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PLEKHA7 signaling is necessary for the growth of mutant KRAS driven colorectal cancer

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Abstract

Plekha7 (Pleckstrin homology [PH] domain containing, family A member 7) regulates the assembly of proteins of the cytoplasmic apical zonula adherens junction (AJ), thus ensuring cell-cell adhesion and tight-junction barrier integrity. Little is known of Plekha7 function in cancer. In colorectal cancer (CRC) Plekha7 expression is elevated compared to adjacent normal tissue levels, increasing with clinical stage. Plekha7 was present at plasma membrane AJ with wild-type KRas (wt-KRas) but was dispersed in cells expressing mutant KRas (mut-KRas). Fluorescence lifetime imaging microscopy (FLIM) indicated a direct Plekha7 interaction with wt-KRas but scantily with mut-KRas. Inhibiting Plekha7 specifically decreased mut-KRas cell signaling, proliferation, attachment, migration, and retarded mut-KRAS CRC tumor growth. Binding of diC8-phosphoinositides (PI) to the PH domain of Plekha7 was relatively low affinity. This may be because a D175 amino acid residue plays a “sentry” role preventing PI(3,4)P_2 and PI(3,4,5)P_3

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Declaration of competing interest

Garth Powis, Lynn Kirkpatrick, Emmanuelle Meuillet, and Martin Indarte hold stock and/or are employees of PHusis Therapeutics. There are no other conflict of interests to declare.

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binding. Molecular or pharmacological inhibition of the Plekha7 PH domain prevented the growth of mut-KRas but not wt-KRas cells. Taken together the studies suggest that Plekha7, in addition to maintaining AJ structure plays a role in mut-KRas signaling and phenotype through interaction of its PH domain with membrane mut-KRas, but not wt-KRas, to increase the efficiency of mut-KRas downstream signaling.

Keywords

Mutant KRAS; PLEKHA7; PH domain

1. Introduction

Plekha7 (Pleckstrin homology domain containing, family A member 7) is a member of the Plekha family of proteins having a characteristic pleckstrin homology (PH) domain consisting of an approximately 120 amino acid structural fold that binds membrane phosphoinositides (PIs) [1]. PH domains are found in a wide range of proteins involved in intracellular signaling and as constituents of the cytoskeleton [2,3]. The most studied role of Plekha7 is as a cytoskeletal adaptor that regulates the assembly of proteins of the cytoplasmic apical zonula adherens junction (AJ) linking cell surface E-cadherin to the microtubule cytoskeleton, thus ensuring cell-cell adhesion and tight junction barrier integrity [4,5]. AJ proteins assembled by Plekha7 include p120-Catenin, Paracingulin, Cingulin, Afadin, and Nezha [6,7]. Binding of Plekha7 to Pdz11 protects Nectin 1/3 transmembrane adhesion proteins from proteolytic degradation, also promoting tight junction stability [8]. A complex formed by Plekha7 and the Adam 10 toxin receptor gives stable toxin pores at the AJ that allows *S. aureus* α-toxin entry to cells [9]. Plekha7 has been reported to recruit Drosha and Dgcr8, core components of the microRNA microprocessor, to apical AJs, and to promote the processing of a subset of miRNAs that suppress anchorage independent cell growth [10,11]. A role for Plekha7 has previously been reported in the regulation of RhoA activity that is important for cross talk between cytoskeletal organization and nuclear signaling mediated by Plekha7 binding partners [12] with Cingulin and Paracingulin sequestering Rho GEFs to tight junctions [8], and cytosolic 120-catenin binding and inhibiting RhoA [13]. RhoA is has recently been shown to coordinate energy metabolism signaling by increasing glucose transport and glycolysis [14] and has been associated with increased colon cancer cell migration and invasion and decreased patient survival [15]. Despite Plekha7's apparent importance for several critical cellular processes PLEKHA7$^{-/-}$ knockout mice do not exhibit developmental abnormalities [16]. Dahl salt-sensitive rats with mutated Plekha7 develop normally, although salt-sensitive hypertension and renal damage are attenuated [17]. Human hypertension [18], and primary angle closure glaucoma [19] have been associated with genetic variants of PLEKHA7. Many human cancers display cytoplasmic and membranous Plekha7 protein, with colorectal cancer (CRC) being among the highest [20]. Increased levels of PLEKHA7 mRNA and protein have been reported to differentiate breast invasive lobular carcinomas from invasive ductal carcinomas [21]. However, other studies found that Plekha7 protein is decreased or lost in high grade breast ductal carcinomas and lobular carcinomas [22]. There are also reports of mis-localized or lost Plekha7 in advanced breast and renal cancer [10], and in inflammatory breast cancer

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[23]. No correlation was found between PLEKHA7 mRNA and tumor type or grade in breast and renal cancer [10], while PLEKHA7 mRNA has been reported to be decreased in high grade compared to low grade epithelial ovarian cancer and to correlates with improved patient outcome [24].

While studying a role for Plekha7 in cellular signaling we unexpectedly found that short term knockdown of Plekha7 inhibited proliferation and tumor growth of CRC cells having mutant KRAS, but was without effect on CRC cells with wild-type KRAS. Herein, we report the biological function and therapeutic implication of inhibiting Plekha7 in mutant KRAS CRC.

