HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2015 May 20.

Published in final edited form as:

Oncogene. 2014 November 20; 33(47): 5457–5466. doi:10.1038/onc.2013.486.

Ras regulates kinesin 13 family members to control cell migration pathways in transformed human bronchial epithelial cells

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Abstract

We show that expression of the microtubule depolymerizing kinesin KIF2C is induced by transformation of immortalized human bronchial epithelial cells by expression of K-Ras^{G12V} and knockdown of p53. Further investigation demonstrates that this is due to the K-Ras/ERK1/2 MAPK pathway, as loss of p53 had little effect on KIF2C expression. In addition to KIF2C, we also found that the related kinesin KIF2A is modestly upregulated in this model system; both proteins are expressed more highly in many lung cancer cell lines compared to normal tissue. As a consequence of their depolymerizing activity, these kinesins increase dynamic instability of microtubules. Depletion of either of these kinesins impairs the ability of cells transformed with mutant K-Ras to migrate and invade matrigel. However, depletion of these kinesins does not reverse the epithelial-mesenchymal transition caused by mutant K-Ras. Our studies indicate that increased expression of microtubule destabilizing factors can occur during oncogenesis to support enhanced migration and invasion of tumor cells.

The Ras family of small GTP binding proteins are essential signaling components that transfer information received from the extracellular environment to elicit responses in the cell with the potential to promote differentiation, proliferation, and survival. Ras proteins cycle between the GDP-bound (inactive) and GTP-bound (active) states. Oncogenic Ras

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*In partial fulfillment of the requirements for the Ph.D. degree

The authors declare no conflict of interest.

The authors have no competing interests with respect to this work.

Supplementary Information accompanies the paper on the Oncogene website at http://www.nature.com/onc

mutations such as V12 are resistant to inactivation by GTPase activating proteins (GAPs), and as a result, remain constitutively in the active state, causing persistent activation of Ras-dependent, downstream effector pathways.

Activating mutations in Ras proteins are present in about 20% of human cancers, with mutations in K-Ras accounting for nearly 85% of the total[1]. In non-small cell lung cancers (NSCLC), K-Ras is mutated in 15–20% of cases, with highest mutation frequency in lung adenocarcinoma (20%–30%)[2]. Epithelial cells expressing mutant K-Ras undergo dramatic morphological changes; they often lose typical epithelial morphology and contact inhibition and become irregularly shaped, consistent with epithelial to mesenchymal transition (EMT) [3,4]. These morphological changes are accompanied by loss of epithelial proteins involved in cell-cell junctions and cell-matrix contacts such as E-cadherin. Conversion to a more migratory phenotype is related to expression of N-cadherin, often used as a marker of cells that have undergone EMT. Supporting the idea that K-Ras induces morphological changes, in certain cell lines morphology can be reverted by blocking pathways downstream of Ras, for example, with farnesyltransferase inhibitors, Anthrax lethal factor, or combinations of kinase inhibitors[5–8], flattening cells and restoring contact inhibition.

KIF2A is a kinesin-13 family member which is important for formation of bipolar spindles during cell division as well as for suppression of collateral branch extension in neurons; both functions are mediated through microtubule depolymerization catalyzed by KIF2A[9, 10]. The closely related kinesin, KIF2C, commonly known as the mitotic centromere-associated kinesin (MCAK), also depolymerizes microtubules in an ATP-dependent manner [11–13]. The depolymerase activity of these KIFs has been demonstrated in a number of ways including in vitro assays with purified proteins, using single molecule microscopy, and analyzing phenotypes of knock out mice[11,12,9]. KIF2C has multiple roles in mitosis from spindle assembly at the centrosome to microtubule turnover at kinetochores [14]. Because of their depolymerizing activity, these kinesins increase dynamic instability of microtubules. Few roles have been ascribed to either protein outside of mitosis. Although KIF2C is thought to be degraded after cell division, it has been implicated in microtubule dynamics during interphase and associates with plus end tips of microtubules[12,15]. KIF2A has also been implicated in organelle localization[16].

In this study, we find that oncogenic K-Ras-induced transformation of human bronchial epithelial cells (HBEC) lacking p53 is accompanied by changes in morphology affecting both microtubule and actin cytoskeletons. Therefore, we hypothesized that regulators of the cytoskeleton may in some way be altered in transformed cells. We find that the kinesin family proteins KIF2A and KIF2C, both microtubule destabilizing, are upregulated in cells that have been transformed with K-Ras^{G12V} and in a fraction of human cancer cell lines. Knocking down either KIF2A or KIF2C reduces the ability of K-Ras^{G12V}-expressing, transformed bronchial epithelial cells to migrate, suggesting that aberrant expression of these proteins during transformation can contribute to the migratory potential of cancer cells.

Results

Expression of oncogenic K-Ras^{G12V} increases expression of the microtubule depolymerases KIF2C and KIF2A

We found that the microtubule depolymerizing kinesins KIF2C and to a lesser extent KIF2A were upregulated in a number of lung and breast cancer cell lines compared to immortalized human bronchial epithelial cells (HBEC) or human mammary epithelial cells (HMEC50) representative of normal tissue (Fig. 1A,F,G, S1A). Lung cell lines A549, Calu-6, H358, HCC515 and H1155 express K-Ras mutations and can be found in the (Cosmic Database (http://www.sanger.ac.uk/cosmic)). We considered the possibility that mutant K-Ras might alter expression of these proteins. Because larger changes in KIF2C expression were noted, we first determined if increased expression of KIF2C can be caused by mutated K-Ras. To do this we used an immortalized HBEC system that has been previously described [17,18]. HBEC from different patients (distinguished by a number) were immortalized by expression of human telomerase reverse transcriptase (hTERT) and CDK4, yielding HBEC3KT, HBEC30KT, etc. These immortalized cells were further altered by stable knockdown of p53 (HBEC3KT53). p53 is commonly mutated or lost in cancers, and loss of wild type p53 is required for HBEC to bypass Ras-induced senescence [2, 19,20]. K-Ras^{G12V} was stably expressed in p53 knockdown cells yielding HBEC3KTR$_L$53 which were used in many of the following studies (Fig. 1B). Expression of KIF2C, almost undetectable in HBEC3KT, was greatly increased in HBEC3KTR$_L$53 grown in serum-containing medium (Fig. 1C, lanes 1 and 6, S1C)[18]. An increase in KIF2A protein was also observed in the K-Ras^{G12V}-transformed cells, but of smaller magnitude. We next tested the effect of growth factor and nutrient withdrawal by placing the cells in Earle's balanced salt solution (EBSS). Removing nutrients and growth factors to slow protein synthesis and energy utilization had little effect on the amount of KIF2C, even after 4 hr of starvation. The increase in KIF2C protein was paralleled by greatly increased KIF2C mRNA in HBEC3KTR$_L$53 compared to HBEC3KT (Fig. 1D), suggesting that transcription is enhanced by expression of mutant K-Ras. An increase in KIF2A mRNA expression observed in cells harboring oncogenic K-Ras reached only a low level of statistical significance (Fig 1E).

To determine if these results with a laboratory-generated model system of mutant K-Ras-transformation might be representative of patient samples, we asked if KIF2C and KIF2A were upregulated in a large group of patient-derived cancer cell lines. Information was obtained from microarray studies that had been performed on 147 lung cancer lines and 59 normal lung cells (Table S1). Statistically significant upregulation of KIF2C was noted in cancer lines compared to normal control cells (Fig. 1F); a less significant increase was also noted for KIF2A (Fig. 1G). No increase or even a small decrease in expression of KIF2B, a related kinesin 13 family member, was observed in the cancer cell lines (Fig. S1B), consistent with the idea that these KIFs have different cellular functions [21].

