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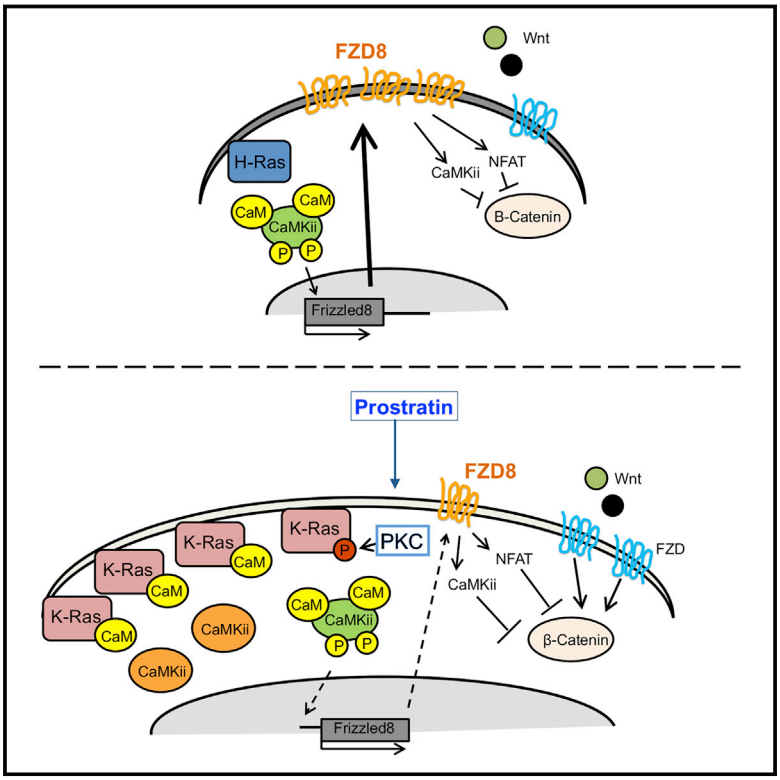
K-Ras Promotes Tumorigenicity through Suppression of Non-canonical Wnt Signaling

Graphical Abstract



Highlights

- Oncogenic K-Ras and H-Ras differ in tumor initiation via non-canonical Wnt signaling
- Suppression of Fzd8-mediated Wnt/Ca^{2+} signaling is essential to K-Ras malignancy
- K-Ras-CaM interaction modulates the Wnt/Ca^{2+} signaling pathway
- Prostratin compromises the K-Ras-CaM interaction and so prevents tumorigenicity

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In Brief

The interaction between K-Ras and calmodulin (CaM) modulates tumor formation through inhibition of CaM kinase activity and suppression of Fzd8-mediated Wnt/Ca^{2+} signaling. This interaction does not occur with H-Ras or N-Ras, and disruption of the K-Ras-CaM interaction by the orally active natural product prostratin represses tumor growth.

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K-Ras Promotes Tumorigenicity through Suppression of Non-canonical Wnt Signaling

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SUMMARY

K-Ras and H-Ras share identical effectors and have similar properties; however, the high degree of tumor-type specificity associated with K-Ras and H-Ras mutations suggests that they have unique roles in oncogenesis. Here, we report that oncogenic K-Ras, but not H-Ras, suppresses non-canonical Wnt/Ca^{2+} signaling, an effect that contributes strongly to its tumorigenic properties. K-Ras does this by binding to calmodulin and so reducing CaMKii activity and expression of Fzd8. Restoring Fzd8 in *K-Ras* mutant pancreatic cells suppresses malignancy, whereas depletion of Fzd8 in H-Ras^{V12}-transformed cells enhances their tumor initiating capacity. Interrupting K-Ras-calmodulin binding using genetic means or by treatment with an orally active protein kinase C (PKC)-activator, prostratin, represses tumorigenesis in *K-Ras* mutant pancreatic cancer cells. These findings provide an alternative way to selectively target this "undruggable" protein.

INTRODUCTION

H-Ras, *N-Ras*, and *K-Ras* genes are frequently mutated in human tumors and affect a multitude of cellular processes. They share a high degree of sequence homology as well as identical regulators and effectors. However, they may also have unique roles in physiological and pathological processes. *K-Ras* mutations occur at high frequency in pancreatic and colorectal cancers and in lung adenocarcinomas, while *N-Ras* and *H-Ras* mutations are far less common (Prior et al., 2012). Furthermore, *K-Ras* deficiency results in embryonic lethality, whereas *N-* and *H-Ras* knockout mice develop normally (Johnson et al., 1997; Koera et al., 1997; Malumbres and Barbacid, 2003). In a model of tumor initiation and differentiation, K-Ras, but not H-Ras or N-Ras, initiated tumors through a mechanism involving stem cell expansion (Quinlan et al., 2008). H-Ras can replace K-Ras when expressed from the endogenous *K-Ras* locus, suggesting that the genetic locus is more important than the isoform of Ras that is expressed (To et al., 2008). However, tumors driven by K-Ras are more metastatic than those driven by H-Ras (Wong et al., 2013). Together, these data suggest a potentially unique role for K-Ras in tumor biology. Here, we report that oncogenic *K-Ras* uniquely elicits a tumorigenic phenotype through down-regulation of non-canonical Wnt/Ca^{2+} signaling. Binding of calmodulin (CaM) to K-Ras, but not to H-Ras or N-Ras, appears to be responsible for this major difference. These data provide an alternative avenue to inhibit this "undruggable" protein. Indeed, treatment of mice with prostratin, a natural product that promotes dissociation of K-Ras from CaM, suppressed tumorigenesis in pancreas cancer models and papillomas driven specifically by oncogenic K-Ras.

RESULTS

K-Ras^{V12} and H-Ras^{V12} Differ in Tumor Initiation Despite Comparable Canonical MAPK and Akt Signaling

To identify distinct properties of H-Ras and K-Ras, we expressed oncogenic H-Ras and K-Ras(4B) in NIH/3T3 cells and looked for phenotypic differences. However, H-Ras^{V12}- and K-Ras^{V12}-transformed cells had almost identical phenotypes: similar morphologies, comparable levels of GTP-loaded Ras (Figure 1A), similar levels of Erk (Figures 1B and S1A), and similar Akt activity (Figures 1B and S1B).

Next, we examined their ability to self-renew at the single-cell level, using a sphere-forming assay (Fang et al., 2005; Fujii et al., 2009; Gou et al., 2007; Ponti et al., 2005; Singh et al., 2003). With limited numbers of seeded cells, K-Ras^{V12}-transformed NIH/3T3 cells showed significantly increased sphere-forming efficiency compared with H-Ras^{V12} (Figure 1C). Re-plating revealed that spheres from K-Ras^{V12}-transformed cells were viable and able to re-initiate exponentially growing cells in 2D culture, whereas H-Ras^{V12}-transformed cells were not (Figure S1C).

We next evaluated their tumorigenic potential, using limited and serial transplantation (Clarke et al., 2006). When 1,000 cells were engrafted subcutaneously, H-Ras^{V12} and K-Ras^{V12} tumors arose at similar rates. However, when the number was reduced to 100, K-Ras^{V12}-transformed cells displayed enhanced tumor initiating rates compared to H-Ras^{V12} cells (Figure 1D, left). We then re-transplanted cells from primary tumors into a second cohort of mice. Upon injection of 500 K-Ras^{V12} cells, all ten injections gave rise to tumors. In contrast, only two out of ten injections of H-Ras^{V12} cells initiated tumors (Figure 1D, right).

To examine whether K-Ras and H-Ras play distinct roles in inducing tumorigenicity in human cancers, we first examined their roles in BxPC3 cells, a pancreatic cancer-cell line expressing wild-type *H-Ras* and *K-Ras*. Knock down of K-Ras, but not H-Ras, significantly reduced sphere-forming efficiency in response to EGF and reduced the size of initiated spheres