2. Materials and methods

2.1. Cells and reagents

CRC cell lines Caco-2, Colo 201, Colo 320DM, DLD-1, HCT8, HCT15, HCT116, HT29, LOVO, LS174T, RKO, NCU-H747, NCI–H716, NCI–H508, SK-CO-1, SW48, SW403, SW480, SW620, SW948, SW1116, SW1417, and WiDR were purchased from the American Type Culture Collection (ATTC Manassas, VA). All cell lines were tested to be *Mycoplasma* free and the identity of each line authenticated before, and at 2 month intervals during study by the Genomics Shared Resource at SBP Cancer Center. HKH-2 cells are a wild-type KRas version of mutant KRas HCT-116 cells with its single mutant KRAS allele deleted [25]. Cells were cultured under recommended conditions with 10% fetal bovine serum in 96-well flat-bottom plates. siRNA transfection was performed with Lipofectamine 2000 and 200 nM of Dharmacon SMARTpool siRNA for 24 h and cell growth measured after 3–4 days using CellTiter 96 (Promega Madison, WI). Anchorage-independent cell growth was measured as colony formation in 0.5% agarose with 10^3 cells per well for up to 4 weeks with fresh medium added every week. For measurement of cell detachment 5×10^5 cells per well were cultured in complete medium in six-well plates coated with Ultra Low Attachment Surface (Corning, Lowell, MA). The number of cells in suspension was measured using the Cell Death Detection ELISA (Roche, Mannheim, Germany). Primary rabbit monoclonal antibodies used for Western blotting were anti: ERK, EGFR, MEK1/2, c-RAF, phospho-Akt (Ser 473), phospho-ERK(Thr202,Tyr220), phospho-EGFR (Tyr1068), and phospho-MEK1/2(Ser 217/221) from Cell Signaling Technology (Danvers, MA), RalA, RalB, and phospho-c-RAF(Ser 338/Tyr340) from EMD Millipore (Billerica, MA), and KRas from Novus Biologicals (Littleton, CO.). diC8- phosphoinositides (PI) were obtained from Echelon Biosciences (Salt Lake City, UT). Cell migration was studied in 6-well tissue-culture plate confluent monolayers scratched with a sterile pipette tip and migration expressed as percentage closure of the scratch. Cell invasion was measure using cell culture filters with 8 μm pores coated on the underside with 8 μg/μL Matrigel® (Sigma-Aldrich). 10^5 cells in medium with 3% BSA were added to upper compartment and after 2 days cells migrating to the on lower side of membrane fixed in 4% paraformaldehyde and stained with crystal violet and counted. Confocal laser scanning microscopy and fluorescence lifetime imaging microscopy (FLIM) was conducted as described in Supplemental Methods 1. Animal studies were carried out in accordance with National Institutes of Health guide for the use and care of laboratory animals (NIH Publication No. 8023, revised 1978).

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Isothermal titration calorimetry (ITC).—The PH domain cDNA, corresponding to residues 164–298 of human Plekha7, was cloned into the BamHI and XhoI restriction sites of the pGEX-6P plasmid, and expressed in *E. coli* BL21 cells. ITC experiments were performed at 23 °C using an iTC200 instrument (MicroCal), with 50 μM Plekha7 PH domain titrated with 0.5–1.0 mM diC8-PI phosphate in 30 mM NaCl, 15 mM Tris pH 7.6, 0.2 mM TCEP buffer. The integrated heat data were processed and analyzed with ORIGIN software (Microcal), and the data fit to a single-site binding model to obtain dissociation constants for each titration.

2.2. Statistical analysis

Quantitative data are represented as the mean and variance of at least three independent experiments. Statistical comparisons were performed using Student's *t*-test or ANOVA test. Differences were considered significant when the p-value was <0.05.

3. Results

3.1. PLEKHA7 inhibition selectively blocks CRC mutant-KRAS but not wild type-KRAS cell growth and phenotype

We first examined the growth of wild-type KRas and mutant KRas lines after Plekha7 siRNA knockdown using a panel of CRC cell lines grown in 2D on plastic. We found that treatment with siRNA to PLEKHA7 markedly inhibited the proliferation of mutant KRas compared to wild-type KRas cells and was independent of the type of KRas mutation (Fig. 1A, Figure S1). We confirmed that Plekha7 knockdown by siRNA lasted at least 4 days (Figure S2). We also found that while siPLEKHA7 and siKRAS both inhibited the growth of mutant KRas HCT-116 cells, in isogenic wild-type KRas HKH-2 cells growth inhibition by siKRAS was attenuated, and not significantly inhibited by siPLEKHA7 (Figure S3). Anchorage independent 3D growth of mutant KRas CRC cells was significantly inhibited by shRNA Plekha7 knockdown and by CRSPR knockout of Plekha7 and both were without effect in wild-type KRas CRC cells (Fig. 1B, Figure S4). We next investigated the effect of Plekha7 inhibition on the phenotype of mutant KRas CRC cells. Detachment of mutant KRas CRC cells from a low adhesion surface was promoted by Plekha7 knockdown which had no effect in wild-type KRas cells (Fig. 2A). Cell invasion of mutant KRas cells through Matrigel® was inhibited by Plekha7 knockdown, but without effect in wild-type KRas cells (Figure S5). The 2D cell migration of mutant KRas HCT-116 cells measured by the scratch assay was significantly inhibited by siPLEKHA7, which did not affect the migration of wild-type KRAS HKH-2 cells (Fig. 2B). A study of the cell cycle effects of shPLEKHA7 showed that there was no effect in wild-type KRas HT-29 cells but in mutant KRas HCT-116 cells there was a block of entry into S cell cycle stage (Figure S6). This was most likely to due to inhibition of Ras-driven activation of Akt and the subsequent inhibition of the CDK inhibitors p27 and p21. Finally, we showed that stable shRNA knockdown of Plekha7 blocked the ability of mutant KRas HCT-116 and DLD-1 cell lines to form tumors *in vivo* but did not affect wild-type KRas HT-29 cell tumor growth (Fig. 2C and Figure S7A). In our hands HCT-116 isogenic HKH-2 cells with wild-type KRas did not form tumors in Nod *scid* mice. Thus, our studies show that inhibition of Plekha7 prevents the effects of mutant KRas

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on *in vitro* cell growth, attachment and invasion, and *in vivo* tumor growth, but is without such effects in wild-type KRas cells.

3.2. PLEKHA7 effects on mutant- KRAS cell signaling

The effects of shRNA knockdown of Plekha7 on signaling in mutant KRas CRC cells showed inhibition of phospho-c-Raf, phospho-Akt (T473), phospho-ERK and phospho-Mek (Fig. 3A and Figure S7B) and a decrease in GTP binding of total Ras protein (Fig. 3B and Figure S7B). We measured Plekha7 and total KRas protein across a panel of CRC cell lines by Western blot and found no difference in levels between wild-type KRas and mutant KRas cells (Fig. 3C). However, a few mutant KRas cell lines and one wild-type KRas line exhibited high levels of KRas protein and in these Plekha7 was also significantly elevated and correlated with the elevated KRas (Fig. 3D). Plekha7 protein showed two bands in nearly all CRC cell lines, probably representing Plekha7 isoforms that have previously been reported [26].