To determine if loss of K-Ras^{G12V} from HBEC3KTR$_L$53 was sufficient to decrease expression of KIF2C, K-Ras was depleted by siRNA. We found that KIF2C expression decreased to a level similar to that in HBEC3KT, indicating that constant expression of oncogenic K-Ras, even after morphological transformation has occurred, maintains elevated

KIF2C expression (Fig. 2A). Expression of KIF2A was not detectably decreased by K-Ras knockdown, perhaps to be expected with the smaller increase caused by K-Ras overexpression. To evaluate the possibility that p53 also affected expression of KIF2C or KIF2A, p53 was transiently knocked down or overexpressed in the HBEC model cell lines. We observed a relatively small effect on expression of either KIF2 protein (Fig. 2B-D).

Expression of oncogenic K-Ras^{G12V} causes morphological changes that alter microtubule and actin cytoskeletons

Cells transformed with oncogenic K-Ras in the context of loss or mutation of p53 and grown in serum are morphologically altered from isogenic cells lacking mutant K-Ras. To examine the effect of oncogenic K-Ras transformation on the microtubule and actin cytoskeleton of HBECs, we compared the morphology of HBEC3KT, HBEC30KT, HBEC3KT53 and HBEC3KTR$_L$53 by immunofluorescence staining for actin and α-tubulin (Fig. 3A,B, S2). HBEC3KT, HBEC30KT, and HBEC3KT53 appear larger and flatter than HBEC3KTR$_L$53. Ras-induced transformation caused inhibition of stress fiber formation[22]; actin appeared less organized, consistent with the irregular cell morphology. HBEC3KTR$_L$53 had fewer microtubule polymers compared to HBEC3KT53, suggesting that microtubules may be more dynamic in cells expressing mutant K-Ras, as would be expected with elevated expression of microtubule depolymerizing kinesins. These changes are also consistent with EMT (see also Fig. 7D,E). Knocking down K-Ras from HBEC3KTR$_L$53 was sufficient to reverse much of the change in morphology of the microtubule and actin cytoskeleton, restoring microtubule polymerization and organized actin stress fibers (Fig. 3C).

Signaling pathways downstream of Ras regulate morphological changes

Because ERK1/2 are known to regulate microtubule and actin dynamics and are activated downstream of Ras [23–25], we hypothesized that inhibition of the ERK1/2 pathway could also revert these phenotypes in HBEC3KTR$_L$53. Indeed, treatment of HBEC3KTR$_L$53 for 48 hr with 100 nM PD0325901, a MEK1/2-specific inhibitor, resulted in longer microtubules and reappearance of stress fibers (Fig. 4A), similar to effects of K-Ras knockdown. The effect of depletion of either KIF2A or KIF2C from HBEC3KTR$_L$53 was also similar to the effects on microtubules of K-Ras knockdown (Fig. S3). To demonstrate that prolonged exposure to this MEK inhibitor is not toxic to the cells, cells treated with PD0325901 for 48 hr were placed in fresh serum-containing medium for 30 min which activated ERK1/2 (Fig. 4B). Furthermore, no change in viability was noted following prolonged PD0325901 treatment (Fig. S4A). Phosphatidylinositol 3-kinase (PI3K) is another important Ras effector that promotes cytoskeleton transformation changes in part through the small Rho family GTPase Rac [26]. To determine if this Ras-activated, growth-promoting pathway is also required for these cytoskeletal changes, we inhibited the PI3K pathway with 10 μM LY294002. This PI3K inhibitor did not cause microtubule spreading or flattening of cells, but did elicit some shape changes, e.g., further elongation (Fig. 4A). These data suggest the relative importance of the ERK1/2 pathway in influencing actin and microtubule cytoskeleton organization in this oncogenic Ras-transformed system.

Because the cytoskeletal changes in the transformed system seemed to be mediated by ERK1/2, we evaluated the importance of this pathway for elevated KIF2C expression.

Inhibition of ERK1/2 activation by exposure of cells to PD0325901 for 48 hr, but not with comparable treatment with a PI3K inhibitor, LY294002, decreased KIF2C protein in HBEC3KTR$_L$53 (Fig. 4C) and to a lesser extent in HBEC3KT53 cells (Fig. S4B,C). Similar to inhibiting PI3K, inhibiting mTOR, another growth-promoting pathway, with the small molecule rapamycin did not affect KIF2C expression to the same extent as inhibition of ERK1/2 activation (Fig. 4D). Inhibition of ERK1/2 activation for 72 hr also reduced KIF2A protein by approximately 30% (Fig. 4E,F).

Inhibition of MEK1/2 with PD0325901 significantly reduced KIF2C mRNA, not only in HBEC3KTR$_L$53 but also in cells that do not express mutant K-Ras, HBEC3KT and HBEC3KT53 (Fig. 5A-C). Expression of constitutively active MEK1 caused a small increase in KIF2C protein (Fig. S5). As was the case with KIF2A protein, KIF2A mRNA also decreased with reduced ERK1/2 activitation (Fig. 5D-F); however, the reduction upon treatment with PD0325901 did not reach statistical significance in some cell lines as it did in HBEC3KTR$_L$53. Interestingly, inhibition of PI3K in HBEC3KT53 results in a significant upregulation of KIF2C mRNA (Fig. 5G-I). In contrast, a closely related kinesin 13 family protein, KIF2B was upregulated by MEK inhibition under the same circumstances, suggesting that functions of specific kinesin molecules are differentially sensitive to regulation through this pathway (Fig. S4D-F). KIF2B is also not upregulated in cancer lines (Fig. S1B).

Changes in expression of KIF2C or KIF2A do not alter cell cycle progression in HBEC3KTR$_L$53

KIF2A and KIF2C have well defined but distinct roles in mitosis [21]. Because KIF2C and KIF2A were upregulated in cancer, we wondered if their functions in regulating the cell cycle were disturbed. Therefore, we examined the cell cycle profiles following individual knockdown of KIF2A or KIF2C (Fig. 6 A,B). DNA content was measured following depletion of kinesins for 96 hr to analyze cell cycle profiles. There appeared to be no difference in the number of cells in G2/M phase when cells were depleted of KIF2A or KIF2C. Unlike an earlier study suggesting a difference in mitotic index in U2OS cells[10], we did not observe mitotic accumulation of HBEC3KTR$_L$53 following knockdown of KIF2A or KIF2C. As a positive control, exposure of HBEC3KTR$_L$53 to 200 nM taxol overnight caused marked accumulation of cells in mitosis (Fig. S6). Thus, under these conditions, residual KIF2A and KIF2C or other compensating proteins are apparently sufficient to prevent abnormalities.

KIF2A and KIF2C facilitate migration of transformed cells

Microtubule dynamics have long been implicated in the migratory ability of cells [27,28]. Therefore, we hypothesized that knockdown of KIF2A or KIF2C could have an effect on cell migration by disturbing microtubule dynamics. As anticipated, HBEC3KTR$_L$53 cells migrated significantly more than HBEC3KT (Fig. 7A). We knocked down KIF2A, KIF2C or both using siRNA in HBEC3KT and HBEC3KTR$_L$53 and found that depletion of either or both KIF2A and KIF2C reduced migration of HBEC3KT and HBEC3KTR$_L$53 through membranes compared to cells treated with a nontargeting siRNA (Fig. 7A,C). Knock down of these kinesins also reduced the bility of HBEC3KTR$_L$53 to invade through matrigel (Fig.

7B,C). HBEC3KT showed little or no ability to invade. HBEC3KT53 express E-cadherin exclusively. On the other hand, HBEC3KTR$_L$53 have lost E-cadherin and instead express N-cadherin. Depletion of either KIF2C or KIF2A does not alter this pattern of cadherin expression. Thus, KIF2A or 2C knockdown did reverse the changes in microtubule organization but did not reverse EMT caused by mutant K-Ras as assessed by expression of E-cadherin (Fig. 7D,E, S3). Importantly, loss of either or both KIF2A and KIF2C reduced the migration and invasiveness of HBEC3KT transformed with oncogenic K-Ras.