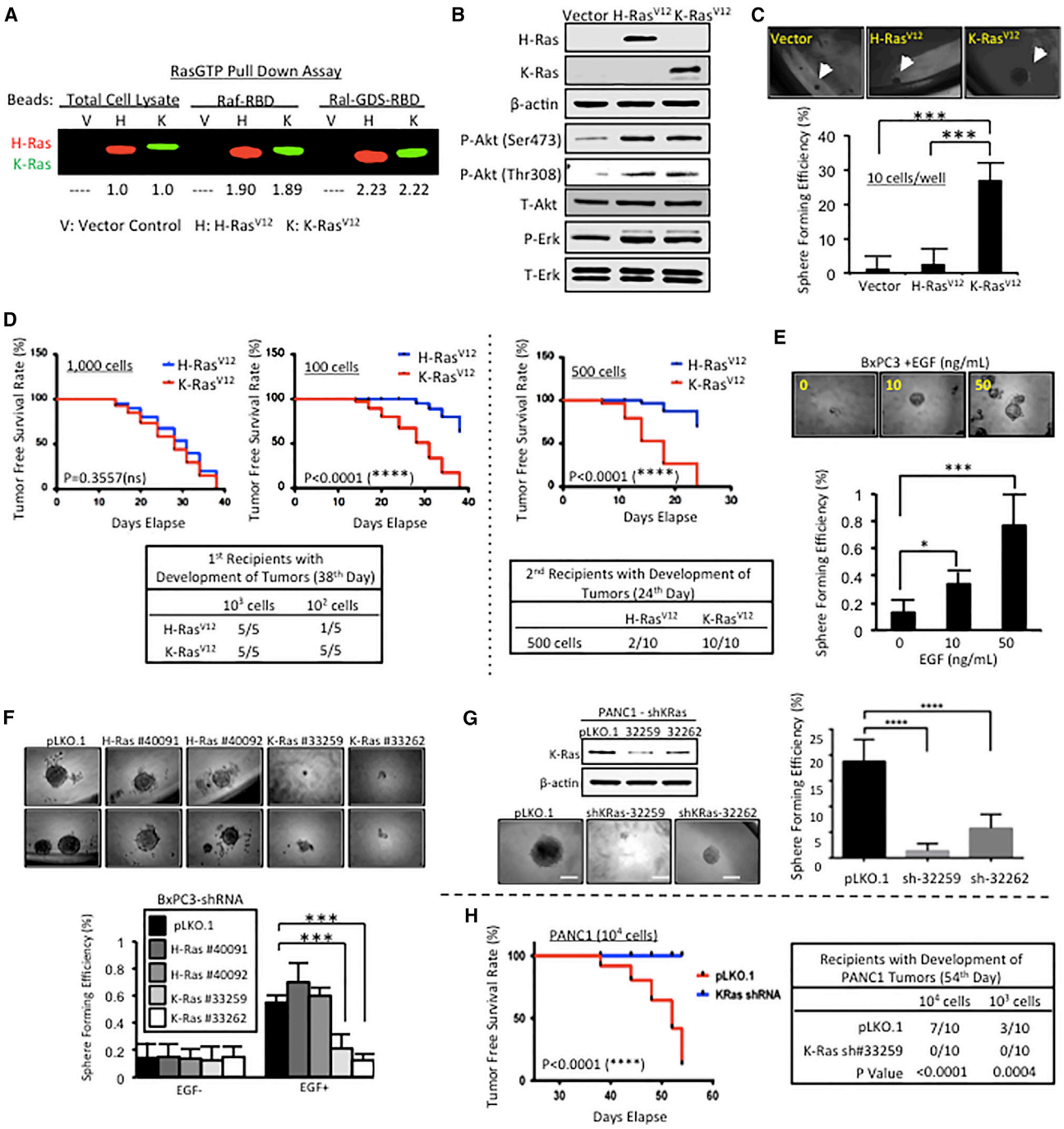

Figure 1. K-Ras^{V12} and H-Ras^{V12} Have Differential Tumor-Initiating Properties, Despite Comparable Canonical Signaling Outputs

(A) Comparable levels of total Ras proteins and GTP-bound Ras as measured by Raf-RBD or Ral-GDS-RBD pull-down assays.

(B) Comparable levels of phosphorylated Erk and Akt in cells transformed by H-Ras^{V12} or K-Ras^{V12}.

(C) K-Ras^{V12}-transformed NIH/3T3 cells presented increased sphere formation. Top: morphology of spheres formed. Bottom: sphere formation efficiency (n = 6).

(D) The tumor initiating abilities of H-Ras^{V12}-transformed and K-Ras^{V12}-transformed NIH/3T3 cells when the number of injected cells was 1,000 (top left) or 100 (top right). K-Ras^{V12}-transformed cells presented increased tumor initiating capacity, in comparison with H-Ras^{V12}-transformed cells, when the number of cells injected became limited (bottom table). Right: cells derived from K-Ras^{V12} driven tumors had enhanced tumor recapitulating ability than H-Ras^{V12}.

(E) Promotion of BxPC3 sphere formation by EGF. Top: morphology of spheres formed. Bottom: sphere formation efficiency as calculated by the number of spheres normalized by the number of cells seeded (n = 6).

(F) Knockdown of K-Ras, but not H-Ras, attenuated EGF stimulation of BxPC3 sphere-forming efficiency (n = 6).

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(Figures 1E, 1F, and S1D–S1F). We next determined whether oncogenic *K-Ras* is required for the maintenance of "stemness" in human pancreatic tumor cells PANC2.13 and PANC1, both of which express oncogenic *K-Ras.* Upon knock down of *K-Ras*, both cell lines showed a differentiated morphology, reduced expression of CD44 and CD24 (Figures S1G–S1I), and reduced ability to form spheres with re-plating potential (Figures 1G and S1J). PANC1 is K-Ras-independent in 2D culture (Scholl et al., 2009; Singh et al., 2009; Wei et al., 2012). However, using a limited number of transplanted cells, K-Ras knockdown significantly reduced their rate of tumor initiation (Figure 1H), suggesting that oncogenic K-Ras mediates tumorigenicity through a function, distinct from its role in maintaining viability in 2D.

K-Ras Suppresses *Frizzled 8* and CaMKii Activity

We next investigated the mechanisms through which K-Ras promotes tumorigenicity and stem-ness more efficiently than H-Ras, despite comparable levels of canonical Ras signaling. We used PCR arrays to profile stem cell-related gene expression mediated by K-Ras or H-Ras (Figure 2A; Tables S1 and S2). Three stem cell-related genes were expressed with a >4-fold change between H-Ras^{V12}- and K-Ras^{V12}-transformed NIH/3T3 cells (Figure 2B; Table S2): *Bmpr1b* was upregulated in K-Ras^{V12}-transformed cells, whereas *Gli2* and *Frizzled 8* (*Fzd8*) were downregulated. We did not pursue *bmpr1b* due to its low expression, or *Gli2*, because it plays different roles in mouse and human cells (Sasaki et al., 1999).

Frizzled proteins are G protein-coupled receptors involved in Wnt signaling. Fzd1, Fzd4, and Fzd10 activate the canonical Wnt/β-catenin pathway, whereas, Fzd8 is a major mediator of non-canonical Wnt/Ca^{2+} signaling in stem cells (Sugimura et al., 2012). The non-canonical Wnt/Ca^{2+} pathway involves activation of CaMKii and the transcription factor NF-AT and inhibition of β-catenin/TCF signaling (Saneyoshi et al., 2002; Semenov et al., 2007; Sugimura and Li, 2010). Figure 2C confirms that Fzd8 was downregulated in K-Ras^{V12}-transformed cells compared with H-Ras^{V12}-transformed cells and vector controls. K-Ras^{V12}-transformed cells also had reduced levels of activated CaMKii, as indicated by phosphorylation at Threonine-286 (Figure 2C). Activation and nuclear translocation of NF-AT was reduced in NIH/3T3-K-Ras^{V12} cells (Figure 2C). The phosphorylated form of β-catenin was also reduced in these cells (Figure 2C).

The non-canonical Wnt/Ca^{2+}pathway and CaMKii suppress canonical Wnt signaling by blocking β-catenin/TCF4 interaction (Semenov et al., 2007; Sugimura and Li, 2010). In K-Ras^{V12}-transformed cells, where CaMKii was barely phosphorylated, the β-catenin/TCF4 interaction was significantly increased compared with H-Ras^{V12}-transformed cells, in which CaMKii activity was elevated (Figure 2D). Furthermore, reduced activation of CaMKii and NF-AT in NIH/3T3-K-Ras^{V12} cells led to increased nuclear localization of β-catenin, whereas in vector controls and NIH/3T3-H-Ras^{V12} cells, in which CaMKii and NF-AT were highly activated, nuclear β-catenin was barely detectable (Figure 2C). TOPFlash assay confirmed that the transcriptional activity of β-catenin was greatly elevated in NIH/3T3-K-Ras^{V12} cells (Figure 2E). Consequentially, the mRNA expressions of β-catenin-target genes, *c-Myc* and *TCF1*, were upregulated in K-Ras^{V12}-transformed cell in comparison with control or H-Ras^{V12}-transformed cells (Figure S2A).

Next, we asked whether non-canonical Wnt/Ca^{2+} signaling differs in tumors driven by H-Ras or K-Ras in vivo. We compared tumors from wild-type mice with tumors from a genetically engineered mouse model devoid of endogenous H-Ras, but expressing wild-type *H-Ras* from the endogenous *K-Ras* locus. *K-Ras* or *H-Ras* mutations were then induced by topical treatment with 7,12-dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol-13-acetate (DMBA/TPA). This model allows comparison of K-Ras and H-Ras oncogenes under control of the same endogenous regulatory elements in the same cellular background (Potenza et al., 2005; To et al., 2008). Figure 2G shows that mutant *H-Ras* driven skin tumors had elevated level of Fzd8 protein and increased levels of phosphorylated-CaMKii compared to skin tumors with *K-Ras* mutations This shows that this unique K-Ras-mediated signaling cannot be recapitulated by H-Ras even when it is knocked in at the *K-Ras* locus.

Canonical and non-canonical Wnt signaling pathways are both regulated by binding of WNT ligands to frizzled receptors. WNT3a preferentially activates Wnt/β-catenin signaling, whereas WNT5a activates non-canonical Wnt signaling (Weekes and Winn, 2011). To assess whether different WNT ligands are involved in modulating the distinct Wnt/Ca^{2+} signaling activities of oncogenic H-Ras and K-Ras, we evaluated the expression levels and functions of WNT-5a and WNT-3a in Ras^{V12}-transformed NIH/3T3 cells. As shown in the Figure S2B, oncogenic Ras^{V12}-transformed cells expressed more WNT-3a and WNT-5a protein than control cells, but there was no difference in expression levels between H-Ras^{V12} and K-Ras^{V12}. Furthermore, addition of WNT ligands did not alter CaMKii activity, β-catenin/TCF/LEF transcriptional activity, or sphere-forming efficiency in H-Ras^{V12} or K- Ras^{V12}-transformed cells (Figures S2C–S2E). The divergence between H-Ras and K-Ras in non-canonical Wnt/Ca^{2+} signaling is therefore not dependent upon the presence of WNT ligands.

We next determined the effect of K-Ras on non-canonical Wnt/Ca^{2+} signaling in human cancer-derived cell lines. In PANC2.13 cells, K-Ras knockdown increased Fzd8 expression, increased phosphorylation of CaMKii (Figure 2H), and reduced β-catenin activity (Figure 2I). We therefore conclude that oncogenic K-Ras, but not H-Ras, represses Fzd8 expression and CaMKii activity, a major effector of the Wnt/Ca^{2+} pathway, in both mouse fibroblasts, mouse tumors, and in human cancer cells.