3.3. Plekha7 subcellular localization and association with KRas

Confocal microscopy showed that while both wild-type KRas HKH-2 cells and mutant KRas HCT-116 cells have a similar cell-junction punctate pattern of Plekha7 localization at the plasma membrane, the mutant KRas cells also exhibited dispersed Plekha7 in the cytoplasm (Figure 4A and S8E). Similarly, wild-type KRas expressing HEK293T cells showed only plasma membrane localized Plekha7 (Figure S8A and E) but when expressed together with mutant KRas, cytoplasmic clusters were also evident (Figure S8B and F). To evaluate the proximity between KRas and Plekha7, we used fluorescence lifetime imaging microscopy (FLIM) [27] to measure fluorescence resonance energy transfer (FRET) between GFP-tagged Plekha7 and RFP-tagged KRas. FRET is indicated by a shortening of the average fluorescence lifetime. The FLIM results showed Plekha7 and wild-type KRas, both at the plasma membrane, are within 10 nm of each other indicating a potential direct interaction The mutant KRas on the other hand showed very limited interaction indicated by higher average fluorescence lifetime (Fig. 4B and Figure S8C and D). The lack of interaction is not surprising given the dispersed intracellular vesicular localization of Plekha7 in cells expressing the mutant KRas. This redistribution places Plekha7 away from the plasma membrane and membrane bound mutant KRas resulting in low FRET. The presence of interacting and non-interacting clusters (Fig. 4C) suggests a dynamic fast movement of molecules within the plasma membrane and potential interaction with KRas with fast off rate kinetics. It is currently not clear how mutant KRas exert this its effect on Plekha7 but collective data clearly shows mutant KRas specifically disperses Plekha7 away from the plasma membrane into the cytoplasm.

3.4. PLEKHA7 in patient colon cancer samples

We next examined Plekha7 protein staining of 21 normal human colon mucosa samples and 56 CRC tumor samples on tissue microarray (MDACC) finding increased Plekha7 expression in tumor compared to normal mucosa (Fig. 4C and D), and that Plekha7 staining showed a significant increase with increased clinical stage (Fig. 4E).

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3.5. Phosphoinositide binding by the Plekha7 PH domain

We employed isothermal calorimetry (ITC) to investigate the binding of soluble diC8-PIs to the Plekha7 PH domain finding a relative lack of specificity and low affinity with Kds of 4 μM for PI(3,5)P_2 and PI(4,5)P_2, and 8 μM for PI(3,4)P_2 and PI(3,4,5)P_3 (Fig. 5A and B, and Figure S9). To better understand the binding, the Plekha7 PH domain was modeled from the published crystal structure of Plekha4 PH domain (PDB 1UPR) which is 52% identical and almost 100% identical at the inositol phosphate binding sites to Plekha7, the only difference being substitution of M179 with Val in Plekha4. The published structure of Plekha4 PH contains bound I (1,3,4,5)P_4 whose position is distorted by crystal lattice contacts, so that consensus positions of the Plekha7 ligand were defined by superpositions of the I (1,3,4,5)P_4 binding of the PH domains of the related Grp, Anln, Tapp1 and Dapp1 (Figure S10). Modeling of the electrostatically charged surface of Plekha7 PH with bound PI(3,4,5)P_3 is shown in Fig. 5C. Sequence alignment revealed that the PH domains of Plekha7, Plekha4 and Anln contain a negatively charged Asp residue in the inositol phosphate binding loop (D175-L181 of Plekha7), with the other PH domains having GLY which gives specificity towards PI(3,4,5)P_3 or PI(3,4)P_2 binding. We therefore investigated whether D175 might play a “sentry” role to prevent PI(4,5)P_2 binding by preparing mutant Plekha7 (D175K) and comparing its specificity with the wild-type protein finding a 10 fold increase in affinity towards PI(3,4)P_2 and PI(3,4,5)P_3 (Fig. 5B). When we modeled the subsequently published structure of Plekha7 PH wild-type [28] and D175K mutant we found that the side chain of D175 did not interact with either P_3 or P_5 of the inositol ligand, but bound to an axially oriented 5-OH of inositol, thus weakening the binding of both I (1,4,5)P_3 and I (1,3,4,5)P_4 to the wild-type Plekha7 compared to the Plekha7 (D175K) (Fig. 5D). The affinity of PI(4,5)P_2 did not change probably because its P_5 is bound predominantly by hydrogen bonds to uncharged serine residues, while the affinity of PI(3,4)P_2 and PI(3,4,5)P_3 for Plekha7 (D175K) was increased because P3 is bound through ionic bonds to Plekha7 K173 and R185..

To further investigate the role of the PH domain of Plekha7 on cell proliferation we transfected CRSPR Plekha7 knockout mutant KRas HCT-116 cells with wild-type Plekha7 which did not increase growth in wild-type or knockout cells (Fig. 5E and F). There was a similar lack of effect of wild-type Plekha7 on proliferation in RKO cells which we had found (Fig. 3C) lacks detectable Plekha7 (Figure S11). Transfection with a Plekha7 with four point mutations in the PH domain at K173I, S177E, K183L and R25Q, which we found by ITC to be necessary to completely block PI binding, markedly inhibited growth in wild-type Plekha7 cells There was less inhibition in Plekha7 knockout cells, which were already growth inhibited by loss of Plekha7 (Fig S4). Plekha7 with an N-terminus c-Src myristylation sequence to mis-localize the Plekha7 protein at the plasma membrane had similar growth inhibitory effects. Thus, PI binding by the PH domain of Plekha7 appears to be important for the stimulation of mutant KRAS cell growth, as does correct membrane localization of Plekha7.

3.6. Inhibitors of PLEKHA7 PH domain

The crystal structure of the Plekha7 PH domain was used to screen a library of PH domain probe inhibitors in a search for an inhibitor of the Plekha7 PH domain, which

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were then tested for binding against the expressed PH domain of Plekha7 (Figure S12A and 12B). We chose PHT-7704 (2-((4-(2,2- dimethylbenzo[d][1,3]dioxol-5-yl)thiazol-2-yl)methyl)-4-(2-hydroxyethyl)phthalazin-1(2H)-one) and DPIEL a promiscuous inhibitor of PH domain binding to the plasma membrane previously identified by us [29]. Both compounds showed selective inhibition of mutant KRas CRC cell growth without affecting the growth of wild type KRas CRC cells. Interestingly PHT-7704 also inhibited the growth of mutant BRaf CRC cells, although not other wild-type KRas cells (Figure S12C), something that was not seen with Plekha7 molecular inhibition, suggesting possible additional targets for PHT-7704 related to mutant BRaf signaling.

