The membrane-associated form of cyclin D1 enhances cellular invasion

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Abstract
The essential G1-cyclin, CCND1, is a collaborative nuclear oncogene that is frequently overexpressed in cancer. D-type cyclins bind and activate CDK4 and CDK6 thereby contributing to G1-S cell-cycle progression. In addition to the nucleus, herein cyclin D1 was also located in the cytoplasmic membrane. In contrast with the nuclear-localized form of cyclin D1 (cyclin D1NL), the cytoplasmic membrane-localized form of cyclin D1 (cyclin D1MEM) induced transwell migration and the velocity of cellular migration. The cyclin D1MEM was sufficient to induce G1-S cell-cycle progression, cellular proliferation, and colony formation. The cyclin D1MEM was sufficient to induce phosphorylation of the serine threonine kinase Akt (Ser473) and augmented extranuclear localized 17β-estradiol dendrimer conjugate (EDC)-mediated phosphorylation of Akt (Ser473). These studies suggest distinct subcellular compartments of cell cycle proteins may convey distinct functions.

Introduction
The cyclin D1 (CCND1) gene, encodes the regulatory subunit of a holoenzyme that phosphorylates and inactivates the retinoblastoma protein (pRB), in order to promote cell cycle progression1-3. Newly synthesized cyclin D1 associates with CDK4/6 to form the holoenzyme that phosphorylates pRB, releasing E2F family transcription factors and inducing a gene expression network contributing to G1/S entry. Early studies demonstrated that cyclin D1 functions as a nuclear collaborative oncogene4. In this regard a cyclin D1 cDNA clone contributed to cellular transformation by complementing a transformation defective adenovirus E1A oncogene4. The requirement for cyclin D1 in oncogenic transformation has been established through cyclin D1 anti-sense5,6 and genetic deletion studies in the mouse7-9. Furthermore, cyclin D1 targeted to the mammary gland was sufficient for the induction of mammary tumorigenesis10,11. Clinical studies have shown a correlation between cyclin D1 expression and tumorigenesis and increased cyclin D1 expression is associated with tumor invasion and metastasis12-15.

A growing body of evidence provides support for an extranuclear function of cyclin D1. Cyclin D1 is actively synthesized and located exclusively in an extranuclear location in hibernating hematopoietic stem cells (HSC)16, in postmitotic neurons17, cardiomyocytes18, and hepatocytes19. The cytoplasmic sequestration of cyclin D1 is important to maintain the non-proliferative state as nuclear enforced expression using a nuclear-localized form of cyclin D1 forces the cell into a proliferative state18. Cyclin D1 has been identified in the cytoplasmic membrane20-22 and shown to bind and regulate the function of several cytoplasmic membrane-associated proteins including PACSIN II (Protein kinase C and Casein kinase Substrate In Neurons protein 2)23 also known as syndapin), Filamin A24 and paxillin21.
The association of cyclin D1 with cytoplasmic membrane proteins is consistent with prior studies demonstrating other components of the cell-cycle control apparatus are located in the cytoplasmic membrane including p27Kip1 and p16INK4a. Although the physiological function of cytoplasmic membrane-associated cell-cycle components was previously not well understood, p16INK4a and CDK6 colocalize in membrane ruffles of spreading cells and functioned upstream of αvβ3-dependent activation of PKC to regulate matrix-dependent cell migration. Cyclin D1-deficient mouse embryo fibroblasts (MEFs) and mammary epithelial cells exhibit increased adhesion and decreased motility compared with wild-type MEFs. Transduction of cyclin D1−/− cells with a human or murine cyclin D1 cDNA, reversed this adhesive phenotype, promoting cell migration. The induction of cell migration by cyclin D1 correlated with the reduction of Rho GTPase activity. Mutational analysis demonstrated that cyclin D1 reduction of cellular adhesion and induction of cellular migration were independent of the pRB- and p160 coactivator-binding domains. Cyclin E knockin of cyclin D1−/− MEFs rescued the DNA synthetic defect of cyclin D1−/− MEFs but did not rescue the migration defect suggesting the pRB binding of cyclins and the promigratory function may be dissociable.