Canonical Wnt/β-catenin signaling is modulated by the non-canonical Wnt/Ca^{2+} pathway in K-Ras^{V12}-transformed cells

(G) Knockdown of mutant K-Ras repressed PANC1 sphere formation efficiency. Top left: western blot confirmed the knockdown efficiency. Bottom left and right: PANC1 with K-Ras shRNA expression formed spheres in smaller sizes and numbers when compared to vector control (n = 6).
(H) Knockdown of mutant K-Ras reduced PANC1 tumor-initiating capacity. Left: tumor free survival curve. Right: tumor formation frequency.
NS, no significance. *p < 0.05; ***p < 0.001; ****p < 0.0001.
See also Figure S1.

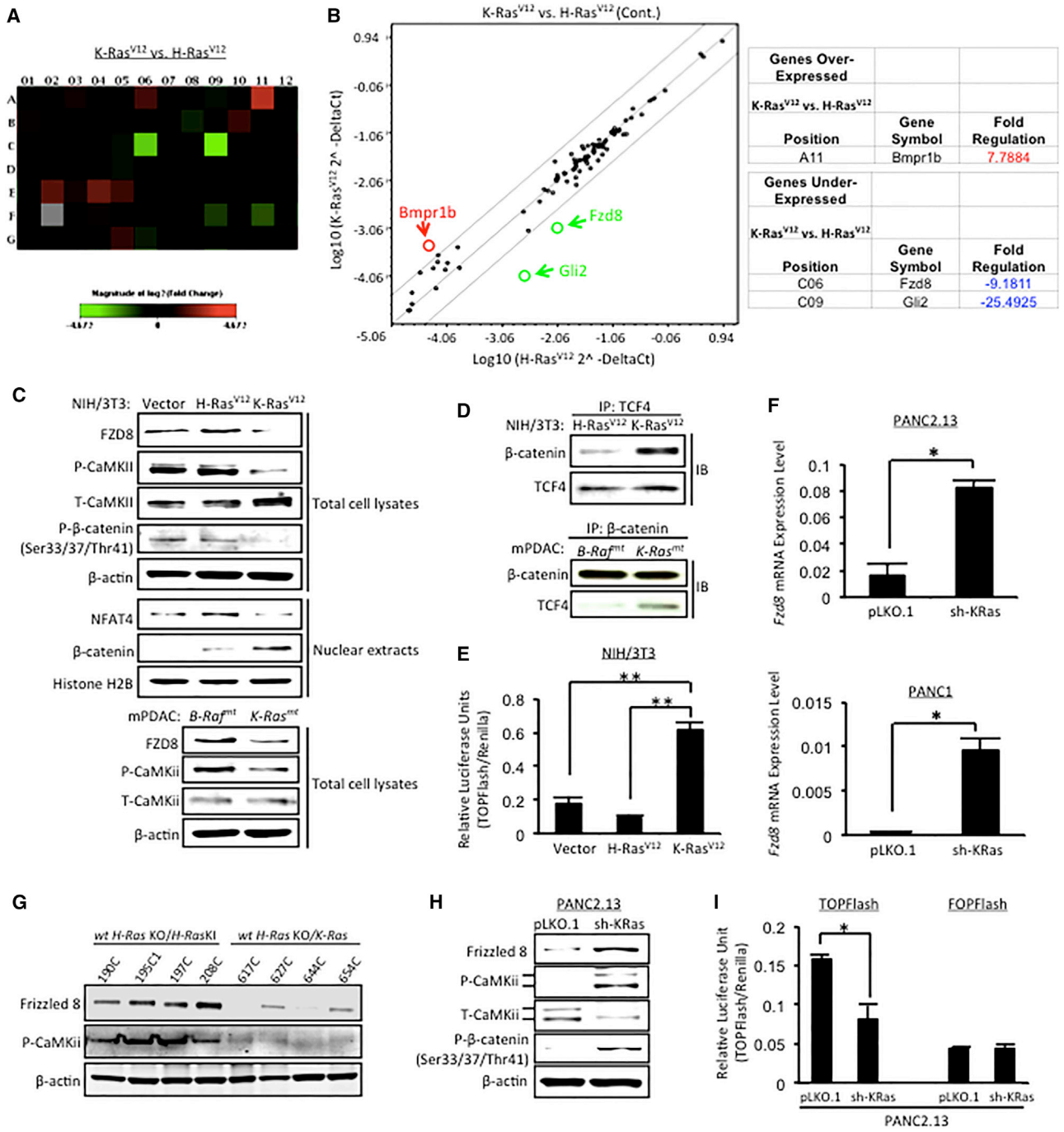

Figure 2. K-Ras, but Not H-Ras, Suppresses Fzd8

(A) Heat map of stem cell factors differentially expressed in H-Ras^{V12} and K-Ras^{V12}-transformed NIH/3T3 cells as evaluated by qPCRarray (n = 3).

(B) Scatter plot (left) and identification of *Bmpr1b*, *Fzd8*, and *Gli2* as genes differentially expressed in H-Ras^{V12} and K-Ras^{V12}-transformed NIH/3T3 cells.

(C) Reduced Fzd8 expression and Wnt/Ca^{2+} signaling in K-Ras-transformed NIH/3T3 cells when compared with the vector control or H-Ras^{V12}-transformed cells (Top eight panels) and in mouse PDAC cells with oncogenic K-Ras mutation when compared with those with mutant Raf (Bottom four panels).

(D) Increased TCF4 and β-catenin complexes in K-Ras^{V12}-transformed NIH/3T3 cells (Top panel) and in mouse PDAC cells with K-Ras mutations (Bottom panel) when compared to those with H-Ras^{v12} or B-Raf, respectively.

(E) Increased TCF/β-catenin activities in K-Ras^{V12}-transformed NIH/3T3 cells as compared to the vector control or H-Ras^{V12}-transformed cells (n = 4).

(F) Knockdown of K-Ras led to increased *Fzd8* expression at mRNA level in PANC2.13 and PANC1 cells (n = 3).

(G) Reduction in the levels of Fzd8 expression and CaMKii phosphorylation in skin tumors harboring WT H-Ras KO with mutations in either Kras or HrasKI alleles.

(legend continued on next page)

and in pancreatic cancer cells containing oncogenic K-Ras (above). This suggests that activation of canonical Wnt/β-catenin may be responsible for stem-like properties induced by K-Ras. However, in colorectal cancers, the canonical Wnt/β-catenin signaling pathway is strongly activated through inactivation of the *APC* gene or mutation of β-catenin potentially rendering K-Ras irrelevant with regard to stem-ness in these cells. Knock down of K-Ras promoted expression of Fzd8 and the activation of NF-AT and CaMKii in multiple colon cancer cell lines regardless of the status of wild-type or mutant *APC* (Figure S3A). As expected from our model, knock down of K-Ras significantly repressed β-catenin/TCF/LEF transcriptional activity in SW480 (mutant *APC*), which express wild-type β-catenin, yet not in either HCT15 (mutant *APC*) or HCT116 (wild-type *APC*) that have a gain-of-function mutation in β-catenin (Figure S3B). However, although repression of K-Ras expression by short hairpin RNA (shRNA) did not alter β-catenin/TCF/LEF transcriptional activity, or even the proliferation rates, in HCT15 and HCT116 cells, it still inhibited their sphere formation ability in 3D culture (Figures S3C and S3D). Therefore, K-Ras-mediated stem-ness is independent from canonical Wnt/β-catenin/TCF/LEF transcriptional activity. To confirm this, we treated NIH/3T3 cells transformed by H-Ras^{V12} or K-Ras^{V12} with the tankyrase inhibitors JW55 and JW67 and with cardionogen1. JW55 and JW67 function as potent inhibitors of canonical Wnt/β-catenin signaling pathway by degrading β-catenin, and cardionogen1 inhibits the β-catenin/TCF/LEF transcriptional activity. Tankyrase inhibitors repressed β-catenin/TCF/LEF transcriptional activity in Ras^{V12}-transformed cells (Figure S3E), but this did not affect their growth in 3D culture and formation of spheres (Figure S3F). These suggest that β-catenin/TCF/LEF transcriptional activity is affected by non-canonical Wnt/Ca^{2+} signaling activity, but K-Ras driven stem-ness is independent from the canonical Wnt/β-catenin signaling pathway and β-catenin/TCF/LEF activation.

Inhibition of CaMKii Enhances Sphere Formation by H-Ras^{V12} Cells

To determine whether suppression of CaMKii observed in K-Ras-transformed cells is responsible for the acquisition of stem-ness properties, we treated NIH/3T3-H-Ras^{V12} with KN-93, a selective CaMKii inhibitor (Figure 3A). Inhibition of CaMKii reduced Fzd8 expression (Figure 3B) and increased β-catenin transcriptional activity, confirming the inhibitory effects of Wnt/Ca^{2+}/CaMKii signaling on the canonical Wnt pathway (Figure 3C). Moreover, KN-93 treatment enhanced the sphere-forming efficiency and the size of spheroid colonies in NIH/3T3-H-Ras^{V12} cells (Figure 3D). Downregulation of CaMKii activity is therefore essential for induction of malignant features observed in K-Ras-transformed cells.

Knockdown of Fzd8 Induces Tumorigenicity in H-Ras^{V12} Cells

To further determine the role of Fzd8 in Wnt/Ca^{2+} signaling, we knocked Fzd8 down in NIH/3T3-H-Ras^{V12} cells (Figures 3A and 3E). We observed reduced phospho-CaMKii level and enhanced β-catenin activity (Figures 3E and 3F). Formation of spheres with re-plating ability was also enhanced upon Fzd8 knockdown (Figure 3G). Importantly, mice injected subcutaneously with 50 NIH/3T3-H-Ras^{V12} cells showed significantly reduced tumor-free survival following shRNA suppression of Fzd8 (Figure 3H). Thus, suppression of Fzd8 expression pheno-copies the effects of oncogenic K-Ras in these stem-ness assays.