4. Discussion

Plekha7 is a member of the PH domain family of proteins and is best known as a scaffold that regulates the assembly of proteins of the cytoplasmic AJ ensuring cell-cell adhesion and tight junction barrier integrity [4,5]. Surprisingly, despite this well documented structural role of Plekha7 it is not essential, and when Plekha7 is depleted there is only modest inhibition of AJ barrier function [5], and Plekha7$^{-/-}$ knockout mice and rats develop normally [12,13]. PH domains are frequently found in proteins involved is intracellular signaling [1]. We therefore investigated the relatively unexplored role of Plekha7 as a regulator of cellular signaling.

For our study we chose CRC where we found Plekha7 in patient tumor levels to be elevated compared to normal colon tissue, with tumor levels increasing with clinical stage. KRas mutation occurs in up to 45% of CRC where it is an early event during transformation [30]. Evolutionarily conservative hotspot mutations at KRAS codons 12, 13, 61, and 146, result either in a protein which is irreversibly bound to GTP (codons 12, 13 and 61), or rapidly exchanges GDP for GTP (codon 146), in both cases leading to permanently activated KRas signaling. Active GTP-bound KRas interacts with a range of effectors to activate multiple downstream pathways, the best studied of which are the Raf-MEK-ERK mitogen-activated protein kinase (MAPK) and phosphotidylinositol 3-kinase (PI3K)-AKT serine/threonine protein kinase effector pathways. A feature of all RAS (H, N and KRAS) is that they self-assemble into nanoclusters, small (6–20 nm diameter) short lived ($t1/2 < 0.4s$) plasma membrane tethered microdomains where RAS is associated with other membrane-associated proteins, effectors and scaffolding proteins [[31], [32], [33]].

In our studies with Plekha7 we found that inhibiting Plekha7 in mutant KRas CRC cells decreases KRas downstream signaling associated with decreased, cell proliferation, attachment and migration, and inhibition of tumor growth. However, inhibition of Plekha7 had no such effects in wild-type KRas CRC cells. Our findings suggest that Plekha7, in addition to a role in maintaining AJ structure, plays a role in maintaining mutant KRAS signaling and phenotype in CRC cells. Confocal imaging showed that mut-KRas displaces Plekha7 from the plasma membrane to cytoplasmic clusters. Our FLIM imaging indicates a there is a direct interaction of Plekha7 with wild-type KRas on plasma membrane. However, due to a large dispersion of Plekha7 from the plasma membrane to intracellular vesicles, we postulate that an insufficient concentration of Plekha7 is present on the plasma membrane to for FLIM-FRET detection. The nature of these intracellular particles which we think

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are Plekha7 phase condensates generated by mut-KRas are the subject of a detailed future investigation. KRas in plasma membrane clusters has been suggested to provide a more efficient platform for downstream signaling than monomeric KRas [33]. It is possible that cytoplasmic mutant KRas and Plekha7 clusters could contribute to the increased efficiency of cytoplasmic mutant KRas downstream signaling caused by Plekha7. Furthermore, under normal condition Plekha7 contributes to maintenance of AJ structure and cell-cell cohesion, and the dispersion of Plekha7 to cytoplasmic signaling clusters with mutant KRas might contribute to decreased cancer cell cohesion and metastasis, a phenotype of mutant-KRas cells.

PH domains share a common fold and some sequence homology [1,3]. A characteristic of PH domains involved in intracellular signaling is binding to membrane PIs, although their affinities and selectivity for PIs vary considerably [3,31]. Using ITC we showed that the Plekha7 PH domain binds to soluble diC8-PIs with relatively low affinity with Kds of 4 μM for $PI(3,5)P_2$ and $PI(4,5)P_2$, and 8 μM for $PI(3,4)P_2$ and $PI(3,4,5)P_3$ and that a D175 residue appears to play a “sentry” role to specifically prevent the binding of $PI(3,4)P_2$ and $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ is produced by PI-3-kinase, a key second messenger in pathways related to cell survival and metabolism, while dephosphorylation of $PI(3,4,5)P_3$ by phosphatases produces $PI(3,4)P_2$ [32]. It could be that Plekha7 is molecularly desensitized by its D175 residue from playing a role in PI-3-kinase signaling. Our findings suggest that PI binding by the PH domain of Plekha7 is important for Plekha7’s ability to stimulate mutant KRas cell growth. An important caveat is that binding of Plekha7 to PIs in lipid membranes may be different to binding in free solution [28]. We also found that the inhibited growth of mutant KRas CRC cells lacking Plekha7 was not restored by reintroduction of wild-type Plekha7, and was inhibited by Plekha7 with a mutated PH domain so as to be unable to bind PIs. These features suggest that Plekha7 has properties of a scaffold protein where membrane phosphoinositide binding by the PH domain also plays a role, such that its expression at concentrations in excess of its binding partners sequester these partners in incomplete complexes, leading to combinatorial inhibition of signaling activity [34,35]. Similar effects were encountered with Cnk1 another PH domain scaffold protein regulator of mutant KRas signaling [36]. Indeed, we observed a pull-down association between Plekha7 and Cnk1 (Fig. S13), and it is possible that together with Rho they form a macro membrane signaling platform.

Thus, in our studies we have shown that Plekha7, in addition to maintaining AJ structure, plays a role in mutant KRas signaling and phenotype through interaction with the plasma membrane to increase the efficiency of mutant KRas downstream signaling. The Plekha7 PH domain might be a useful drug target to inhibit all the forms of mutant-KRas in tumors, but more work is needed to develop inhibitor agents with greater selectivity and optimized pharmacokinetic properties.