Although cyclin D1 binds cytoplasmic membrane-associated proteins and correlative studies have suggested that cyclin D1 may promote cellular migration, no studies have selectively uncoupled the functional activity of the nuclear vs. cytoplasmic cyclin D1 pools. The current studies were conducted in order to determine the function of cyclin D1 when localized to either the cellular membrane or the nucleus.

**Results**

**Cyclin D1 is located at the cytoplasmic membrane**

The endogenous cytoplasmic membrane-associated protein PACSIN II was shown to bind cyclin D1 in liver tissue and cyclin D1 bound to PACSIN II and paxillin (Pxn) in 3T3 cells. In order to characterize the function of membrane-associated cyclin D1, studies were conducted in the human diploid fibroblast cell line (MRC-5) and human breast cancer samples. Using immunohistochemistry, endogenous cyclin D1 was identified at the MRC-5 cellular leading edge, in proximity with PACSIN II (Fig. 1a, Fig. S1). Paxillin (Pxn) is a structural and regulatory component of FAs and is also found along the cell membrane. Cyclin D1 was identified co-staining with tyrosine phosphorylated paxillin (Paxillin-PTyr118) and F-actin. Focal contacts are identified by the tyrosine phosphorylated Paxillin. Co-staining of PACSIN II with tyrosine phosphorylated Paxillin (Y118) with merged staining shown by yellow arrows.
the location of cyclin D1 in human breast cancer we compared membrane-associated cyclin D1 in patients with IBC and other breast cancers. Samples from 6 IBC patients and 17 non-IBC patients were stained for cyclin D1 and analyzed by a clinical pathologist (Fig. S3A, B). The subcellular distribution was assigned using the standard Aperio digital analysis algorithm for cell-membrane staining. The entire slide was scanned enabling analysis of >1000 cells per sample. Five of six IBCs stained for membrane associated cyclin D1, whereas only 2/17 non-IBC stained for membrane-associated cyclin D1. Cytoplasmic-membrane-associated cyclin D1 was observed in 5 of 6 IBC patient samples and 2 of 17 non-IBC patient samples had membrane-associated cyclin D1 (Fig. S3). All 6 IBC and 16 of 17 non-IBC patients had nuclear localized cyclin D1. We next conducted immunofluorescent studies for cyclin D1 in non-IBC patients in order to provide more sensitive detection of membrane-associated cyclin D1. Costaining of cancer cells with a pan-cytokeratin antibody and underexposing the immunofluorescence (IF) signal provides an effective way of delineating the cellular boundaries, revealing tumor cores with clusters of cells displaying membrane-associated cyclin D1. In a tissue microarray of 50 ERα-positive breast cancers examined, membrane-associated cyclin D1 was detected in four cases (Fig. S3C–F).