Roles of Fzd8 in K-Ras-Driven Malignancy

Next, we tested whether downregulation of Fzd8 is required for NIH/3T3-K-Ras^{V12} cells to promote tumor formation. Restoration of Fzd8 expression in NIH/3T3-K-Ras^{V12} cells enhanced levels of phosphorylated CaMKii (Figure 4A) and reduced β-catenin/TCF/LEF activity (Figure 4B). Furthermore, restoration of Fzd8 significantly reduced sphere formation in NIH/3T3-K-Ras^{V12} cells (Figure 4C). Fzd8 overexpression also abolished tumor formation in nude mice (Figure 4D). Interestingly, exogenously added WNT3a or WNT5a ligand did not affect the increased phospho-CaMKii and inhibited β-catenin activity caused by the overexpression of Fzd8 in NIH 3T3-K-Ras^{V12} cells (Figures S4A and S4B). These suggest that the altered Wnt/Ca^{2+} signaling pathway and β-catenin activity resulting of Fzd8 overexpression cannot be rescued by canonical or non-canonical Wnt pathway ligands.

When Fzd8 was overexpressed in PANC2.13 cells, we observed an increase in phospho-CaMKii level, with a concurrent reduction in the expression of CD44 and CD24 (Figure S4C). When compared with control cells, Fzd8-overexpressing PANC2.13 cells displayed significant downregulation of multiple β-catenin targeted genes, including *CCND-1*, *LEF1*, and c-*Myc* (Herbst et al., 2014), consistent with repressed β-catenin transcriptional activity (Figure S4D). Overexpression of Fzd8 in PANC1 cells, resulted in elevated NF-AT transcriptional activity, decreased activity of β-catenin, and reduced expression of CD44 and CD24 (Figures 4E and S4E). Overexpression of Fzd8 in these pancreatic cancer lines induced differentiation-like morphological changes, phenocopying those observed upon K-Ras knockdown (Figure S4F). Furthermore, nude mice with subcutaneous xeno-transplants of Fzd8-overexpressing PANC1 had increased tumor-free survival rates (Figure 4F). Thus, restoration of Fzd8 expression, which enhances Wnt/Ca^{2+} signaling and suppresses canonical Wnt signaling, reduces tumor formation by K-Ras^{V12}-transformed cells or pancreatic tumor cells possessing oncogenic *K-Ras*.

Human Fzd8 is normally expressed in brain, heart, kidney, and muscle, as well as in the pancreas (Saitoh et al., 2001). Immunohistochemistry of four human pancreatic tissue arrays revealed that while Fzd8 expression was abundant in normal pancreatic acini, islet, and ductal cells, its expression was frequently lost in malignant pancreatic adenocarcinomas (Figures 4G and S4G). Tissue array T143, B1, B2, B5, and B6 revealed no reduction in Fzd8 expression in islet cell tumors, which are mostly

(H) Knockdown of K-Ras increased Fzd8 protein level, non-canonical Wnt signaling (p-CaMKii), and increased phosphorylation of β-catenin.
(I) Knockdown of K-Ras in PANC2.13 cells reduced canonical Wnt signaling as revealed by TOPFlash assay (n = 4).
*$p < 0.05$; **$p < 0.01$.
See also Figures S2 and S3 and Tables S1 and S2.

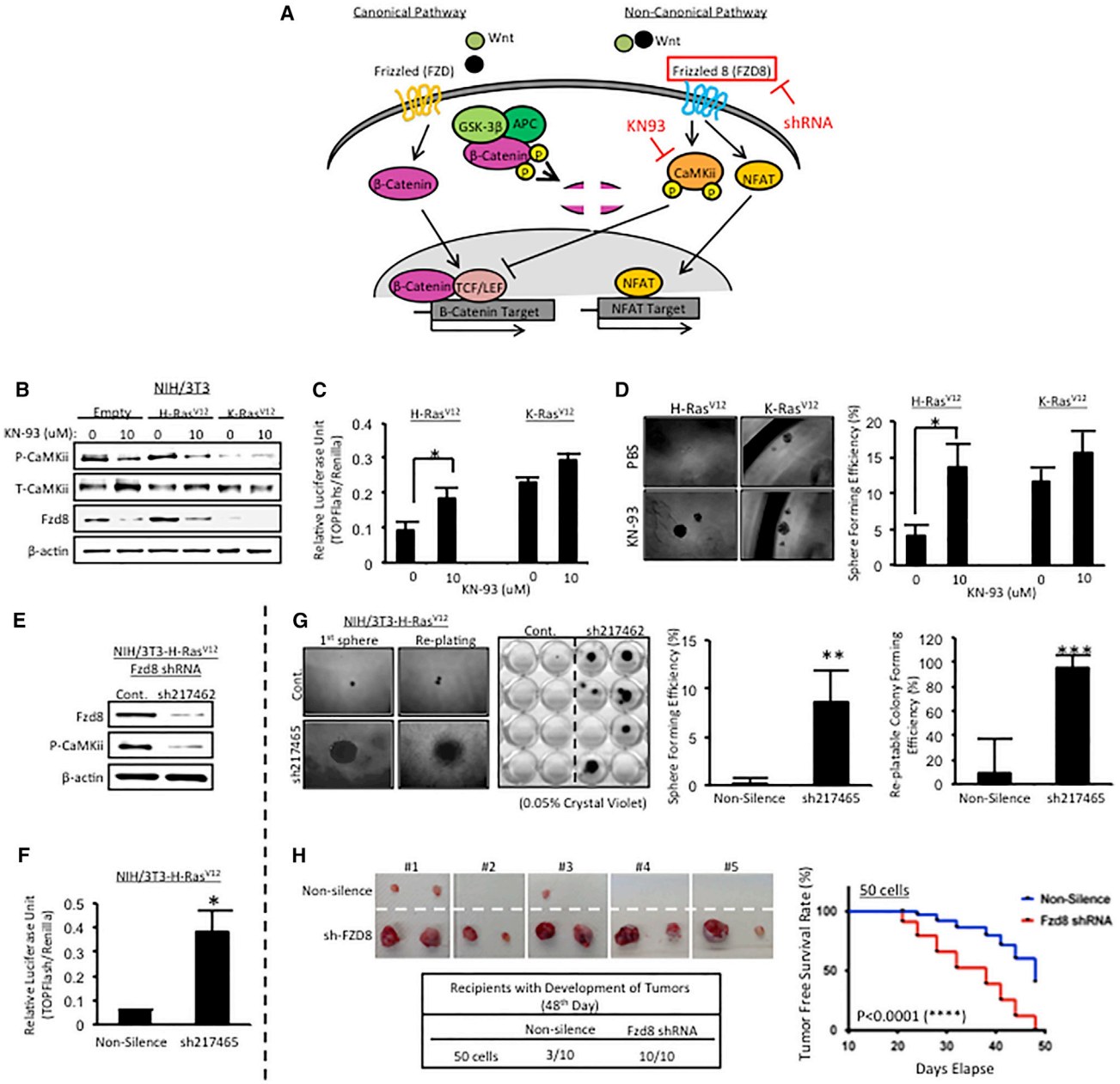

Figure 3. Fzd8-Mediated Non-canonical Wnt/Ca^{2+} Signaling Suppresses the Tumor-Promoting Properties of H-Ras^{V12}-Transformed NIH/3T3 Cells

(A) Schematic illustration of Fzd8 in non-canonical Wnt/Ca^{2+} signaling pathway and its crosstalk with canonical Wnt signaling. Small molecule, KN-93, and shRNA against Fzd8 were used to block CaMKii activity and Fzd8 expression for following experiments.

(B) Inhibition of CaMKii by KN-93 reduced phosphorylation of CaMKii and reduced the expression of Fzd8.

(C) KN-93 treatment stimulated β-catenin transcriptional activities in H-Ras^{V12}-transformed NIH/3T3 cells (n = 4).

(D) Inhibition of CaMKii by KN-93 enhanced sphere formation in H-Ras^{V12}-transformed NIH/3T3 cells, but not in K-Ras^{V12}-transformed NIH/3T3 cells (n = 6).

(E and F) Knockdown of Fzd8 in H-Ras^{V12}-transformed NIH/3T3 cells. Reduced phospho-CaMKii levels (D) and stimulated β-catenin transcriptional activities (n = 4) (E).

(G) Knockdown of Fzd8 in H-Ras^{V12}-transformed NIH/3T3 cells promoted sphere formation and re-plating efficiency (n = 6).

(H) Knockdown of Fzd8 in H-Ras^{V12}-transformed NIH/3T3 cells enhanced their tumor initiating abilities.

*p < 0.05; **p < 0.01; ***p < 0.001.