Discussion

We find that KIF2C and to a lesser extent KIF2A, two related microtubule-depolymerizing kinesins, are upregulated in transformed lung epithelial cells and contribute to the ability of cells transformed with mutant K-Ras to migrate. A number of kinesins including KIF2A have been implicated in cancers either from cell-based studies or through cancer genome analysis, most in the context of their mitotic roles [29–33]. Nearly four dozen human kinesins are known[34], making the delineation of their individual functions difficult at best. Some transport cargo on microtubules in a plus-end direction, and others transport cargo in a minus-end direction, while a smaller number, including KIF2C, KIF2A, and KIF2B, depolymerize microtubules increasing dynamic instability[11,12,35]. Why two of these related kinesins are utilized by K-Ras and the extent to which the actions of these depolymerases in K-Ras-transformed cells reflect on their physiological actions in normal cells are questions that remain to be answered. Reduced migration of HBEC and Ras-transformed HBEC upon KIF2A and 2C knockdown suggests that some of their actions are common in normal and cancer cells.

The full transforming potential and tumor formation caused by mutant K-Ras depend on its activation of interacting downstream effector pathways. Activated K-Ras contributes to many properties of successful cancers; these include enhanced proliferation under suboptimal conditions, evasion of cell death, escape from the primary tumor and invasion and metastasis to distant sites. A variety of observations have suggested that Ras-transformation increases microtubule dynamics[36]. Changes in microtubule dynamics may have multiple consequences, but among them is modulating migratory capacity of cells. For example, H-Ras transformed MCF10a cells exhibit fewer acetylated microtubules; a reduction in this post-translational modification is thought to indicate a decrease in microtubule stability[23]. Mechanistic studies have focused largely on microtubule stabilizing factors, such as discs large 1 (Dlg1), RASSF1A and adenomatous polyposis coli (APC) which control cell motility and are frequently lost in human cancers [37–39]. Our studies indicate that increased expression of microtubule destabilizing factors can also occur during oncogenesis to support enhanced cell migration and invasion.

The Raf/ERK1/2 and PI3K pathways are major effectors of Ras transformation and have powerful actions on the cytoskeleton. ERK1/2 were first described as microtubule-associated protein kinases and regulate aspects of both microtubule and actin lattices [24,25,40–43]. In Ras-transformed Swiss-3T3 fibroblasts, sustained ERK-MAPK signaling prevents actin stress fiber formation and promotes migration by downregulating Rho-kinase [22,44]. In agreement with this finding, inhibition of ERK1/2 activation by

PD0325901 restored actin stress fibers in our system and restored microtubule polymers as well. Ras-induction of KIF2C was suppressed through inhibition of ERK1/2 activation, resulting in expression of KIF2C comparable to its amount in normal immortalized HBEC. A less dramatic change was also noted in KIF2A. We conclude that the ERK1/2 pathway is a major Ras effector controlling expression of these kinesins. These findings suggest that ERK1/2 may be more important in controlling morphology in transformed cells than recognized previously.

Mutation of K-Ras induces changes in gene expression and morphology of cancer cells that have been evaluated in a number of ways, leading to the conclusion that changes in many genes downstream of Ras contribute to cancer phenotypes [2, 45–49]. Among prominent examples, Zeb1 participates in the induction of EMT by suppressing expression of E-cadherin [48,50]. Knockdown of K-Ras in cancer cells has revealed genes whose expression is reversible upon loss of mutant K-Ras and those that have become Ras independent, such as Zeb1 [4, 48]. In the transformed model we studied, knockdown of K-Ras or inhibition of ERK1/2 activation did reduce KIF2C and KIF2A expression, suggesting that these genes retain Ras dependence for expression.

PI3K influences the actin cytoskeleton to confer a migratory advantage on cancer cells. The mechanism includes the stimulation of Rac through regulation of T-lymphoma invasion and metastasis gene 1 (Tiam), which functions as a specific guanine nucleotide exchange factor (GEF) for Rac1 [51]. Additionally, the regulatory subunit of PI3K, p85α, can activate Cdc42 and subsequently regulate actin dynamics and migration [52]. Though we hypothesized that some of the changes in the actin cytoskeleton as a consequence of K-Ras^{G12V} transformation would be reversed with the inhibition of PI3K pathway, we found that in our system this pathway had a smaller than expected effect on the actin phenotype. Prolonged inhibition of this pathway also had a minor effect on microtubule organization and only a small effect on KIF2C expression. Thus, in this model of nonsmall cell lung cancer, ERK1/2 are dominant regulators of morphology and of kinesins involved in microtubule dynamics.

Finally, we determined that KIF2C and KIF2A are not only upregulated in our laboratory-generated system of K-Ras oncogenic transformation but in many cancer lines isolated from lung cancer patients. Knockdown of KIF2C and KIF2A decreased the ability of HBEC3KTR$_L$53 to migrate and invade, suggesting the utility of targeting Ras-mediated pathways that promote different aspects of cancer biology for therapeutic advantage. Because of its critical functions in regulating the cytoskeleton, KIF2C has been proposed as a potential new cancer drug target for breast, colorectal and pancreatic cancers [53]. Further studies are necessary to investigate the role of KIF2C and KIF2A in cancer invation and metastasis.

Methods

Cell Culture

Immortalized HBEC3KT, HBEC30KT and HBEC3KT53 cells were cultured in Keratinocyte serum free medium (KSFM) (Invitrogen) supplemented with 5 ng/mL epidermal growth factor and 50 μg/mL bovine pituitary extract according to manufacturer's

recommendations. HBEC3KTR$_L$53 were cultured in RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum (vol/vol) and 2 mM L-glutamine. Cells were grown at 37°C in a humidified atmosphere of 5% CO_2. MDA-MB-231, HCC38, HCC1143, Htb126, and MCF7 cells were provided by M.A. White (Dept Cell Biology) and T47D and HCC1428 cells were provided by G.W. Pearson (Simmons Comprehensive Cancer Center, Dept Pharmacology). SUM149PT and SUM190PT cells were purchased from Asterand (Detroit, MI) and grown according to provider culture conditions. HCC1937 cells were a gift of A. Gazdar (Hamon Cancer Center, Dept Pathology). HME50 cells were derived from the non-cancerous breast tissue of a female diagnosed with Li-Fraumeni syndrome as previously described [54]. The missense p53 mutation (M133T) in HME50 was sequence verified. Breast cancer cell lines were cultured in Dulbecco's modified Eagle's medium or RPMI-1640 with 10% fetal calf serum. HME50 was cultured in serum-free conditions as described elsewhere [55].

Microarray

Our cell line panel consists of 118 NSCLCs, 29 SCLCs, 30 HBECs (immortalized human bronchial epithelial cells), and 29 HSAECs (immortalized human small airway epithelial cells). All cell lines were DNA-fingerprinted and mycoplasma-tested. Total RNA (500 ng) was prepared using the RNeasy Midi kit (Qiagen, Valencia, CA) and checked for quality and concentration using the Bio-Rad Experion Bioanalyzer (Hercules, CA). cRNA labeling was done with Ambion Illumina TotalPrep RNA Amplification kit (IL1791). Amplified and labeled cRNA probes (1.5 ug) were hybridized to Illumina HumanWG-6 V3 Expression BeadChip (BD-101-0203) overnight at 58 degree C, then washed, blocked and detected by streptavidin-Cy3 per manufacturer's protocol. After drying, the chips were scanned by Illumina iScan system. Bead-level data were obtained, and pre-processed using the R package mbcb for background correction and probe summarization [56]. Pre-processed data were then quantile-normalized and log-transformed. All array data are deposited on GEO (GSE32036).

siRNA

Cells were transfected for from 48 to 96 hr as indicated with dsRNA oligonucleotides using Lipofectamine RNAiMax according to manufacturer's protocol (Invitrogen). The following target sequences for KIF2A were used: GAAAACGACCACUCAAUAA (Thermo Scientific) and GACCCTCCTTCAAGAGATA (Thermo Scientific); KIF2C: GCAAGCAACAGGUGCAAGU (Thermo Scientific) and GGCAUAAGCUCCUGUGAAU (Thermo Scientific); Ras: GGAGGGCUUUCUUUGUGUA (Thermo Scientific); p53: GGAGAAUAUUUCACCCUUC (Thermo Scientific). In several experiments in which duplicate lanes are shown, duplicate wells of cells were treated with the same oligonucleotides.