Supplementary Material

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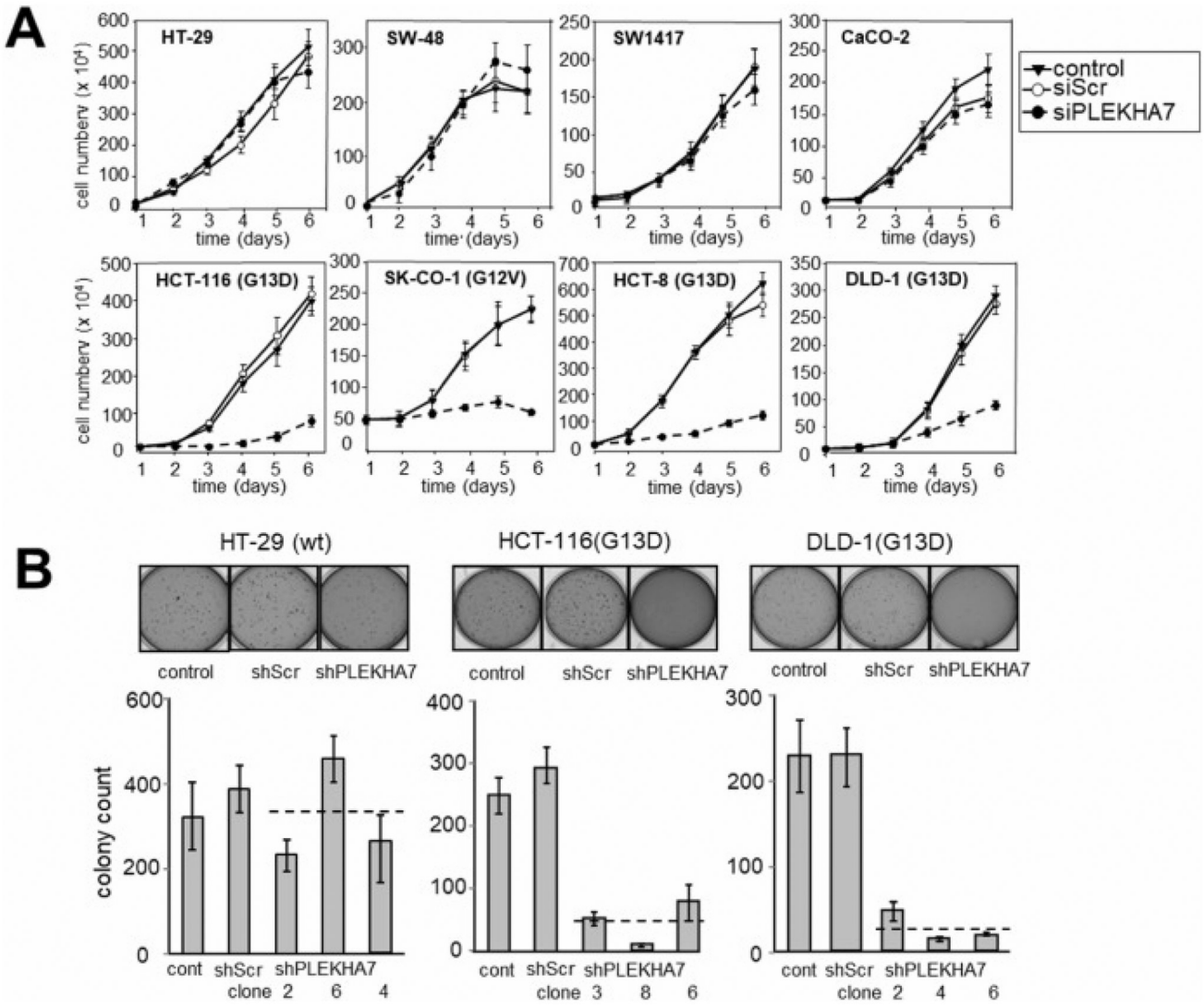

Fig. 1.

PLEKHA7 inhibition blocks growth of mutant-KRas but not wild type KRas colorectal cancer cells. **A**, CRC cells lines were untreated, or treated with scrambled siRNA (siScr) or siPLEKHA7 for 24 h, and then cell proliferation measured on plastic surfaces for 4 days. Values are the mean of 3 determinations and bars are SD. **B**, Anchorage independent soft agarose colony formation over 4 weeks for shPLEKHA7 knockdown in 3 for each, clonal CRC wild-type KRAS (HT-29) and mutant KRAS HCT-116 and DLD-1 cell lines was compared to control untreated (control) and shScr treated CRC cells. The upper panel shows culture dishes with typical colony formation for each cell line condition. The lower panel shows colony counts as the mean of 4 determinations and bars are SD. * is P = < 0.05 for shPLEKHA7 treated cells compared to shScr cells. The dashed line is the mean colony count of the 3 clones for each cell line.

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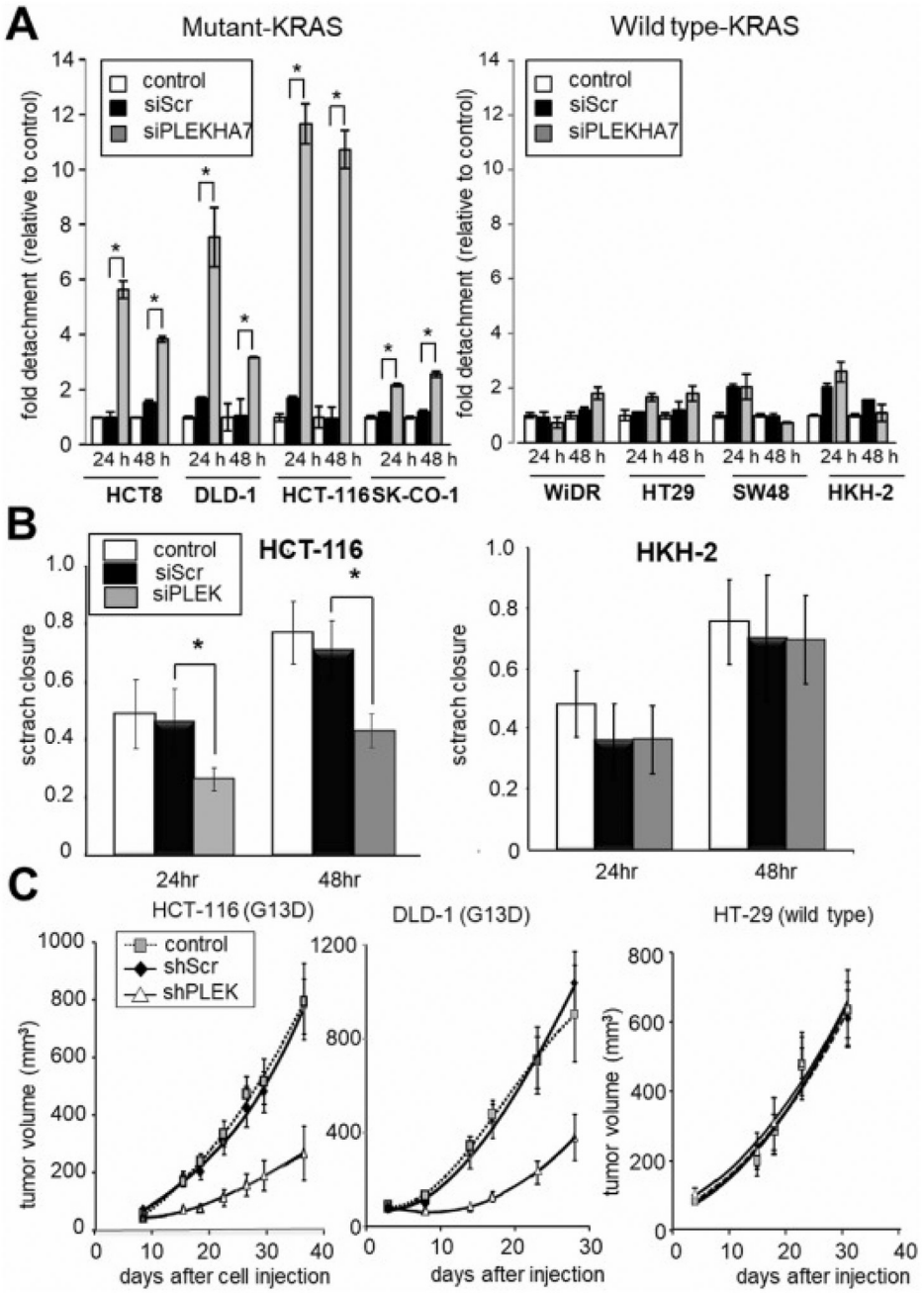