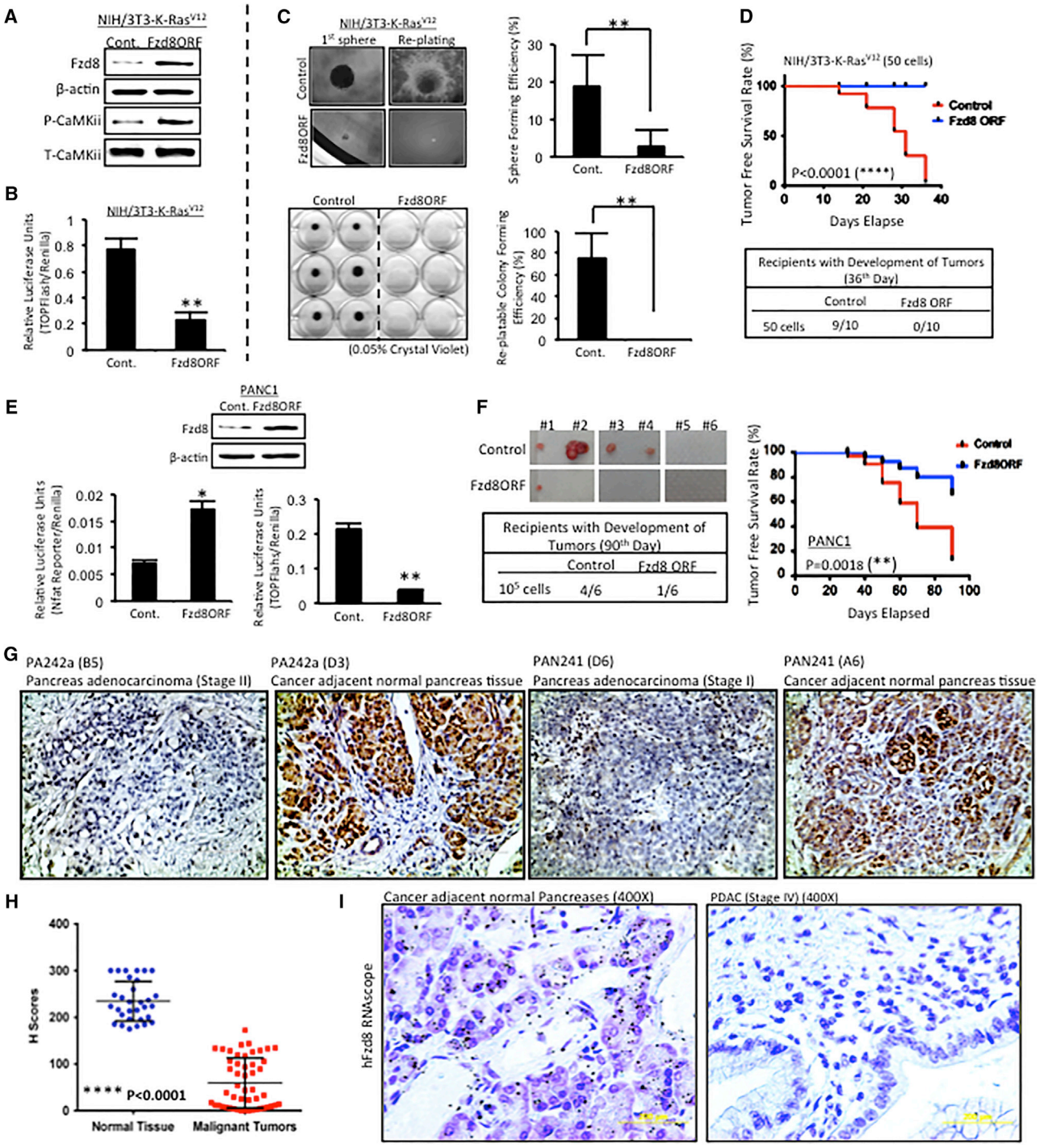

Figure 4. Downregulation of Fzd8 Is Required for K-Ras to Enhance Tumor Initiation

(A and B) Restoration of Fzd8 expression in K-Ras^{V12}-transformed NIH/3T3 cells enhanced Wnt/Ca^{2+} signaling (A) and reduced β-catenin transcriptional activities (B) (n = 4).

(C) Restoration of Fzd8 in K-Ras^{V12}-transformed NIH/3T3 cells reduced sphere formation and re-plating efficiency (n = 6).

(D) Restoration of Fzd8 reduced tumor initiation capacity of K-Ras^{V12}-transformed NIH/3T3 cells.

(E) Fzd8 restoration in PANC1 cells enhanced Wnt/Ca^{2+} signaling as revealed by NF-AT transcriptional activities (n = 4) and reduced β-catenin activities (n = 4).

(F) Fzd8 restoration reduced the tumor initiating ability of PANC1 cells.

(G) Downregulation of Fzd8 in human pancreatic tumor tissues: micrographs of tissue sections immunostained for Fzd8 in human pancreatic cancer adjacent normal tissue and adenocarcinomas (magnification = 200×).

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benign and in which K-Ras is rarely mutated (Figure S4G). In addition, Fzd8 expression was strongly repressed in stage I pancreatic adenocarcinomas (Figure 4G), in which oncogenic activation of *K-Ras* has most likely already occurred. H-scoring further provided semiquantitative analysis indicating Fzd8 was significantly repressed in human malignant pancreatic specimens when compared with normal pancreases including cancer-adjacent normal tissues (Figure 4H; Data S1).

To further confirm the expression of Fzd8 in human pancreas tissues, we used an in situ hybridization method, RNAscope (Wang et al., 2012). As shown in Figure 4I, normal pancreases and cancer-adjacent normal tissues hybridized with the probes specifically for human Fzd8, whereas malignant pancreatic tissue showed no detectible Fzd8 expression. Moreover, Oncomine software (Life Technologies), showed that Fzd8 was significantly downregulated in human pancreatic ductal adenocarcinomas and multiple types of human cancer (Figure S4H). Taken together, we conclude that expression of Fzd8 is repressed in multiple cancers and suggest it may have a role as a tumor suppressor.

Wnt/Ca^{2+} Signaling Is Modulated by K-Ras-Calmodulin Interaction

Calmodulin (CaM) binds preferentially to GTP-bound K-Ras4B, but not to H-Ras, N-Ras, or K-Ras4A (Klee and Vanaman, 1982; Schulman, 1993; Villalonga et al., 2001) (Figure 5A). This specific interaction between CaM and K-Ras can change its subcellular localization and thus reduce the pool of CaM available to activate CaMKii and its subsequent non-canonical Wnt/Ca^{2+} signaling. The hypervariable region of K-Ras4B is essential for CaM binding, and phosphorylation of Ser181 of K-Ras4B abolishes this interaction (Lopez-Alcalá et al., 2008; Villalonga et al., 2001). We generated retroviruses encoding either a mutant (S181D) that mimics phosphorylation or mutant (S181A) form of K-Ras^{V12} that cannot undergo phosphorylation and introduced these mutants into NIH/3T3 cells (Figure S5A). K-Ras^{V12}-S181D did not co-immunoprecipitate with CaM, whereas wild-type and the S181A mutant maintained interaction with CaM under identical conditions (Figure 5B). K-Ras^{V12}-S181D-expressing cells showed increased levels of phospho-CaMKii and marked increase in Fzd8 promoter activity, as well as increased expression of Fzd8, in comparison with K-Ras^{V12}- or K-Ras^{V12}-S181A-transformed cells (Figures 5C–5E). Even though they exhibited comparable levels of K-Ras protein expression and phospho-Erk, K-Ras^{V12}-S181D-infected NIH/3T3 cells showed elevated levels of active CaMKii when compared with K-Ras^{V12}- or K-Ras^{V12}-S181A-transformed cells (Figure 5E). K-Ras^{V12}-S181D-expressing cells further showed increased transcriptional activity of NF-AT, another major down-stream mediator of Wnt/Ca^{2+} signaling pathway, and repression of β-catenin transcriptional activity (Figure 5F). These data show that K-Ras regulates Fzd8-mediated non-canonical Wnt/Ca^{2+} signaling and sequential canonical Wnt/β-catenin signaling by specific interaction with CaM. In contrast, oncogenic H-Ras-transformed tumor cells contain sufficient free CaM to activate CaMKii. N-Ras, like H-Ras, is unable to bind to CaM (Figure S5C). As a result, cells transformed by N-Ras resemble those transformed by H-Ras, including the phosphorylation of CaMKii, elevated expression of Fzd8, and decreased β-catenin/TCF/LEF transcriptional activity (Figures S5D–S5F).

Taken together, these data suggest that the differential interactions between CaM and Ras proteins are the key components that distinguish down-stream CaMKii activity and Fzd8 expression and that disrupting the interaction between K-Ras and CaM by stimulating phosphorylation of S181 is an attractive approach to suppress oncogenic K-Ras-driven malignancy.

Phosphorylation of K-Ras by Treatment with Prostratin Compromises Its Binding to CaM and Suppresses Tumorigenicity

Protein kinase C (PKC) regulates K-Ras by phosphorylation of S181 (Bivona et al., 2006). While typical phorbol esters, such as PMA, activate PKC and promote tumor formation, an atypical PKC activator, prostratin (12-deoxyphorbol-13-acetate), is not a tumor promoter (Szallasi et al., 1993; Zayed et al., 1984). Here, we determined whether this non-tumor promoting PKC activator could be used as an anti-cancer agent to reduce K-Ras-mediated malignancy (Figure 5G).

Prostratin activates PKC in a dose-dependent manner (Figure S6A) and abolishes the interaction between K-Ras and CaM in Ras^{V12}-transformed cells and human pancreatic cancer cell lines (Figure 5H) and increased levels of phospho-CaMKii (Figures 5H and 5I). Prostratin also increased expression of *Fzd8* and reduced expression of the β-catenin targeted gene, *LEF1* (Figure S6B). Of note, prostratin did not alter the activity of CaMKii in cells transformed by H-Ras^{V12} or K-Ras^{V12}-S181D (Figure 5I). Moreover, K-Ras^{V12}, but not H-Ras^{V12} or K-Ras^{V12}-S181D-transformed NIH/3T3 cells, were sensitive to prostratin and showed dramatically reduced cell viability (Figure 5J). These in vitro responses led us to ask whether prostratin could be used to treat K-Ras-driven malignancies. Indeed, prostratin administered either orally or intra-peritoneally strongly suppressed tumorigenicity of K-Ras^{V12}-transformed cells with no evidence of systemic toxicity (Figure S6C). A single, small subcutaneous tumor derived from K-Ras^{V12}-NIH/3T3 cells in the presence of prostratin showed greatly increased phospho-CaMKii compared with vehicle control (Figure S6D).

Prostratin Suppresses Tumor Initiation and Growth of Human Pancreatic Cancers

In response to prostratin, human pancreatic cancer cells showed morphological changes, phenocopying those observed with knock down of K-Ras or overexpression of Fzd8 (Figure S7A, left panel). Moreover, treatment of prostratin significantly reduced

(H) H-scores of Fzd8 immunoreactivities in pancreatic tissue arrays including normal and malignant tissues.
(I) RNAscope in situ hybridization probed for human Fzd8 in cancer adjacent pancreatic normal tissue and pancreatic adenocarcinoma.
*$p < 0.05$; **$p < 0.01$.
See also Figure S4.