**Cytoplasmic membrane-targeted cyclin D1 promotes transwell migration and increases cellular migratory velocity**

Cyclin D1 is known to promote cellular migration of fibroblasts and mammary epithelial cells. In order to further characterize the molecular mechanisms by which cyclin D1 governs the induction of cellular migration we conducted subfractionation of nuclear and cytoplasmic cellular fractions from cyclin D1 WT vs cyclin D1 WT/− 3T3 cells (Fig. 2a). Western blot analysis demonstrated enrichment of histone H2A in the nuclear fraction,
α-tubulin in the cytoplasmic fraction and Na⁺/K⁺-ATPase in the membrane-associated fraction as previously described²⁹. Cyclin D1 was identified in each of the subcellular fractions, consistent with prior studies conducted by confocal microscopy²¹,²³. In order to determine the function of the cytoplasmic membrane-localized fraction of cyclin D1, cyclin D1⁻/⁻ 3T3 were transduced with a cyclin D1 expression vector encoding either cyclin D1¹⁰⁷, cyclin D1¹⁰⁸, or cyclin D1¹⁰⁹ (Fig. 2b) and functional analysis were conducted. Cherry-lacR-NLS-CD1¹⁰⁸ which encodes a nuclear localized form of cyclin D1, was previously well characterized³⁰,³¹. Cyclin D1 was cloned at the C-terminus of the Cherry-lacR-NLS vector³²,³³. For cyclin D1¹⁰⁹ the cyclin D1 cDNA was cloned in frame to pECFP-Mem (Clonetech), which encodes a fusion protein consisting of the N-terminal 20 amino acids of neuromodulin, also called GAP-43, and a cyan fluorescent variant of the enhanced green fluorescent protein. The neuromodulin fragment contains a signal for posttranslational palmitoylation of cysteines 3 and 4 that targets ECFP to cellular membranes. Expression of ECFP-Mem in mammalian cells results in strong labeling of the plasma membrane and had been used to target proteins including ERα to the plasma membrane³⁴. Using electroporation the transfection efficiency was >90%. Cyclin D1¹⁰⁷ enhanced transwell migration twofold (Fig. 2c, f), cyclin D1¹⁰⁸ did not enhance transwell migration (Fig. 2d, f) and cyclin D1¹⁰⁹ enhanced transwell migration threefold (Fig. 2e, f).

Transwell migration assays were next conducted in MCF-7 cells that were serum starved to reduce endogenous cyclin D1. Compared with the respective vector control transwell migration was enhanced 6.6-fold by cyclin D1¹⁰⁷, 2.2-fold by cyclin D1¹⁰⁸ and 12.6-fold by cyclin D1¹⁰⁹ (Fig. S4).

Cyclin D1-deficient fibroblasts show the same diameter size as wild-type cells, but attach and spread more rapidly after seeding on fibronectin-coated plates²⁶,²⁸. Herein, time lapse video microscopy demonstrated the induction of cellular velocity by cyclin D1¹⁰⁷ (Fig. 3a, d). Expression of Cyclin D1¹⁰⁹, but not cyclin D1¹⁰⁸, promoted cellular migratory velocity (Fig. 3b–d).

The subcellular distribution of cyclin D1¹⁰⁹ and cyclin D1¹⁰⁸, was further characterized using confocal microscopy (Fig. S5). Transfected cells were examined by confocal microscopy and by Z series reconstruction with the nucleus stained with Hoechst 33342. The cells expressing membrane-associated cyclin D1 showed green fluorescence predominantly at the cellular membrane (Fig. S5A, B), whereas the cyclin D1¹⁰⁸ showed red fluorescence predominantly in the nucleus (Fig. S5C, D).

**Cytoplasmic membrane-targeted cyclin D1 augments DNA synthesis and contact independent growth**

Reintroduction of cyclin D1 into cyclin D1⁻/⁻ fibroblasts may enhance DNA synthesis associated with a reduction in the proportion of cells in the G₀/G₁ phase of the cell cycle. In order to determine the capacity of membrane-targeted cyclin D1 to regular the cell-cycle distribution, fluorescence activated cell sorting (FACS) analysis was conducted. Comparison was made to the
empty control vector because of the potential impact of fluorescent proteins on apoptotic and cell-cycle control proteins. The distribution of cells in each phase of the cell cycle assessed by FACS demonstrated cyclin D1WT, cyclin D1NUC, and cyclin D1MEM enhanced the proportion of cells in the DNA synthetic (S) phase with a doubling of the proportion of cells in S phase by cyclin D1WT (7.2 vs. 18.2%) (Fig. 4a, b), an 80% increase in S phase by cyclin D1NUC (10.7 vs. 18.7%) (Fig. 4c, d) and a doubling of the proportion of cells in S phase by cyclin D1MEM (5.35 vs. 12.2%) (Fig. 4e, f).