Immunofluorescence

Cells were washed with Tris-buffered saline (TBS); fixed with 4% paraformaldehyde (vol/vol) in TBS for 10 min; and washed twice for 5 min with TBS. Cells were permeabilized with 0.1% Triton X-100 for 5 minutes and washed twice with TBS. After incubation with 10% normal goat serum (vol/vol) at room temperature for one hr, cells were incubated with

the indicated antibodies at 4 °C overnight. Cells were washed with TBS, incubated with Alexa fluor-conjugated secondary antibody at room temperature for one hr, washed with TBS, and imaged. Fluorescent Z-stacks (0.2 mm) were acquired and deconvolved using the Deltavision RT deconvolution microscope. α-tubulin antibodies were from Sigma (T6199).

qRT-PCR

Cells were harvested and RNA was extracted using QuickRNA™ (Zymo Research) according to the manufacturer's protocol. RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad). PCR reactions were performed with IQ SYBR Green Supermix (BioRad), and fluorescence was measured using a quantitative real-time thermocycler (ABI 7500). The following primer sets were used: KIF2C (sense 5'-CAGAACTCTTACAGCTTCTTCCC-3' and antisense 5'-CAGTGGACATGCGAGTGGA-3'); KIF2A (sense 5'-TGCAAATAGGGTCAAAGAATTG-3'and antisense 5'-TTGTTCACAAAGAAGTTTTAGATCAT-3'); KIF2B (sense 5'-CTGAAACCCTTGAAGCCACAT-3' and antisense 5'-AGCAGGAGTATGGTCTCCAGG-3'); beta actin(5'-CATGTACGTTGCTATCCAGGC-3' and antisense 5'-CTCCTTAATGTCACGCACGAT-3'). Relative changes in gene expression were calculated by $2^{-\Delta Ct}$, where ΔCt= Ct(KIF2)-Ct(ActinB).

Cell harvest

Cells were washed twice with TBS and lysed on ice with in 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 0.2 mM Na_3VO_4, 100 mM NaF, 50 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1.6 μg/ml aprotinin, 0.1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of N^{α}-p-tosyl-L-lysine chloromethyl ketone, N^{α}-p-tosyl-L-arginine methyl ester, pepstatin A, and leupeptin. Lysates were frozen in N_2 (liquid) and thawed on ice, followed by centrifugation for 15 min at 16,000 × g in a microcentrifuge at 4 °C. Supernatants boiled in Laemmli sample buffer (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue, 50 mM Tris-HCl) and subjected to SDS-PAGE and immunoblotting. Following antibodies were used: KIF2A (Abcam, 37005), KIF2C (Bethyl Laboratories, A300-807A), actin (Fisher Scientific, MAB1501MI), ERK1/2 [57], pERK (Sigma, M8159), p53 (sc-126), Ras (Santa Cruz, sc-166691), E-cadherin (BD Transduction Laboratories, 610181), N-cadherin (BD Transduction Laboratories, 610920), pS6 (Cell Signaling, 5364).

Cell cycle analysis

HBECK3KTR_L53 were transfected with siRNA for 96 hr and collected for flow cytometry. Cells were trypsinized and collected by sedimentation at 1,000×g. Cells were washed in 1X phosphate buffered saline (PBS) and fixed in 70% cold EtOH at −20° C overnight. Cells were washed in 1X PBS and stained with 0.4 ml of propidium iodide/RNase A 1 solution. DNA content was measured by flow cytometry with FACSCalibur (BD Biosciences), and data were analyzed using FlowJo. Cells harvested for immunoblot were washed with 1X PBS and harvested with Laemmli sample buffer. Samples were boiled for 2 min at 95° C, sonicated, and boiled again for 2 min at 95°C. Protein concentration was measured with

BCA Protein Assay (Thermo Scientific Prod # 23235). Cell lysate protein (20 μg) were resolved on gels and processed for immunoblotting as above.

Migration Assays

The HBEC3KTR$_L$53 and HBEC3KT cell lines were used for all migration assays. For the transwell migration assays, 1x10^4 cells were seeded 72 hr post-knockdown of indicated proteins, using Transwell permeable supports (Corning #3422). Cells were seeded in the top chamber in 5% FBS and allowed to migrate along a concentration gradient through a polycarbonate membrane with 8 μm pores to the bottom chamber containing medium with 25% FBS. After 24 hr cells were fixed, stained (with 1% methylene blue, 1% borax solution), and counted. For invasion assays 1.5 x10^5 cells were imbedded in Growth Factor Reduced Matrigel in transwell permeable supports, 72 hr post-knockdown of indicated proteins. Cells were allowed to migrate for 48 hr across membranes with a gradient of 25 % serum in the bottom chamber. Cells were fixed, stained (with 1% methylene blue, 1% borax solution), and counted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Andrés Lorente-Rodríguez and Michael Kalwat in the Cobb lab for comments and suggestions and Dionne Ware for administrative assistance. This work was supported by grants from the National Institutes of Health (R37 DK34128 to MHC and P50CA70907 to JDM), grants from the Cancer Prevention and Research Institute of Texas (CPRIT) to JDM, DOD PROSPECT and Longenbaugh Foundation to JDM, and the Robert A. Welch Foundation I1243 to MHC. JKO was supported by NIGMS Pharmacological Sciences Training Grant 5-T32 GM007062. JEL was supported by NHMRC Biomedical Fellowship (494511)TSANZ/Allen & Hanburys Respiratory Research Fellowship.

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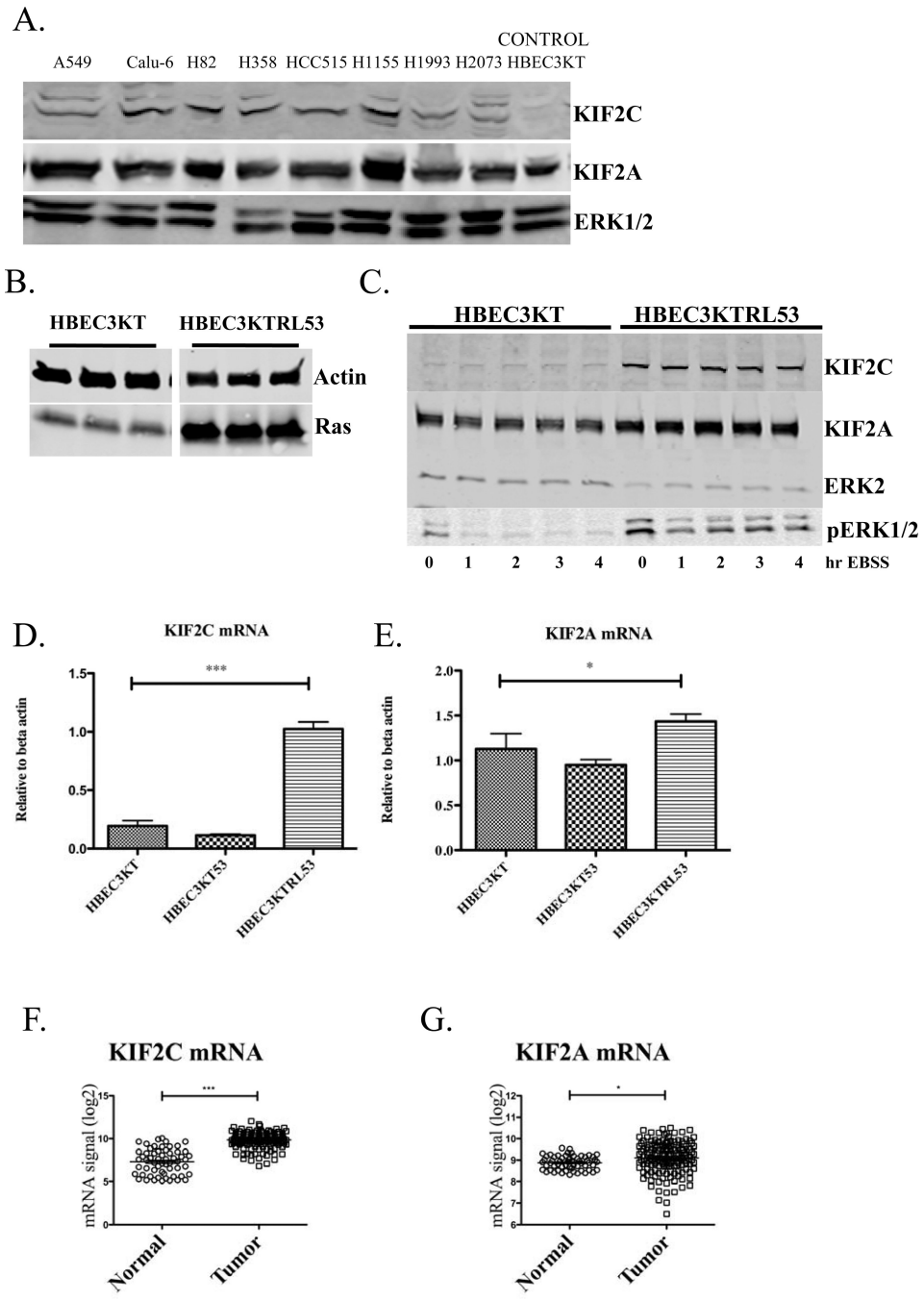