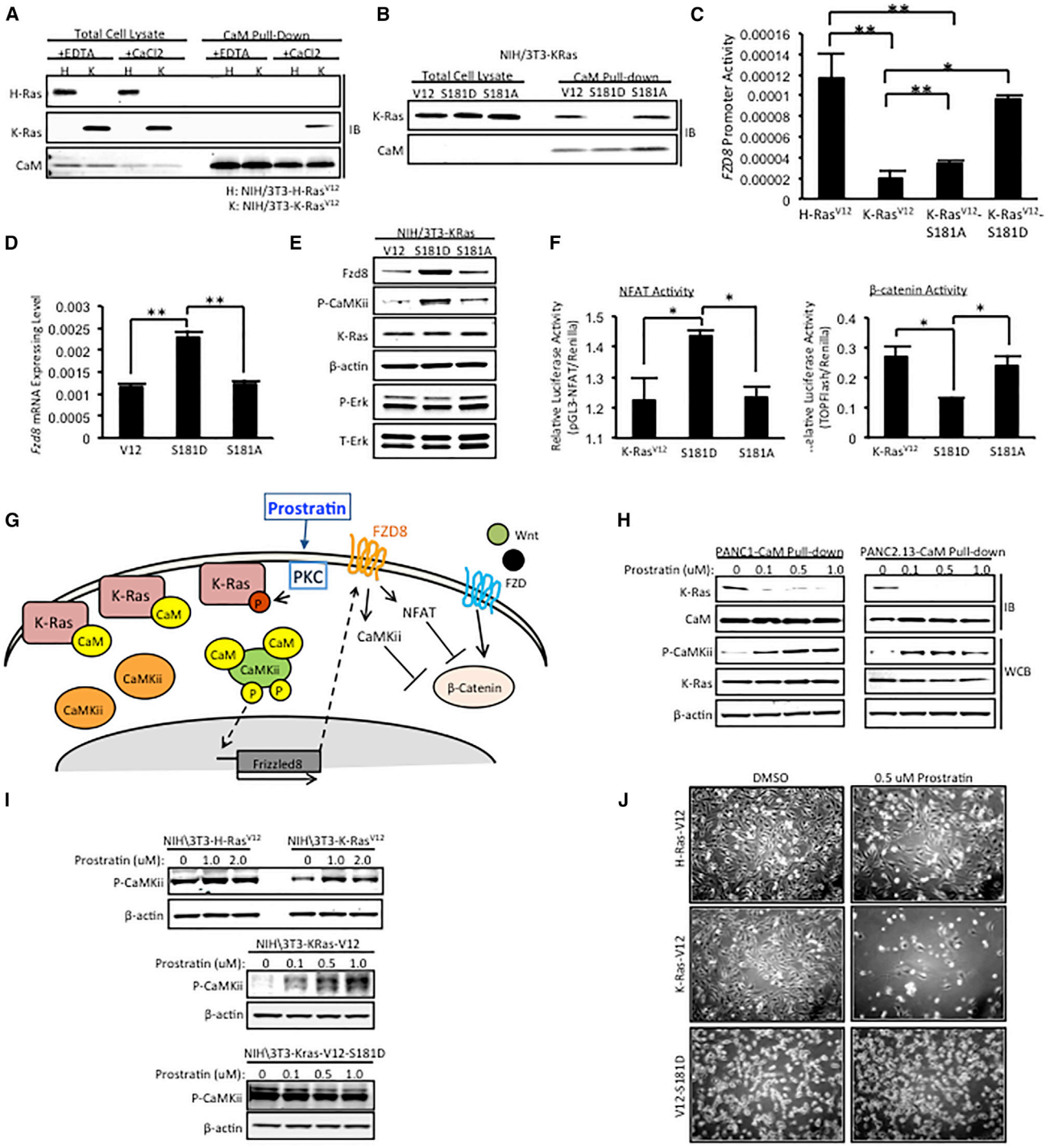

Figure 5. CaM-KRas Interaction Is Essential for Suppression of CaMKii Activity and Fzd8 Expression in K-Ras^{V12}-Transformed NIH/3T3 Cells

(A) CaM interaction with K-Ras^{V12}, but not with H-Ras^{V12}, as revealed by CaM pull-down assay in the presence of EDTA or Ca^{2+}.

(B) Loss of interaction between CaM with K-Ras^{V12}-S181D mutant when compared with K-Ras^{V12} or K-Ras^{V12}-S181A mutant.

(C) K-Ras^{V12}-S181D mutant presented reduced capability to suppress Fzd8 promoter activities when compared with K-Ras^{V12} or K-Ras^{V12}-S181A mutant (n = 4).

(D) Increased Fzd8 expressions in NIH/3T3-K-Ras^{V12}-S181D cells when compared with K-Ras^{V12} or K-Ras^{V12}-S181A group at RNA level (n = 3).

(E) K-Ras^{V12}-S181D-expressing NIH/3T3 cells showed increased levels of Fzd8 expression and phospho-CaMKii when compared with NIH/3T3-K-Ras^{V12} or NIH/3T3-K-Ras^{V12}-S181A cells. There was no significant difference in the levels of K-Ras protein and phospho-Erk among three cell lines.

(F) NIH/3T3-K-Ras^{V12}-S181D cells presented significantly increased NF-AT transcriptional activity (left) and reduced Wnt/β-catenin activity (right) when compared with K-Ras^{V12} or K-Ras^{V12}-S181A group (n = 4).

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both cell viability and proliferation rates of these cells (Figure S7A, right panel).

To test the effects of prostratin on K-Ras-driven human pancreatic cancers in vivo, we first asked whether prostratin can prevent pancreatic tumor formation in a xenograft model. As Figure 6A shows, prostratin significantly reduced the frequency of tumor formation at subcutaneous sites. Moreover, the average size of established tumors was much smaller than tumors in the control group (Figure 6B). Pancreatic tumor cells were labeled with luciferase for detecting tumor formation more accurately. Bioluminescence imaging (BLI) confirmed that treatment with prostratin profoundly suppressed tumor initiation and tumor size (Figure 6C). In addition, xenografted pancreatic tumors treated with prostratin showed reduced tumor growth rates during therapy, as well as reduced expression of Ki67 (Figure 6D).

We next evaluated the effects of prostratin in orthotopic models of human pancreatic cancer cells with mutant *K-Ras*. PKC activity assays revealed that the oral route was preferable for delivering prostratin efficiently into the pancreas relative to the intraperitoneal route (Figure S6E). Immuno-compromised NOD-SCID mice receiving daily oral treatment of prostratin had lower tumor burden in the orthotopic sites compared to control mice. BLI analysis revealed that prostratin reduced the sizes of orthotopic tumors (Figures 6E and S7B). Moreover, prostratin reduced metastasis to the peritoneum in orthotopic pancreatic cancer models (Figures 6E and S7C). As shown in Figures 6F and S7B, H&E staining revealed that most of prostratin-treated mice did not show formation of primary tumors in the pancreas, whereas mice in the control group had obvious orthotopic tumors and normal pancreatic tissue was barely detectable. In addition, orthotopic tumors in the prostratin-treated group expressed much lower Ki67 (Figures 6G and S7B). Our data suggest that prostratin significantly reduces tumor initiation frequency of human pancreatic cancers in xenograft models.

Next, we tested the anti-tumor effects of prostratin on established human pancreatic xenograft tumors, subcutaneously or orthotopically transplanted into immune-compromised mice. Daily oral prostratin treatment started around 10–14 days post-injection, depending on the cell lines and models (Figure 7A). Prostratin showed anti-tumor activity on human pancreatic subcutaneous tumors, defined by reduced growth rate when compared to vehicle-treated tumors (Figure 7A). Additionally, prostratin-treated subcutaneous tumors showed enhanced expression of cleaved caspase 3 (Figure S7D), suggesting it exerts cytotoxic effects on established tumors.

Cell-free DNA (cfDNA) in the bloodstream shows a nearly perfect correlation with primary tumor size following effective therapy or tumor recurrence (Anker et al., 1999; Sozzi et al., 2003). Therefore, the quantification of cfDNA can be a useful tool for monitoring certain cancers, including pancreatic malignancies (Sikora et al., 2015). Here, we used Taqman probes specifically detecting human cfDNA in mice in which human pancreatic cancer cells had been orthotopically implanted (Cheng et al., 2009) (Figure 7B). The level of human cfDNA increased more than six times above baseline on day 14 post-tumor implantation. The concentration of human-specific cfDNA decreased in prostratin-treated animals over time, whereas it showed positive dynamic changes in controls (Figure 7B). These data demonstrate that prostratin significantly represses the burden of human pancreatic cancers in orthotopic xenograft models.

Taken together, our data suggest that prostratin, an activator of atypical PKCs, can efficiently reduce the interaction of K-Ras and CaM, rewire Wnt/Ca^{2+} signaling, and suppress malignancy mediated by oncogenic K-Ras in pancreatic cancers.

Prostratin Specifically Represses K-Ras^{G12V}-Induced Papilloma

While the xenografts described above demonstrate the potential of human tumor cells to respond to prostratin in vivo, the effects of the tumor microenvironment and immune system are not recapitulated in these models. Therefore, we examined the effects of prostratin in a genetically engineered mouse model (GEMM). We used a papilloma model driven by oncogenic H-Ras or K-Ras under the control of a skin stem cell promoter, Lrig1 (Figure 7C) (Jackson et al., 2001; Jaks et al., 2010; Page et al., 2013; Powell et al., 2012; Tuveson et al., 2004). In this GEMM, tamoxifen-inducible Cre recombinase initiates the expression of oncogenic H-Ras or K-Ras, inducing papillomas during wound-healing (Figure 7C).