Cellular proliferation was assessed by MTT activity with comparison made to the control vector. Cellular proliferation was increased by cyclin D1WT (2.4 vs. 3.5-fold), cyclin D1NUC (2.4 vs. 3.1-fold) and cyclin D1MEM (1.6 vs. 2.7-fold) (Fig. 5a). Colony formation as an assay of contact-independent growth, showed an increase in both colony number and colony size with either cyclin D1WT, cyclin D1NUC or cyclin D1MEM (Fig. 5d–l) with a twofold increase in colony number and size with cyclin D1MEM (Fig. 5j–l). In order to determine potential mechanisms by which cyclin D1NUC and cyclin D1MEM may induce proliferative signaling, we assessed the impact of signaling induced using downstream reporter target genes (Fig. S6). Consistent with prior studies, that cyclin D1 repressed the (AOX)3-LUC reporter gene, herein both cyclin D1NUC and cyclin D1MEM repressed the (AOX)3-LUC reporter gene (Fig. S6). The immediate early gene c-Fos-LUC and cyclin D1-LUC were induced approximately twofold more by cyclin D1MEM than by cyclin D1NUC (Fig. S6). These studies show that cyclin D1MEM activates immediate early gene c-Fos and cyclin D1 transcription and suggest that cyclin D1MEM may promote distinct signaling pathways to augment cellular growth.
Cytoplasmic membrane-targeted cyclin D1 augments estrogen-dependent Akt kinase activation via K112

The estrogen receptor α (ERα) is known to convey both genomic and extranuclear activities. The extranuclear estrogen signaling pathway is thought to involve a membrane-associated ERα, which activates PI3-kinase and thereby Akt signaling. Maximal activation of Akt requires phosphorylation on the carboxy-terminal site, S473, by mTORC2. In recent studies, membrane-associated estrogen signaling was shown to occur via cyclin D1. We investigated the impact of expressing cyclin D1 as either total, nuclear, or membrane-tethered.
forms of cyclin D1 (Fig. 6). The human breast cancer cell line (MCF-7) was transduced with expression vectors encoding cyclin D1 targeted to the nucleus (cherry-D1NUC), the cytoplasmic membrane (pECFP-D1MEM) or cyclin D1 expressed in both compartments (Total-MSCV-D1TOT). Cells were treated with E2 (10 nM) for 10 min. Vinculin is a protein loading control. Cells transfected with expression plasmids encoding cyclin D1 under control of the MSCV promoter (cyclin D1MEM) showed the characteristic enrichment of membranous GFP staining (Fig. 7a). Akt1 phosphorylated at Serine473 may be either nuclear or cytoplasmic, related to additional signaling partners45. In the vehicle treated cells, cyclin D1MEM expression was associated with the induction of nuclear p-Ser473-Akt1. EDC treatment of vector control cells increased nuclear p-Ser473-Akt1. EDC treatment of cyclin D1MEM transduced MCF-7 cells correlated with the induction of p-Ser473-Akt1, which was found to be in a cytoplasmic membranous distribution (Fig. 7a). In MCF-7 cells transduced with cyclin D1NUC, cyclin D1-RFP was located primarily in the nucleus. Nuclear localized cyclin D1 (cherry-CD1NUC) did not induce p-Ser473-Akt1 significantly (Fig. 7b). EDC treatment of MCF-7 cells augmented phosphorylation p-Ser473-Akt1, which was primarily nuclear in distribution (Fig. 7b). Careful quantitation evidenced that cyclin D1NUC did not augment EDC-induced nuclear Akt1 Serine473 phosphorylation (Fig. 7b, d). MCF-7 cells transduced with cyclin D1TOT showed nuclear, cytoplasmic, and membrane-associated cyclin D1, and an enhancement of EDC induced Akt1 Serine473 phosphorylation. p-Ser473-Akt1 was located in both the nucleus and membrane (Fig. 7c, d). Thus, the cytoplasmic membrane localized cyclin D1 (PECFP-CD1MEM), but not the nuclear localized form (cherry-CD1NUC), augmented Akt1 phosphorylation at Serine473.
The immediate activation of Akt1 by insulin requires cyclin D1