Fig. 2. PLEKHA7 inhibition prevents a mutant KRas phenotype and tumor growth.

A. PLEKHA7 inhibition promotes detachment of mutant-KRas but not wild type-KRas CRC cells from plastic surfaces. Cell were untreated (control) or treated with scrambled siRNA (siScr) or siPLEKHA7 and detachment measured after 24 hr. Left panel mutant KRas CRC cells; right panel wild-type KRas CRC cells. Values are the mean of 4 determinations, bars are SD. *p = < 0.05 of siPLEKHA7 treated cells compared to siScr treated cells. **B**, Scratch assay to measure cell motility of parental, siScr and siPLEKHA7 transfected mutant KRas HCT-116 and HKH-2 isogenic wild-type KRas cell lines. Data show the size of the scratch after 24 h and 48 h, relative to 0 h. Values are mean of 4 determinations and bars are SD.

*P = < 0.05 compared to siScr. **C**, Effect of shPLEKHA7 knockdown on tumor growth of mutant KRas HCT-116 and mutant KRas DLD-1 cell xenografts, and HT-29 wild-type KRas xenografts. There were 5 mice in each group (3 female, 2 male) and bars are S.D.

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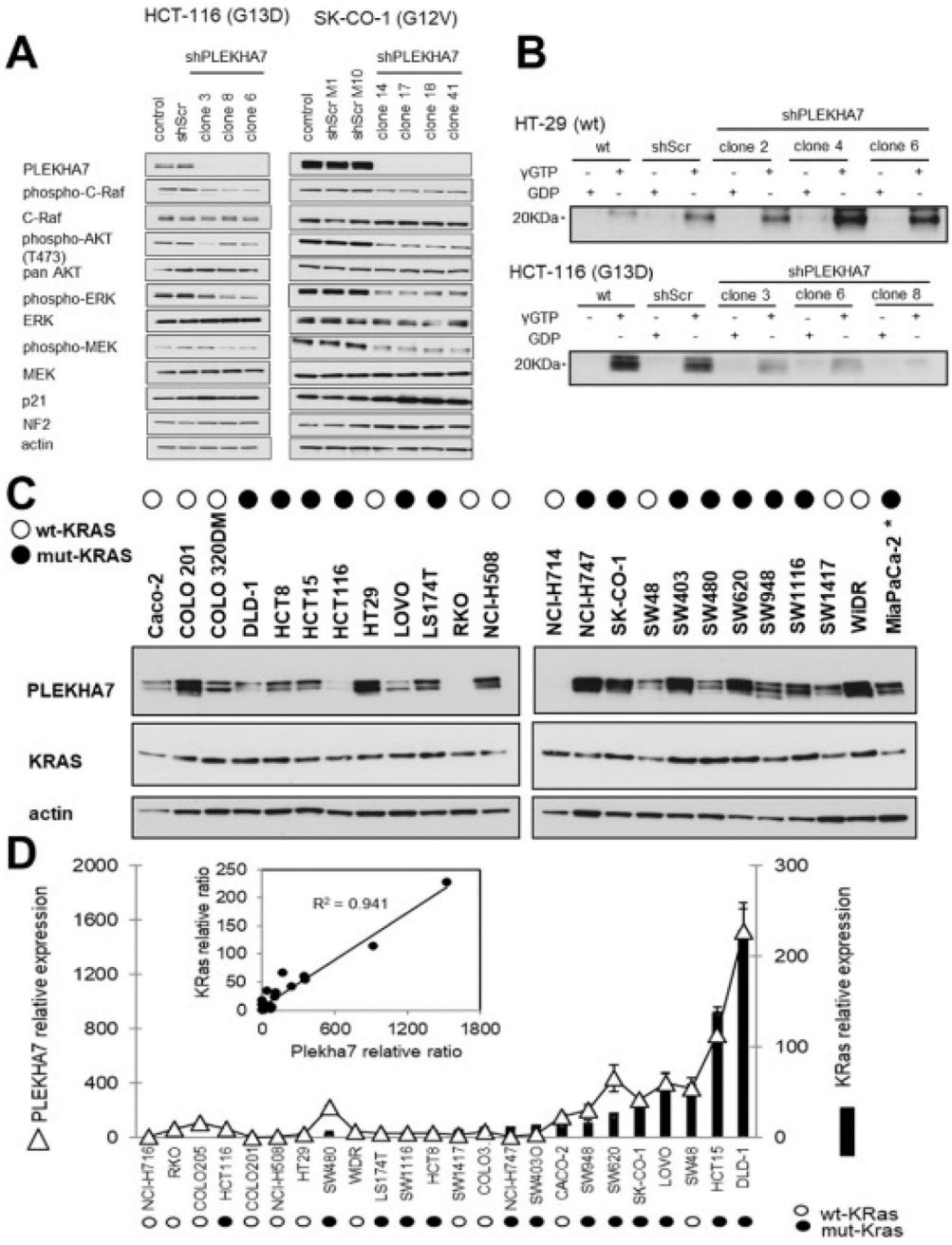

Fig. 3. Effect of PLEKHA7 inhibition on KRas signaling and relationship to KRas expression.