Starting from day 5 post-tamoxifen induction and 2 days before wounding, daily oral prostratin treatment was administered to Lrig1-Cre/ER/LSL/K-Ras^{G12D} and Lrig1-Cre/ER/LSL/H-Ras^{G12V} mice (Figure 7C). Six of six Lrig1-Cre/ER/LSL-K-Ras^{G12D} mice receiving placebo treatment had detectable papillomas within 7 days post-wounding; however, only two of six Lrig1-Cre/ER/LSL/K-Ras^{G12D} mice treated with prostratin showed papilloma formation, at a delayed point in time (between 14 and 26 days post-wounding) (Figure 7D, bottom panel). In addition, prostratin significantly reduced the invasive signatures and the growth rate of K-Ras^{G12D}-induced papillomas (Figures 7E and S7E). Lrig1-Cre/ER/LSL/H-Ras^{G12V} mice showed no significant difference in tumor initiation rates and tumor sizes between control (7/9) and prostratin-treated group (8/9) (Figures 7D, S7F, and S7G). In conclusion, prostratin significantly

(G) Schematic illustration of CaM-K-Ras interaction in K-Ras-mediated repression of Fzd8 expression and Fzd8-promoted stem-ness. Prostratin is proposed to interfere the interaction through phosphorylation of K-Ras by the activation of PKC.

(H) CaM interaction with K-Ras^{V12} was suppressed by the treatments of prostratin, as revealed by CaM pull-down assay. WCB, whole cell lysate; IB, immunoblotting.

(I) Elevated activation of CaMKii by prostratin treatments in NIH/3T3 cells transformed by K-Ras^{V12}, not K-Ras^{V12}-S181A mutant or H-Ras^{V12}.

(J) Cell morphologies of NIH/3T3 cells transformed by K-Ras^{V12}, K-Ras^{V12}-S181A mutant, and H-Ras^{V12} in the response to prostratin treatments. DMSO was used as the vehicle control.

*$p < 0.05$; **$p < 0.01$.

See also Figures S5 and S6.

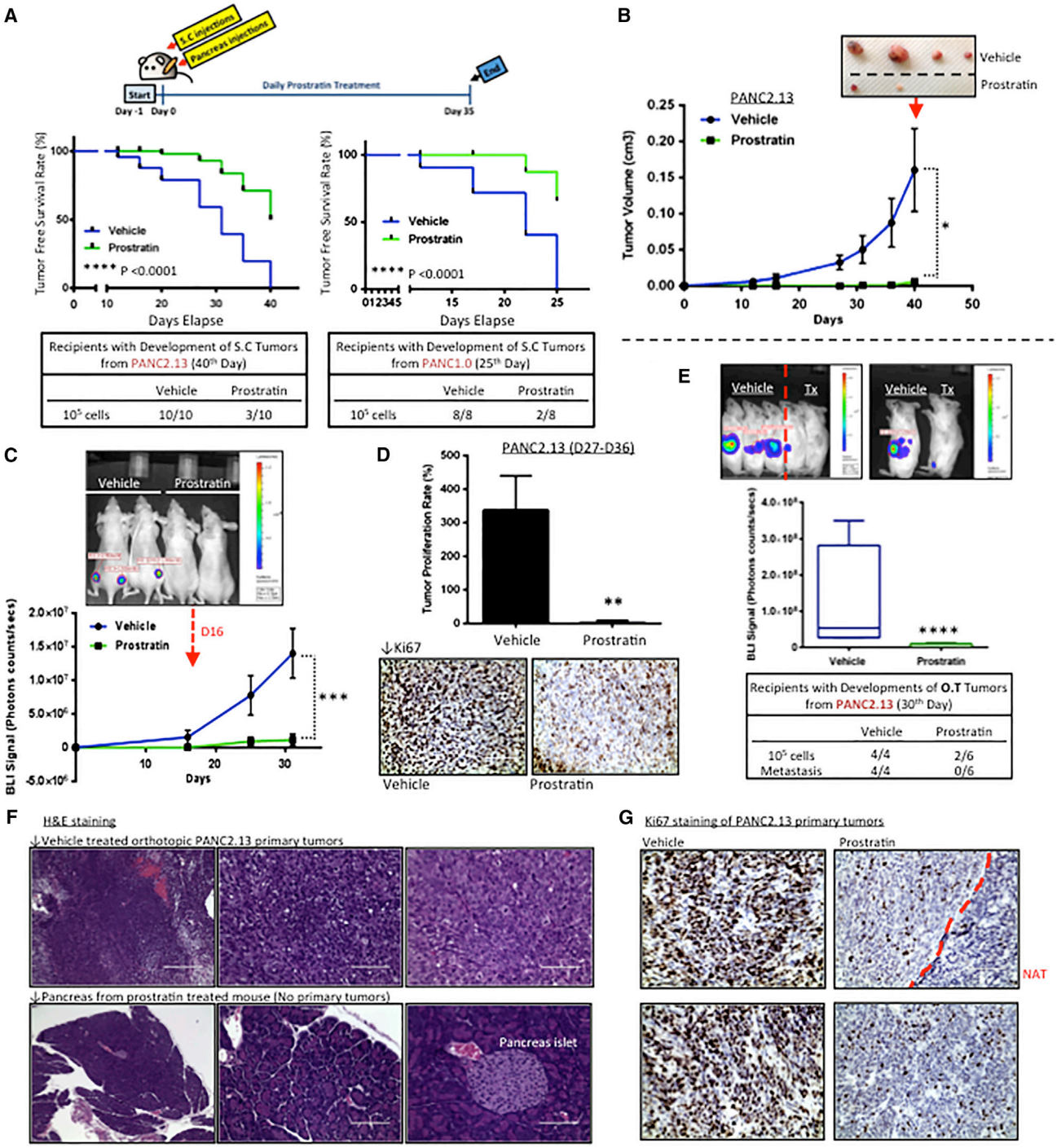

Figure 6. Prostratin Prevented Tumor Initiation of Human Pancreatic Cancers with Mutant *K-Ras*

(A) Tumor initiation rates of subcutaneously injected PANC1 and PANC2.13 in the response to either vehicle or prostratin treatments. Top panel: schematic illustration of experimental design. Oral prostratin administration was given 1 day before tumor implantation. Nude mice were used for subcutaneous injections, and SCID mice were used for orthotopic implantation.

(B) Tumor growth curve of the subcutaneous tumors derived from PANC2.13 in the response to drug treatments (n = 10).

(C) Bioluminescence imaging (BLI) signaling changes of the subcutaneous tumors derived from PANC2.13 in the response to drug treatments (n = 10).

(D) Tumor proliferation rate and Ki67 staining of the subcutaneous tumors derived from PANC2.13 in the response to drug treatments. Tumor proliferation rate (D27–D36) = (size of tumor on D36 − size of tumor on D27)/size of tumor on D36 × 100.

(E) Tumor initiation rate and bioluminescence imaging (BLI) signaling activity of the orthotopic tumors derived from PANC2.13 in the response to drug treatments.

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reduced the formation of papillomas driven by K-Ras^{G12D}, whereas it showed no effect on H-Ras^{G12V}-induced tumors.

All the described experiments in xenograft and GEMM models are approved by UCSF IACUC (protocol number: AN092211-03D).

DISCUSSION

Historically, the high degree of sequence homology, coupled with similar ability of mutant *H-Ras*, *N-Ras*, and *K-Ras* oncogenes to transform cells in culture and to activate common cellular signaling pathways, supported the idea that these three *Ras* gene products are functionally redundant (Bos, 1989; Castellano and Santos, 2011; Chesa et al., 1987; Furth et al., 1987; Leon et al., 1987). Here, we report that *H-Ras* and *K-Ras* actually differ in their abilities to induce tumor initiation and that this is directly related to the ability of K-Ras to suppress the Fzd8-mediated non-canonical Wnt/Ca^{2+} signaling pathway. One consequence of suppressing this pathway is the induction of canonical Wnt signaling. However, our data, and those of others, suggest that this induction is not sufficient to promote malignancy, at least in pancreatic tissue.

To be noted, two key down-stream effectors of non-canonical Wnt/Ca^{2+} signaling, NF-AT and CaMKii, are involved in the regulation of expression of multiple genes (Krebs, 1998; Rao et al., 1997). For example, NF-AT is a transcription factor and has been predicted to directly bind to the promoter regions of multiple genes that are associated with tumorigenesis, such as *TNF* and *MMP9*. The functions of NF-AT on down-stream genes and their potential crosstalk with K-Ras need to be investigated further.

CaMKii is regulated by binding to CaM (Bachs et al., 1994; Klee and Vanaman, 1982; Stewart et al., 1982). Interestingly, CaM also binds to K-Ras4B, but not to N-Ras, H-Ras, or K-Ras4A (Villalonga et al., 2001). Furthermore, the binding of K-Ras to CaM is attenuated by phosphorylation of Ser181 by PKC. The K-Ras^{V12} S181D variant lost the ability to suppress CaMKii activity and Fzd8 expression, suggesting that the interaction between K-Ras and CaM, which is GTP-dependent, is an important pathway for *K-Ras* to inhibit Fzd8-mediated non-canonical Wnt/Ca^{2+} signaling. Therefore, blocking this specific interaction between K-Ras and CaM may provide a novel approach to target K-Ras selectively.

Here, we report that activation of PKC by prostratin leads to the dissociation of K-Ras and CaM, activation of non-canonical Wnt/Ca^{2+} signaling, and suppression of K-Ras-mediated malignancy. Our finding leads to an open question: while the activation of PKC isozymes by phorbol esters has long been considered to promote tumorigenesis, what drives the difference between PKC activation by prostratin and other typical activators, such as PMA?

PKC has been implicated in tumorigenesis for over 30 years (Castagna et al., 1982). PKC is a family of related isoforms, categorized as conventional (α, βI, βII, and γ), novel (δ, ε, η, and θ), and atypical (ζ, λ/ι) (Basu and Pal, 2010), which exhibit overlapping as well as opposing functions (Steinberg, 2008). For example, PKCδ is believed to function as a tumor suppressor since downregulation rather than activation of PKCδ has been associated with tumor promotion (Lu et al., 1997). Surprisingly, the majority of cancer-associated mutations in PKC subgroups are loss-of-function (Antal et al., 2015), and several mutations are dominant negative, that suppress global PKC signaling (Antal et al., 2015). This establishes a hypothesis: PKC isozymes generally function as tumor suppressors and, therefore, anti-cancer therapies should focus on restoring, not inhibiting PKC activity. This suggestion had also been made by Bivona et al. (2006) based on their observations that PKC-mediated phosphorylation of K-Ras at Serine-181 affects K-Ras activity.

