et al., 2013): PXD003223.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.03.029.

A video abstract is available at http://dx.doi.org/10.1016/j.cell.2016.03.029#mmc4.

**AUTHOR CONTRIBUTIONS**

C.J.T. conceived the project, performed all proteomic-signaling experiments, and wrote the paper. S.L. performed PSC secretome studies, M.D. and G.P. performed PDA mitochondrial flux analysis. K.M.M. and J.D.W. provided LC-MS/MS support. I.C.N., H.S.L., and C.J.M. performed FACS RNA-seq. D.A.L. oversaw the project. C.J. conceived the project, oversaw the project, and wrote the paper.

**ACKNOWLEDGMENTS**

C.J.T. is funded by a Sir Henry Wellcome Fellowship (098847/Z/12/Z). C.J. is funded by a Cancer Research UK Career Establishment Award (C37293/ A12905) and a Cancer Research UK Institute Award (A19258). D.L. is funded by NIH grants U54-CA112967 and R01-CA96504. This research was also supported by the Rosetrees Trust (M286). The authors would like to acknowledge colleagues at The ICR Oncogene Team, CRUK Manchester Institute Systems Oncology Team, Dr. John Brognard, Dr. Owen Sansom, and Dr. Jennifer Morton for valuable input. We would also like to acknowledge Prof. Ronald DePinho, Dr. Marina Pasca di Magliano, and Prof. Raúl Urrutia for their generous sharing of reagents. In particular, we would like to acknowledge Prof. Chris Marshall for essential support and mentorship.

Received: September 18, 2015

Revised: February 5, 2016

Accepted: March 17, 2016

Published: April 14, 2016; corrected online June 12, 2016

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