Fig. 1. Expression of kinesin 13 family proteins in cancer cell lines

(A) KIF2A and KIF2C were detected by immunoblotting of lysate proteins from A549, Calu-6, H82, H358, HCC515, H1155, H1993, H2073, HBEC3KT analyzed on denaturing gels as in *Methods*. Immunoreactivity was detected with the LI-COR Odyssey infrared system. (B) K-Ras was immunoblotted in lysates from HBEC3KT and HBEC3KTR$_L$53. These lanes are from ends of the same gel; irrelevant lanes in the middle were removed, indicated by a space. (C) HBEC3KT and HBEC3KTR$_L$53 cells were starved in Earle's Balanced Salt Solution (EBSS) for the specified times. Lysate proteins were resolved on a

gel followed by immunoblotting with the indicated antibodies. (D) RNA was isolated from HBEC3KT, HBEC3KT53 and HBEC3KTR$_L$53. KIF2C gene expression was measured by qPCR. $p < 0.0001$ by ANOVA (one-way analysis of variance). Tukey's Multiple Comparison Test showed $P < 0.05$ for comparison between HBEC3KT vs HBEC3KTR$_L$53 and HBEC3KT53 vs HBEC3KTR$_L$53. (E) KIF2A gene expression with $p = 0.045$ by ANOVA. Tukey's Multiple Comparison Test showed $P < 0.05$ for comparison between HBEC3KT53 vs HBEC3KTR$_L$53. Microarray profile of tumor and normal cell lines for (F) KIF2C; $p < 0.0001$ by two-tailed t test (G) KIF2A; $p = 0.0022$ by two-tailed t test.

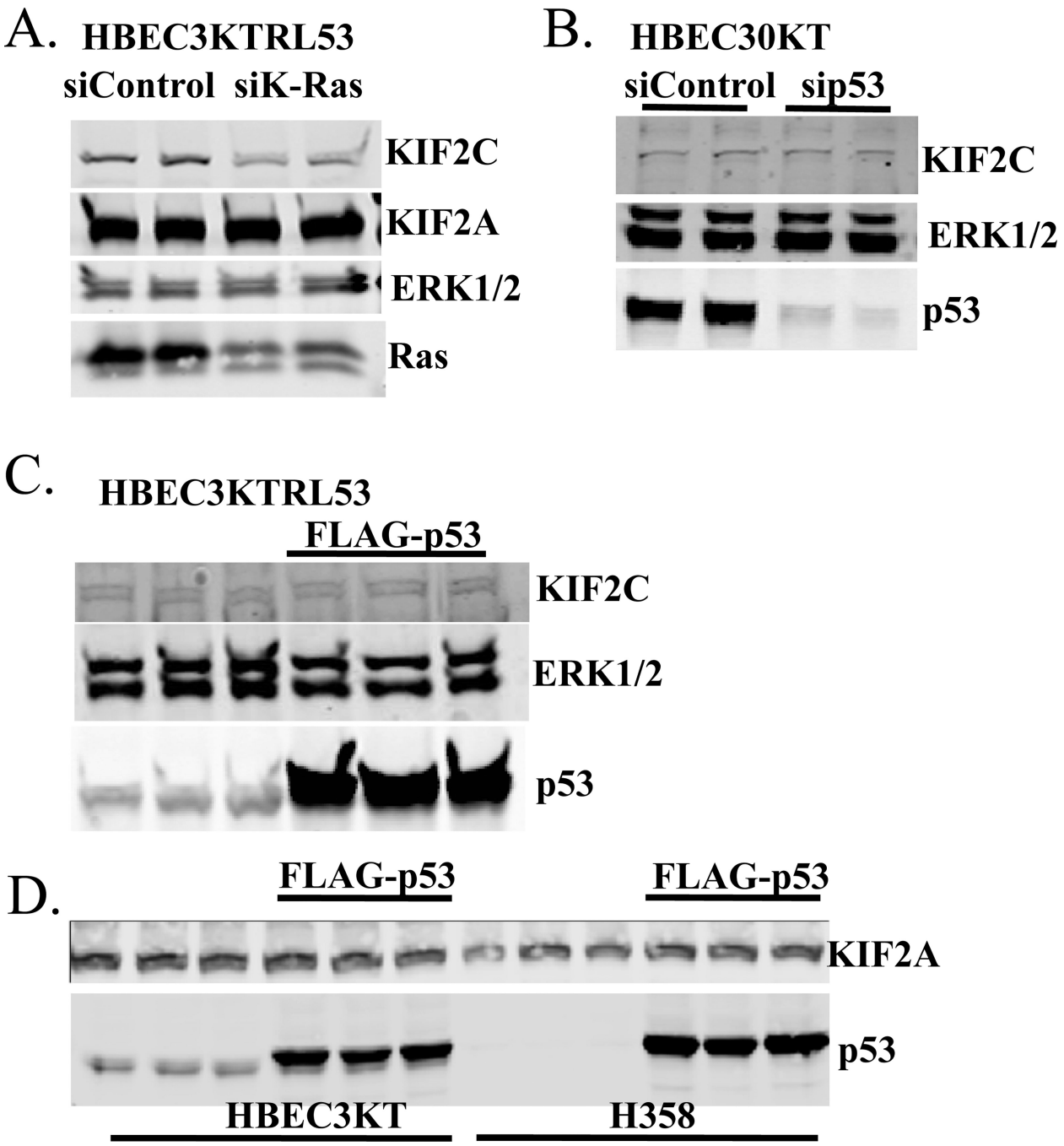

Fig. 2. K-Ras^{G12V} transformation affects KIF2C expression more than loss of p53

(A) KIF2C and KIF2A were immunoblotted in HBEC3KTR$_L$53 following depletion of K-Ras with siRNA compared to a non-targeting control siRNA. (B) KIF2C was immunoblotted in HBEC30KT following depletion of p53 with siRNA compared to non-targeting control siRNA. (C) KIF2C was immunoblotted in lysates of HBEC3KTR$_L$53 or (D) KIF2A was immunoblotted in HBEC3KT and H358 in each case following re-expression of p53.