Interestingly, PMA and prostratin have been shown to differ substantially in their biological activities (activation versus subcellular translocation) on PKCα and PKCδ (Márquez et al., 2008), potentially explaining their distinct properties on tumor promotion. Moreover, although K-Ras is known to be phosphorylated by PKC, the specific functions of different PKC isozymes on the phosphorylation of S181 within the polybasic region of K-Ras remain to be defined. Further investigation of the specific PKC isoforms required for K-Ras phosphorylation and regulation by prostratin will be needed to clarify these unanswered questions and to develop more potent inhibitors for blocking K-Ras-CaM interactions.

In summary, K-Ras suppresses Wnt/Ca^{2+} signaling pathway by direct binding with CaM, leading to the reduced CaMKii activity and downregulating Fzd8 expression. Downregulation of Fzd8 expression by K-Ras leads to a sustained suppression of Wnt/Ca^{2+} signaling, which causes increased tumorigenicity. The isoform-specific activity of K-Ras, described here, can be exploited to block K-Ras oncogenic activity without affecting other Ras isoforms.

EXPERIMENTAL PROCEDURES

Cell Lines

NIH/3T3, BxPC3, PANC1, PANC2.13, SW480, LS180, HCT15, and HCT116 cells were from ATCC. Mouse pancreatic adenocarcinoma cells were from Dr. Collisson (University of California, San Francisco). Mouse cell lines were grown in DMEM with 10% FBS (or calf serum [CS] for NIH/3T3 cells) at 37°C, 5% CO_2. Human colon cancer cell lines were cultured in RPMI-1640 medium with 10% FBS. Human pancreatic cancer cell lines were maintained in RPMI-1640 medium supplemented with 15% FBS and human recombinant insulin (GIBCO 12585-014).

Animal Studies

All experiments were approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee (IACUC). Ras^{V12}-transformed NIH/3T3 cells were subcutaneously injected in female nude mice (Nu/Nu) at 50, 100, or 1,000 cells per flank. Palpable tumors were measured twice a week. Mice were divided into five/group. Pancreatic adenocarcinoma cells-derived *Kras*$^{LSL-G12D}$ mice were provided by Dr. Collisson and genotyped

(F) H&E staining of normal mouse pancreases and orthotopic tumors derived from PANC2.13.
(G) Ki67 staining of orthotopic tumors derived from PANC2.13 in the response to drug treatments.
*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Data are means ± SEM for (B) and (C).
See also Figure S6.

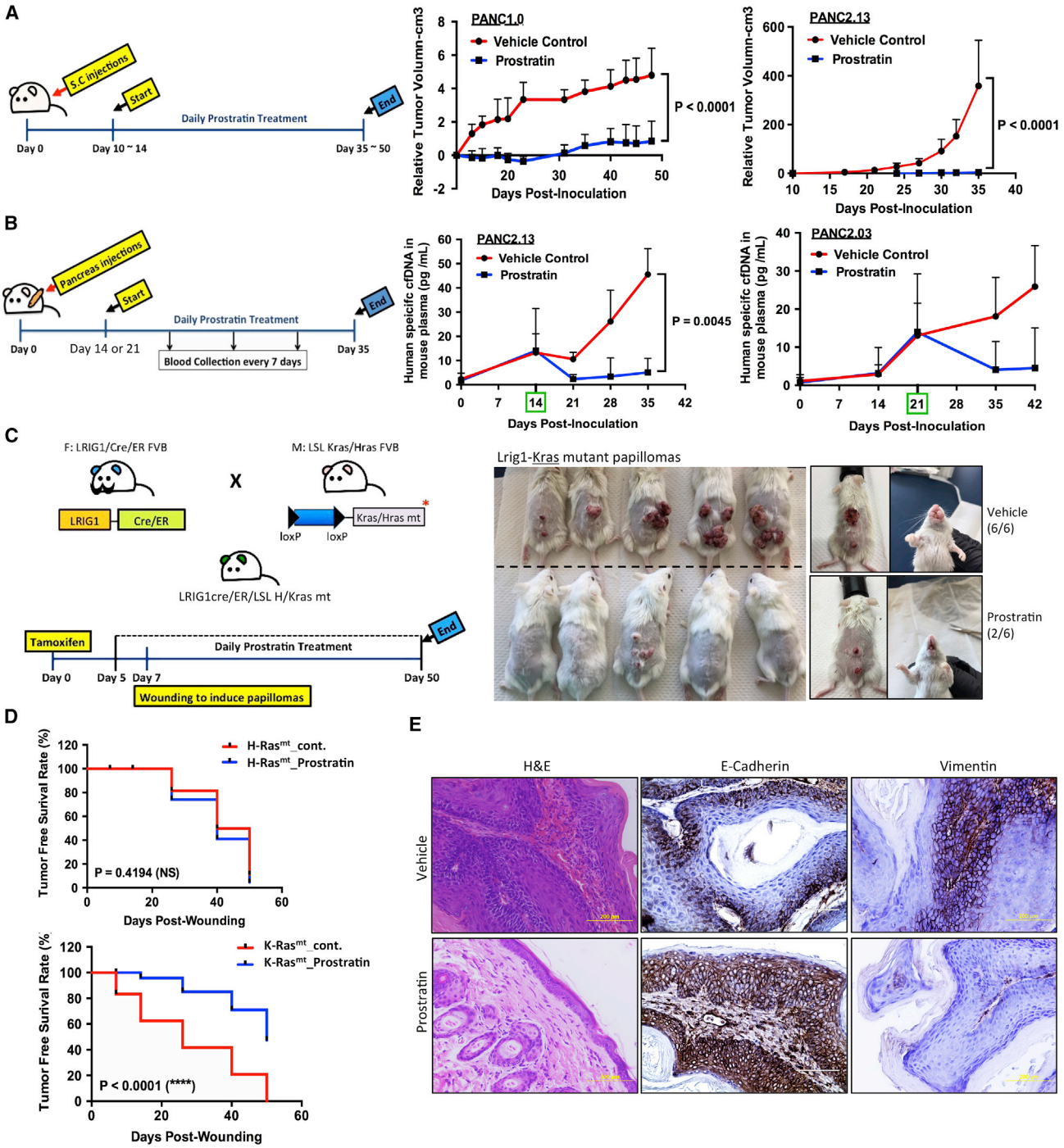

Figure 7. Prostratin Represses In Vivo Malignancy Driven by Oncogenic K-Ras

(A) Prostratin showed anti-tumor effects on established subcutaneous tumors derived from 0.5 × 10^6 cells of PANC1 or PANC2.13 (n = 7; data are means ± SEM).

(B) Prostratin suppressed orthotopic tumor burdens measured by cfDNA values (n = 5 for PANC2.13 group; n = 6 for PANC2.03 group).

(C) Prostratin reduced the incidence of papilloma formations in LRIG1cre/ER/LSL-Ras^{mutant} GEMM. Left: schematic illustration of the generation of papilloma in LRIG1cre/ER/LSL-Ras^{mutant} mice. Right: the pictures of mice carrying K-Ras^{G12D}-induced papillomas with vehicle or prostratin treatment.

(D) Prostratin affected papilloma initiation differently in LRIG1cre/ER/LSL-H- and K-Ras^{mutant} mice.

(E) H&E staining and IHC stained for E-cadherin and vimentin in K-Ras^{G12D}-induced papillomas with vehicle (top) or prostratin treatment (bottom).

See also Figure S7.

as described (Collisson et al., 2011; Dankort et al., 2007; Hingorani et al., 2003). One hundred cells were orthotopically implanted in 6- to 8-week-old FVB/n mice in 20 μl 50% Matrigel using a 28.5-gauge needle. Mice were monitored for 1 month and were euthanized when distressed.

Skin tumors were induced by the two-stage chemical carcinogenesis protocol using DMBA and TPA (Balmain and Pragnell, 1983). Histologically confirmed skin carcinomas were processed for molecular analyses by conventional methods. Mutations in Kras and HrasKI alleles were identified by direct sequencing (Karnoub and Weinberg, 2008; To et al., 2008).

Prostratin for animal treatment was from Santa Cruz Biotechnology (sc-203422A). It was administrated daily into either NOD-SCID mice or athymic NUDE mice by oral gavage at 1 mg/kg or intraperitoneal injection at 0.5 mg/kg. Ten percent DMSO, 10% cremophor, and 80% saline solutions were used as the solvent and vehicle control. The toxic effects of prostratin were evaluated by monitoring body weight for at least 30 days.

Lrig1Cre/ER/LSL Hras^{G12V} and *Lrig1Cre/ER/ LSL-Kras^{G12D}* mice have been backcrossed into the *FVB/N* background over multiple generations. Cre recombinase was activated in both groups of mice. A single dose of 4-OH-tamoxifen was topically applied at 8 weeks of age and on day 7. Thereafter, papilloma development was induced by wounding the back of the mice.

DNA Extraction from Mouse Plasma Samples

All blood samples were collected in K_2EDTA containing tubes (BD Microtainer; 365974) and centrifuged at 1,500 × *g* for 10 min. The supernatants were collected from the top of the plasma to eliminate cell contamination. Plasma was stored at −80°C. cfDNA was extracted from 100 μl of plasma using NucleoSpin Plasma XS kit (Macherey-Nagel; 740900). Quantification of human cfDNA in mouse plasma is described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, two tables, and one data file and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.10.041.

AUTHOR CONTRIBUTIONS

M.-T.W. performed most of the experiments. M.H. generated the K-Ras^{V12}-S181D and -S181A mutant constructs. M.D.T., R.D., and A.B. generated the chemically induced skin tumors from the *H-Ras* knockin mice and also provided tumor-bearing Lrig1-Cre/ER-K-RasLSLG12D and Lrig1-H-RasLSLG12V mice. J.G. provided technical assistance. M.-T.W. and F.M. conceived the project, designed experiments, interpreted results, and wrote the manuscript.

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