A, Typical Western blots showing the effect of shPLEKHA7 on cell signaling in cell line clones of mutant KRas HCT-116 and SK-CO-1 CRC cells, compared to untreated and shScr treated cells. **B**, Western blot of total activated γGTP bound Ras in wild-type KRas HT-29 and mutant KRas HCT-116 CRC cells, either untreated, shScr treated or 3 clones each of shPLEKHA7 treated cells. GDP provides a negative control for the binding. **C**, Western blot for a Plekha7 and total KRas in a panel of 24 CRC cell lines with **D**, relative protein levels of Plekha7 and total KRas with the inset panel showing the correlation between the two.

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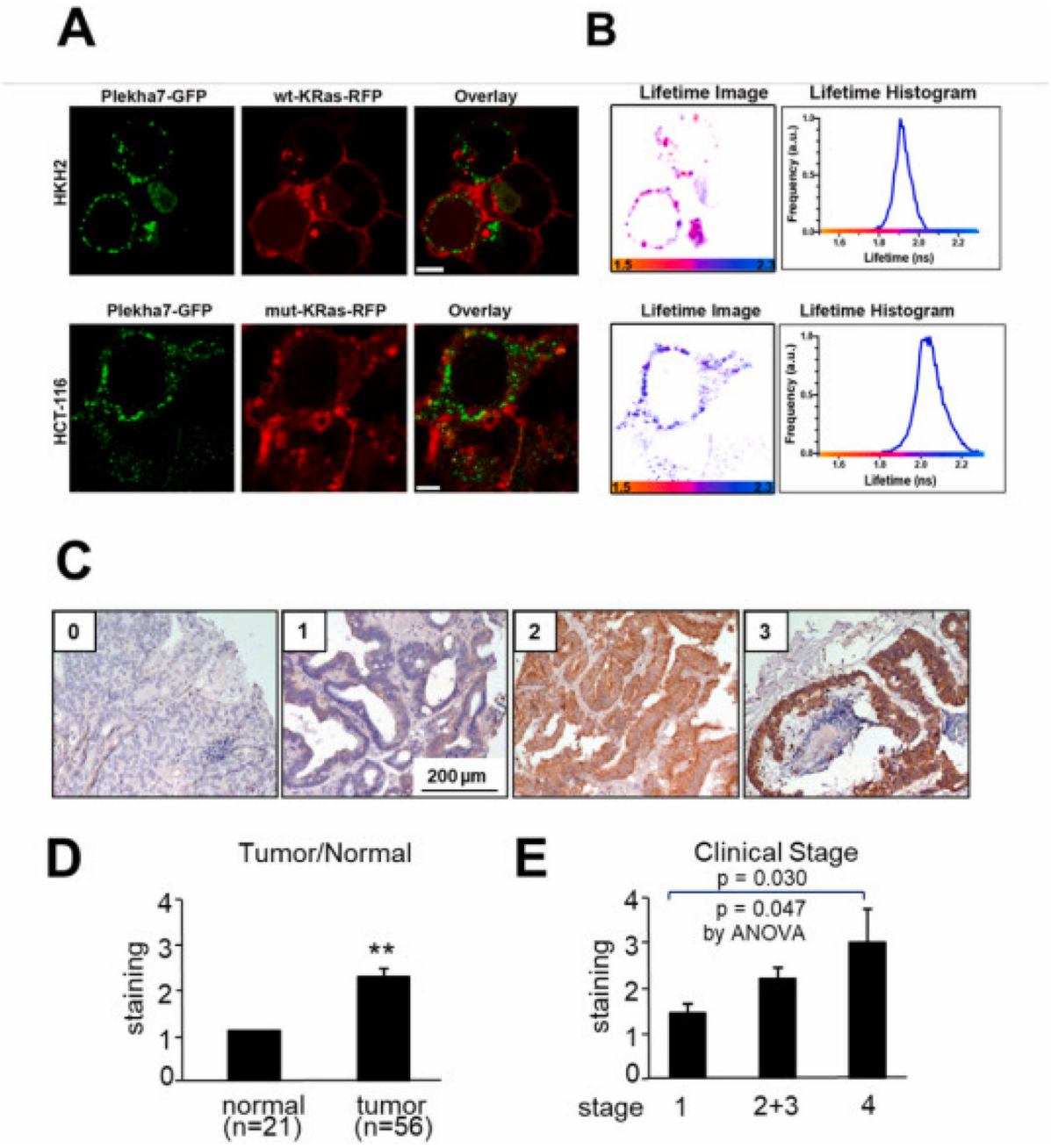

Fig. 4.

Subcellular localization of Plekha7 and KRas and patient Plekha7 tumor levels. **A**, Confocal microscopy of HCT-116 cells expressing the fusion proteins Plekha7-GFP and either wild-type KRas-RFP (HKH-2 top panel), or Plekha7-GFP and mutant (G12D)-KRas-RFP (lower panel). Plekha7-GFP protein was largely localized to the plasma membrane in discrete areas with some cytosolic distribution, while wild-type KRas-RFP and mutant KRas(G12D)-RFP are largely found at the plasma membrane. **B**, Fluorescence lifetime imaging microscopy (FLIM) showing the average fluorescence lifetime distribution for Plekha7- GFP in the presence of wild-type KRas-RFP or mutant (G12D)-KRas-RFP. The lifetime image was

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generated through pixel-by-pixel mapping of the recorded fluorescence lifetime based on the false color scale 1.5–2.3 ns (ns). The lifetime histograms show the normalized frequency of the recorded lifetime for each pixel in the image with the same 1.5–2.3 ns scale. Plekha7-GFP co-transfected with wild type-KRas-RFP showed an average fluorescence lifetime centered around 1.9 ns, which is shorter than the average GFP lifetime (2.1 ns). The 200 ps (ps) shorter lifetime indicates direct interaction. Plekha7-GFP co-transfected with mutant KRas-RFP had a fluorescence lifetime around 2.1 ns, comparable to those observed for GFP alone [35] indicating no direct interaction between mutant KRas and Plekha7. **C**, Typical Plekha7 staining from a MD Anderson tissue microarray of 21 normal human colon mucosa samples and 56 CRC tumor samples. Staining without Plekha7 antibody was assigned a score of 0, and with Plekha7 antibody weak, moderate and strong staining were assigned scores of 1, 2 and 3, respectively. **D**, Plots of tissue staining scores. Values are the mean staining in normal and tumor tissue and bars are S.D., **p = < 0.005. **E**, Plekha7 tumor staining expressed by clinical stage showed a significant increase with increased stage (p = < 0.05 by ANOVA).