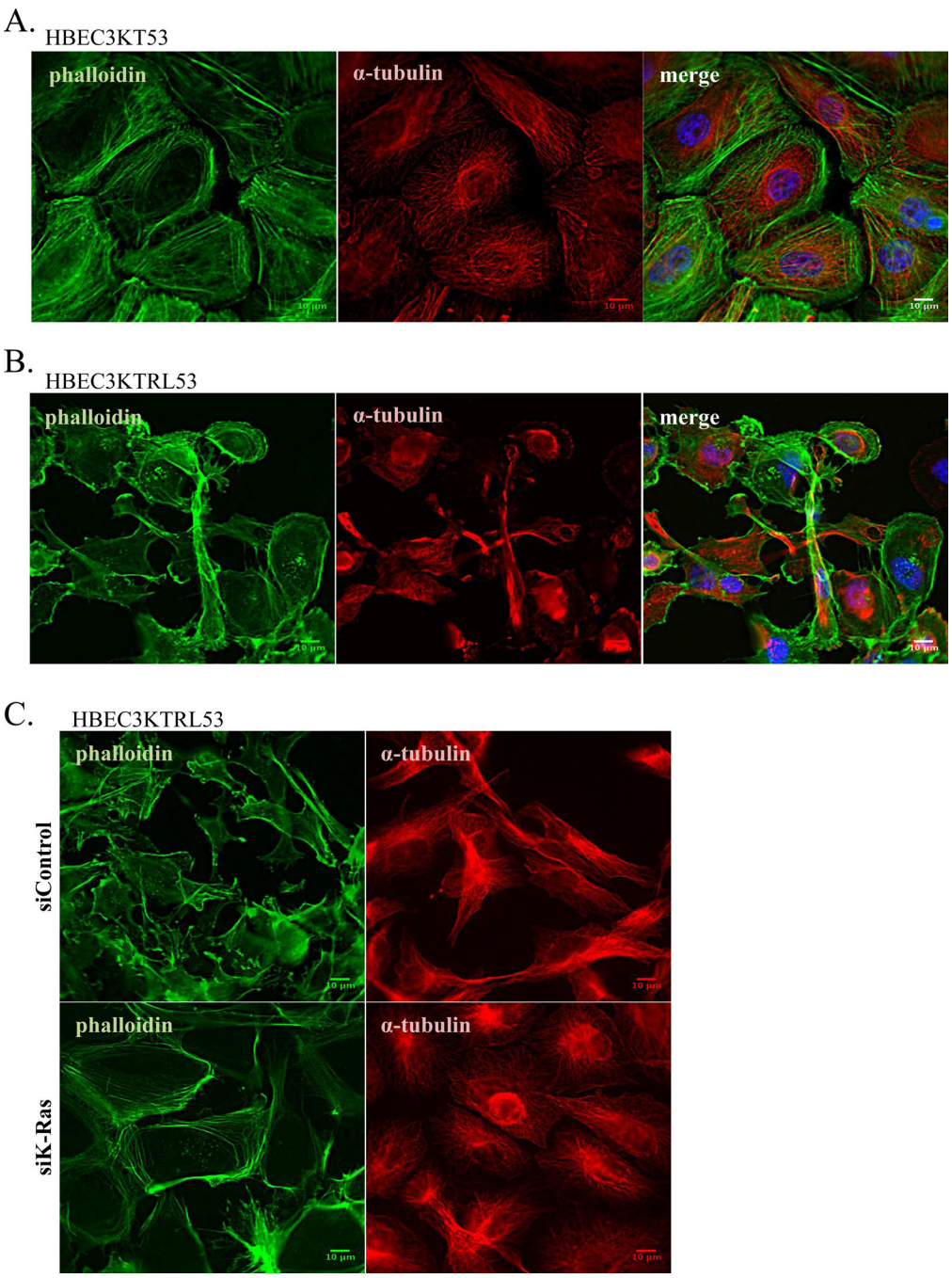

Fig. 3. Cells transformed with K-Ras^{G12V} display changes in microtubule and actin cytoskeletons

Actin was detected using phalloidin (green) and α-tubulin (red) was detected by immunostaining in (A) HBEC3KT53, (B) HBEC3KTR$_L$53, and (C) HBEC3KTR$_L$53. In (C) staining was performed 72 hr after transfection with control (top) or K-Ras siRNA (bottom).

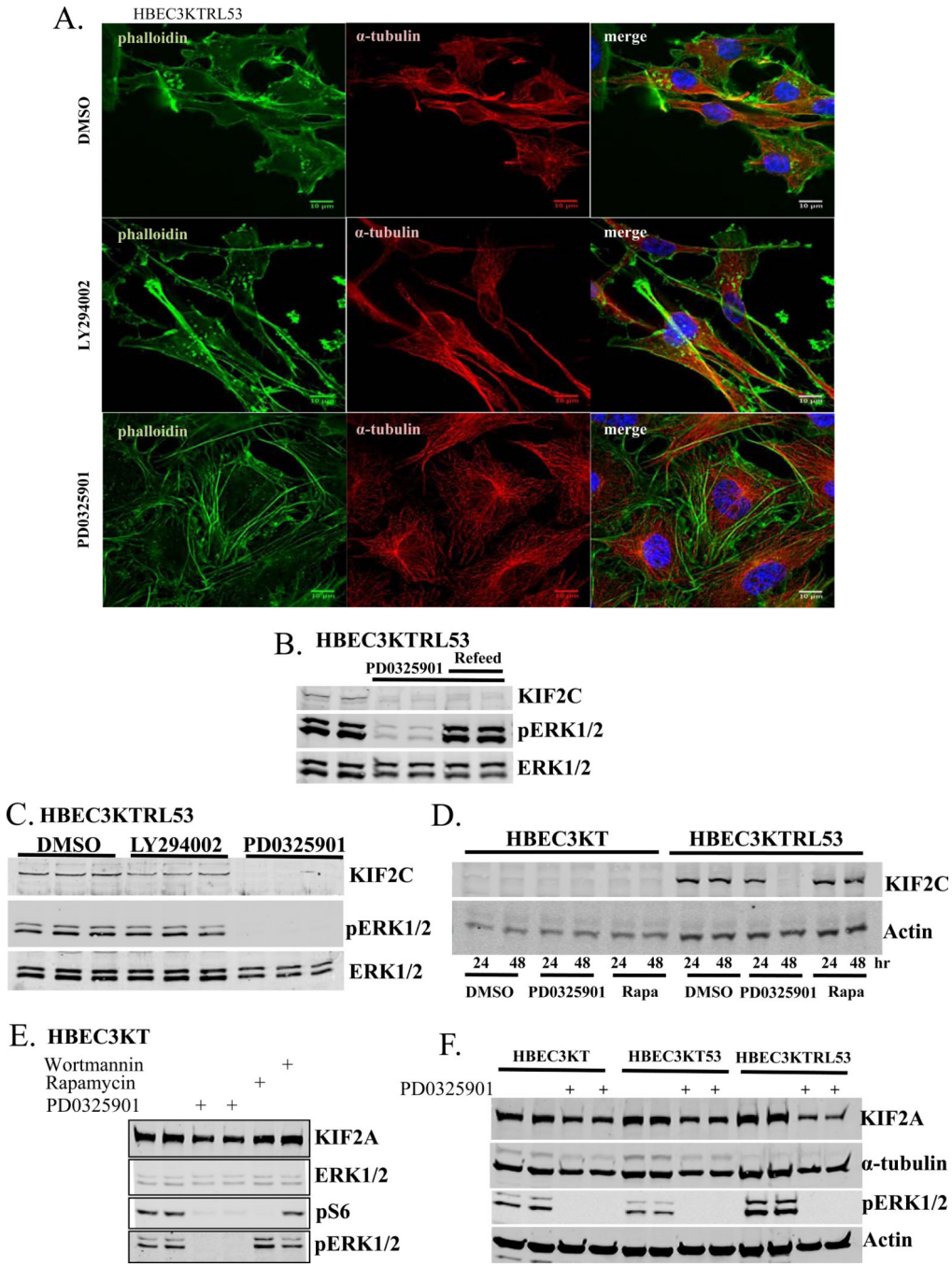

Fig. 4. ERK1/2 activity affects cellular morphology and KIF2C expression

(A) HBEC3KTR$_L$53 were treated with 100 nM PD0325901 or 10 μM LY294002 for 48 hr followed by immunofluorescence with the indicated antibodies. (B) Activation of ERK1/2 was detected by immunoblotting of lysates of HBEC3KTR$_L$53 treated with PD0325901 for 48 hr and stimulated with fresh medium after several washes with PBS to remove the inhibitor. (C) KIF2C was immunoblotted in HBEC3KTR$_L$53 that had been treated for 48 hr with 10 μM LY294002 or 100 nM PD0325901. (D) KIF2C was immunoblotted in HBEC3KT and HBEC3KT53 treated for 24 or 48 hr with 5 μM PD0325901 or 100 nM