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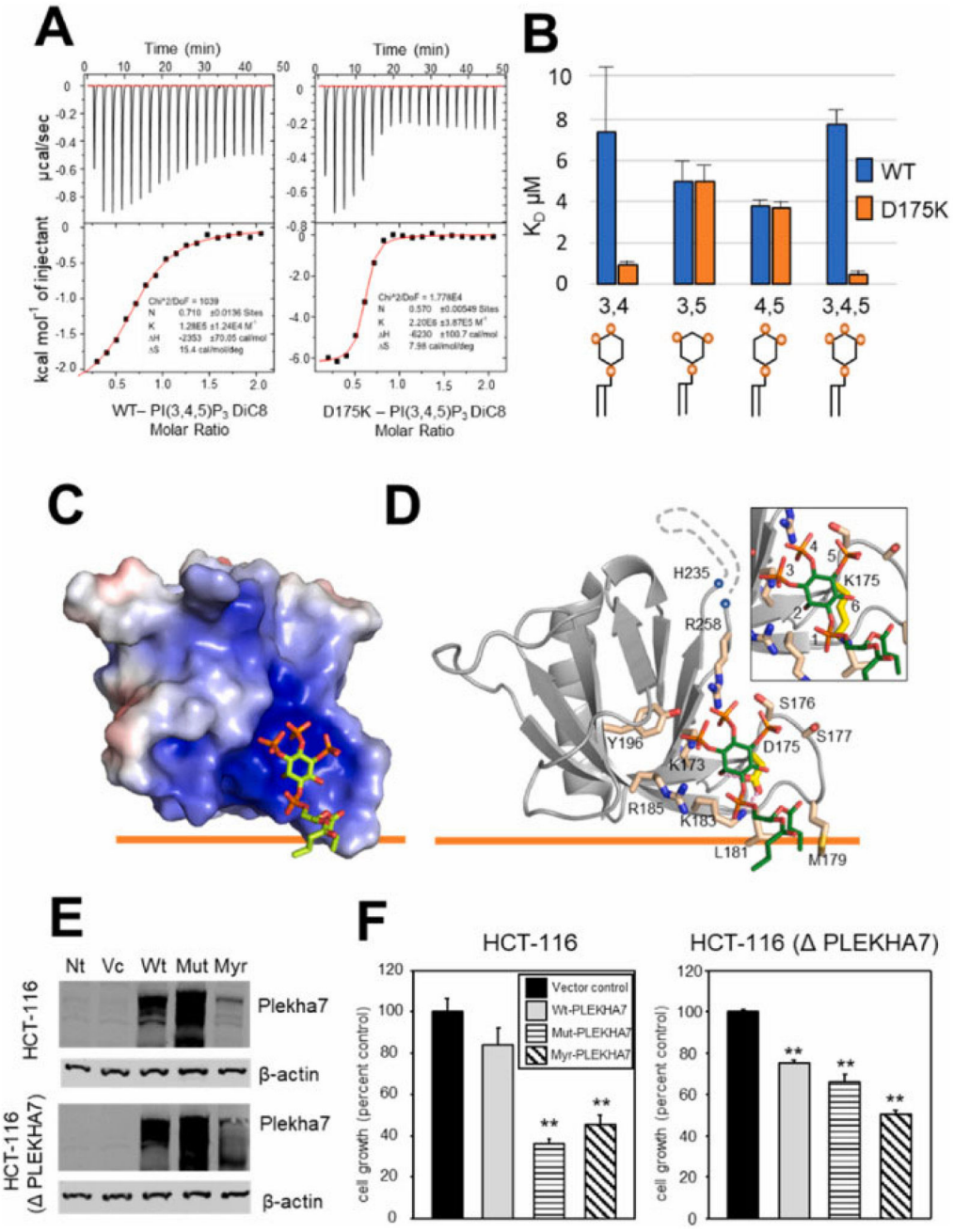

Fig. 5.

Involvement of the Plekha7 PH domain and phosphoinositide binding. A, Typical ITC plots showing titration of wild-type (WT) Plekha7 PH domain and Plekha7 (D175K) PH domain with diC8-PI(3,4,5)P_3 in 30 mM NaCl, Tris pH 7.6, 0.2 mM TCEP buffer. Red lines are the best fits of the data to a single-site binding model. **B**, KD values for binding of Plekha7 PH and Plekha7 (D175K) PH to various diC8- phosphatidylinositides. Values are mean and bars are SD determined by ITC curve fitting. The positions of phosphates on the inositol head group of the diC8 PI is shown schematically below. **C**, Modeling of the electrostatically charged surface (blue is positive charge) of Plekha7 PH at the plasma membrane (orange

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line) with bound PI(3,4,5)P_3 (sticks). **D**, Cartoon presentation of Plekha7 PH domain with bound PI(3,4,5)P_3 (sticks). Boxed insert illustrates phosphatidylinositide binding site of Plekha7 (D175K) PH. **E**. Western blot of Plekha7 CRSPR knockout (Δ Plekha7) HCT-116 cells: non-transfected (Nt), or transfected with vector alone (Vc), wild-type PLEKHA7 (Wt); PLEKHA7 with 4 point mutations, K173I, S177E, K183L and R25Q (mut) to completely block phosphoinositide binding as measured by ITC, and Myr-PLEKHA7 with an N-terminus c-Src myristylation membrane binding sequence MGSSKSKPKDPSQRRR (Myr). **F**, Growth of the HCT-116 and ΔPlekha7 HCT-116 CRC cells measured after 3 days expressed as a percent of the appropriate vector alone control. Bars are S.E. and ** indicate $P < 0.01$ compared to vector control.