rapamycin. (E) KIF2A was immunoblotted in HBEC3KT treated with 100 nM rapamycin, 50 nM wortmannin or 1 μM PD0325901 for 72 hr. (F) KIF2A was immunoblotted in lysates of HBEC3KT, HBEC3KT53 and HBEC3KTR_L53 treated for 72 hr with 1 μM PD0325901.

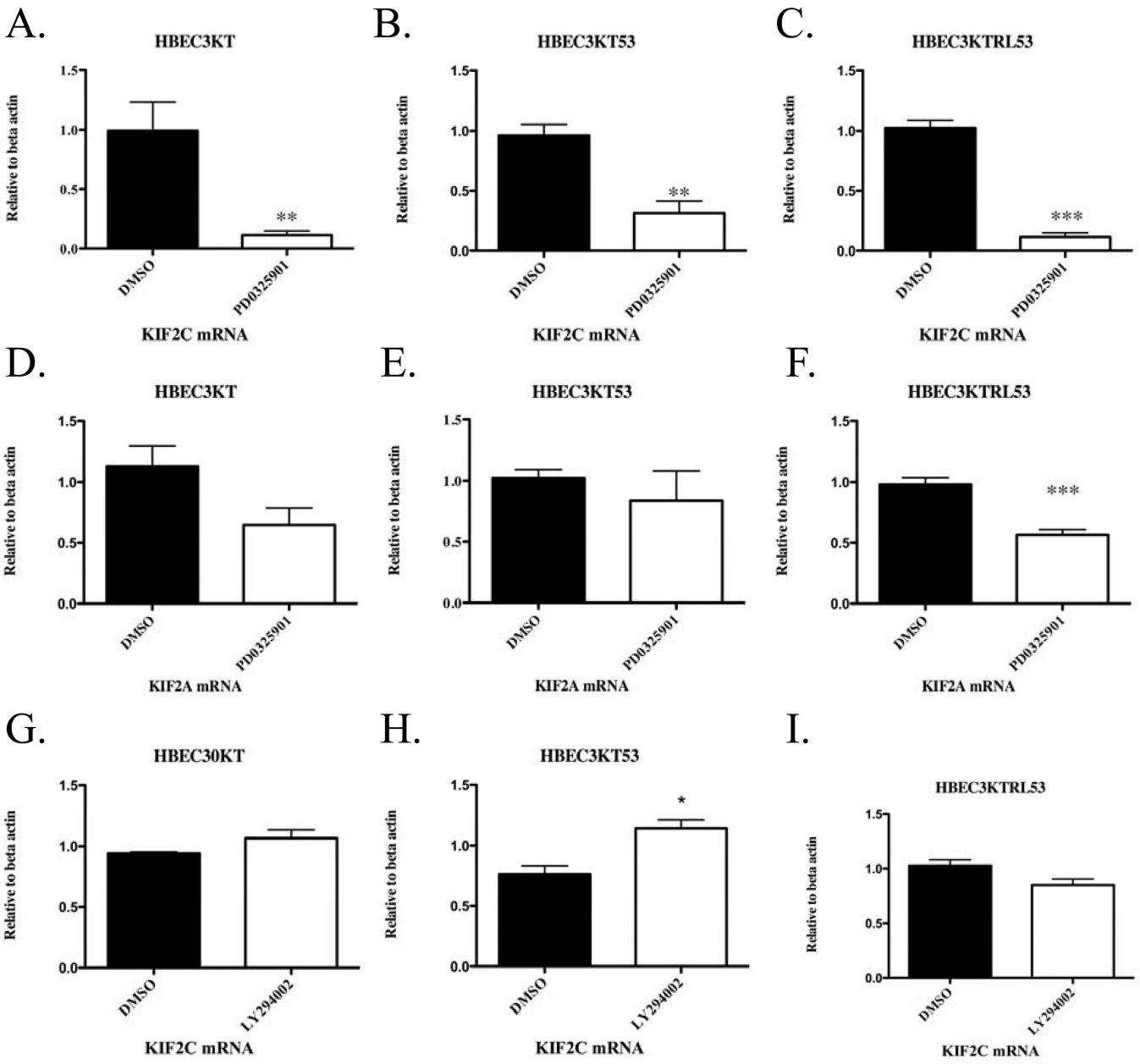

Fig. 5. Inhibiting the ERK1/2 pathway affects KIF2C gene expression more than KIF2A

RNA was isolated from (A) HBEC3KT, (B) HBEC3KT53 and (C) HBEC3KTR$_L$53 treated for 48 hr with 1 μM PD0325901 followed by qPCR measure of KIF2C gene expression. Three different experiments were analyzed by an unpaired t-test resulting in p-value (A) 0.0045 (B) 0.0028 (C) < 0.0001. RNA was isolated from (D) HBEC3KT, (E) HBEC3KT53, or (F) HBEC3KTR$_L$53 and KIF2A gene expression was measured by qPCR after 48 hrs of treatment with PD0325901. Data from three experiments was graphed. Statistical analysis was performed by t-test of control versus treatment: p-value (D) 0.0533, (E) 0.4795 and (F) 0.0301. Treatment of (G) HBEC3KT, (H) HBEC3KT53 and (I) HBEC3KTR$_L$53 with 10 μM LY294002 followed by qPCR for KIF2C gene expression as above. Statistical analysis

was performed by t-test of control versus treatment: p-value (G) 0.1464, (H) 0.0192 and (I) 0.0628.

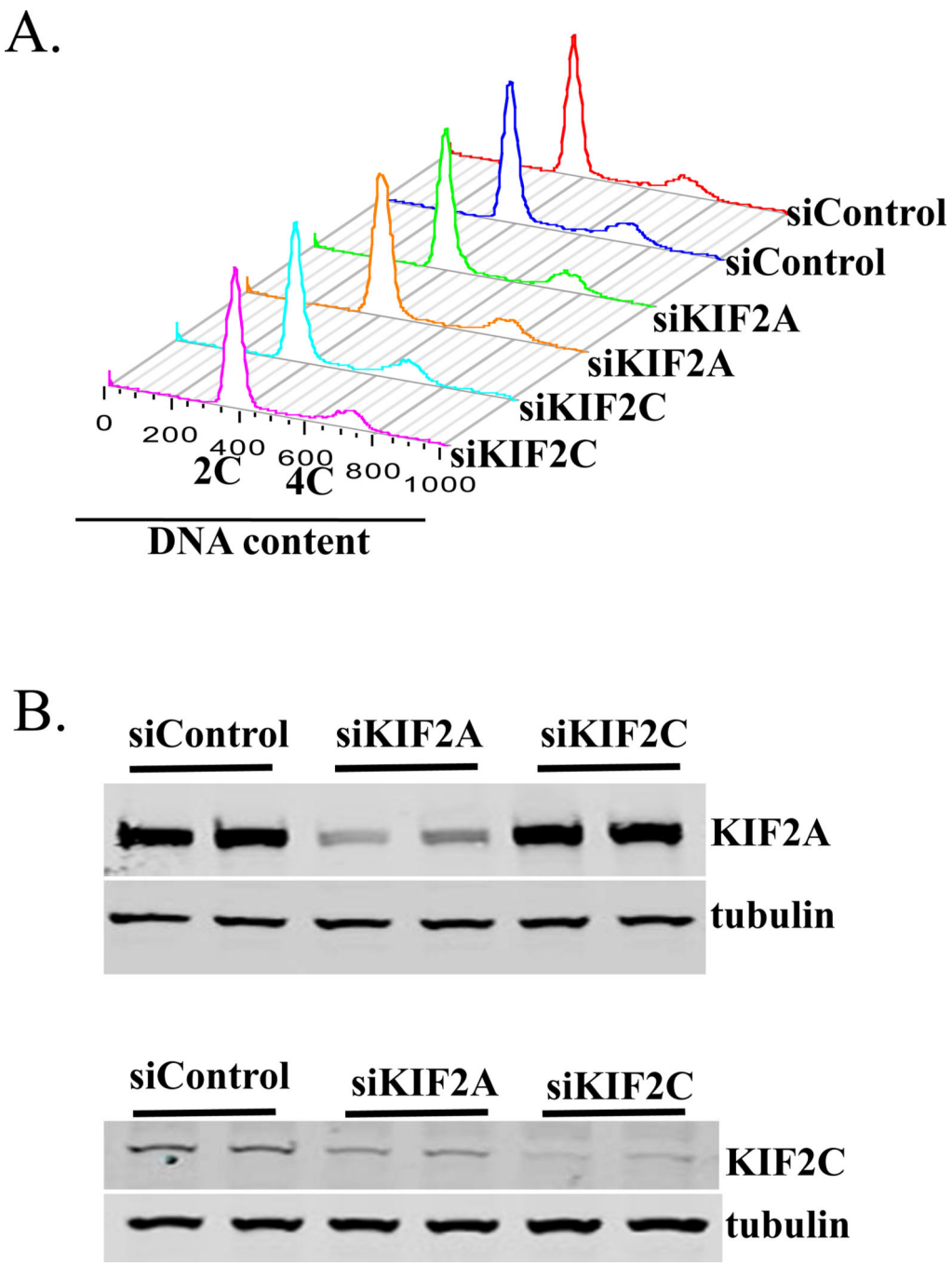

Fig. 6. Depletion of either KIF2A or KIF2C does not change the cell cycle profile in HBEC3KTR$_L$53

HBEC3KTR$_L$53 were harvested for flow cytometry and immunblotting following depletion for 96 hr of KIF2A and KIF2C alone or together. (A) Cells were stained with propidium iodide (PI) and DNA content was measured by flow cytometry with FACSCalibur (BD Biosciences). Data were analyzed using FlowJo. (B) Lysate proteins (20 μg) were resolved on gels and immunoblotted to detect the indicated proteins.

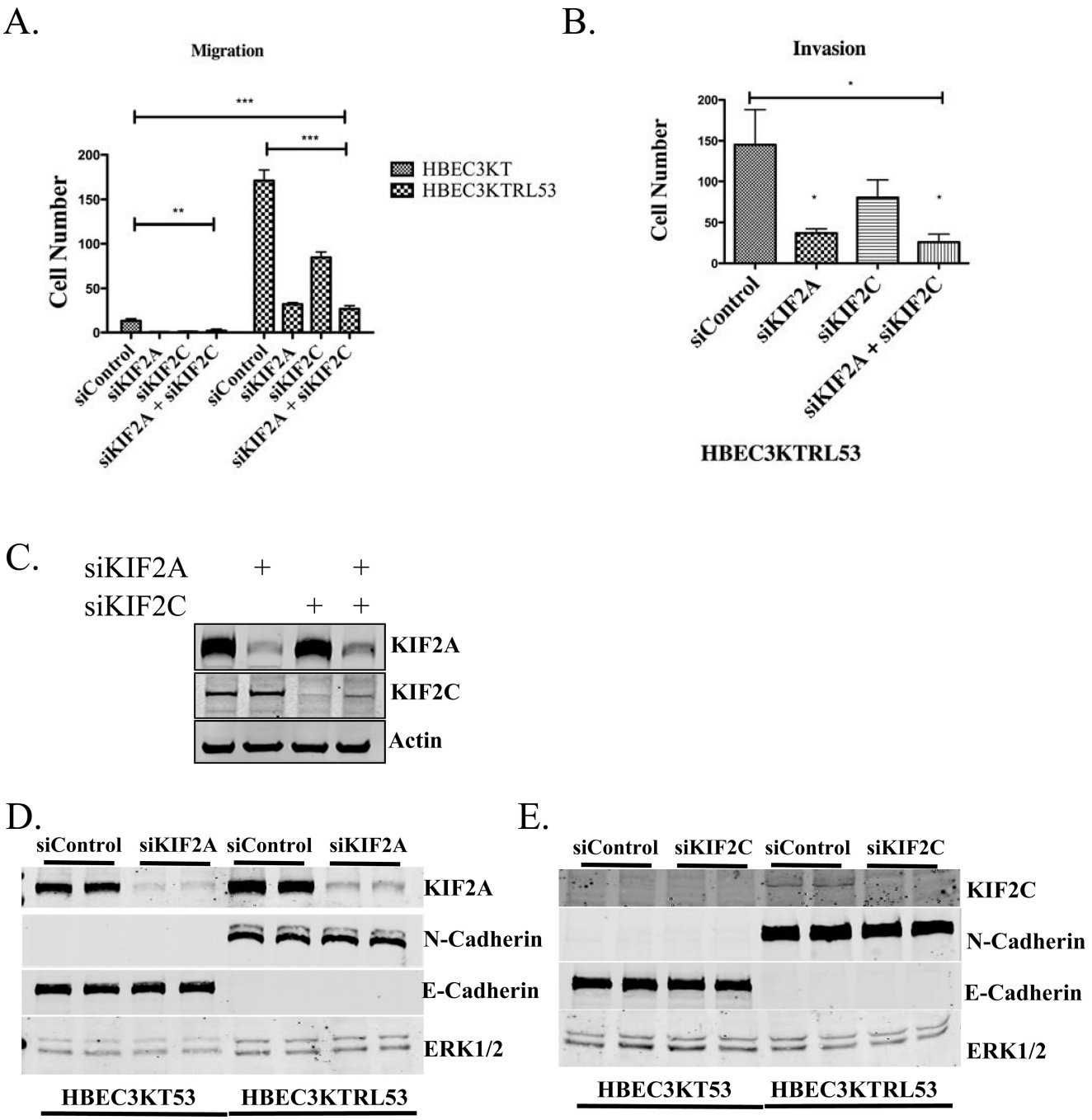

Fig. 7. KIF2A and KIF2 facilitate cell migration

Cell migration was measured in HBEC3KT and HBEC3KTR$_L$53 after depletion of KIF2A or KIF2C: (A) transwell migration assay (B) Invasion by HBEC3KTR$_L$53 after depletion of KIF2A or KIF2C through Matrigel. (A) Analysis by ANOVA shows $p < 0.0001$ for cell type and knockdown difference. Bonferroni Multiple Comparison Analysis shows $p < 0.01$ between siControl and knockdowns in HBEC3KT and $p < 0.0001$ in HBEC3KTRL53. (B) One-way analysis by ANOVA: $p < 0.05$ with Dunnett's Multiple Comparison Test showing statistical significance for siControl vs siKIF2A and siControl vs siKIF2A+siKIF2C (C)

Panel shows efficient knockdown of kinesins for panels (A) and (B). Knockdown of (D) KIF2A or (E) KIF2C followed by immunoblotting of the indicated proteins in HBEC3KT53 and HBEC3KTR$_L$53.