Recent studies identified a dichromic fluorescent (DCF) dye substrate for cellular Akt1 activity. The diserine DCF substrate was shown to serve as a specific substrate for Akt1, which can be used to quantitatively assess the enzyme’s activity in real time. Insulin activation of cellular Akt phosphorylates a single serine residue of the diserine DCF substrate in a time dependent manner, resulting in a spectral shift that can be used to assess longitudinally the stimulation and reversibility of Akt1 activity. The dichromic dye LS456 is phosphorylated by Akt1, but not a variety of other kinases (including PKA, PKC, RSK1, P70S6K, and PI3K). The binding of insulin to its cell surface receptor stimulates phosphoinositide-3 kinase (PI3K), which then induces the second messenger, phosphotidylinositol-3, 4, 5-triphosphate (PIP3). PIP3 activates Akt and additional downstream effectors. As LS456 was shown to serve as a specific substrate for Akt1 in response to 150 nM insulin, we examined the kinetics of insulin-mediated activation of LS456 in cyclin D1−/− MEF compared with wild-type MEFs. Insulin stimulation of Akt1 activity assessed by LS456 was delayed with reduced induction in cyclin D1−/− cells compared with the cyclin D1WT rescued cells (Fig. S7).

Cyclin D1 restrains RhoA activity via K112

In the current studies, cytoplasmic membrane-tethered cyclin D1 augmented cellular migratory velocity and estrogen-dependent induction of Akt1 Ser473 phosphorylation. In prior studies cyclin D1 rescue of cyclin D1−/− MEFs reduced RhoA activity. Although these prior studies suggested that cyclin D1 may augment cellular migration by restraining RhoA activity, Rac1 and Cdc42 can also participate in cellular migration. In order to examine the functional interactions with cyclin D1 and Rho GTPases we deployed the FRET based fluorescent probes for RhoA, Rac, and Cdc42 (Fig. 8a). pRaichu-RhoA consists of a truncated RhoA (aa 1–189), the RhoA-binding domain (RBD) and the FRET pair of CFP and YFP. When RhoA binds to GTP, and thereby the RBD, RhoA recruits CFP in close proximity to YFP, thereby increasing the FRET activity between CFP and YFP. We examined the functional interaction between cyclin D1 and RhoA using FRET. The image from a typical FRET experiment was shown in Fig. 8b. Cells were
co-transfected with pRaichu-RhoA and either cyclin D1WT, cyclin D1KE, or their corresponding vector control. Spectral images in 10 channels from 470 to 566 nm with excitation at 458 nm were simultaneously recorded. YFP was inactivated by photobleaching with a 514 nm laser at 100% power output (Fig. 8b). The emission spectra within the ROI increased in the CFP signal at 481 nm after photobleaching with YFP which has an emission peak at 534 nm (Fig. 8c). FRET efficiency was used to quantitatively compare the difference in RhoA activity among the
cells. (FRET efficiency was defined as $\frac{F_B - F_D}{F_B} \times 100\%$, where $F_B$ is the intensity of the donor (CFP) after photobleaching and $F_D$ is the intensity of the donor before photobleaching, see “Methods”). FRET efficiency was reduced 40% by cyclin D1WT but was not significantly reduced by expression of the cyclin D1KE (Fig. 8d). Similar analysis of FRET for the related Rho family members, Rac1 and Cdc42, failed to elicit changes in FRET efficiency upon re-expression of cyclin D1 wild type. By using FRET, we extend prior studies demonstrating cyclin D1 reduces Rho GTPase activity26, to define the interaction of cyclin D1 occurs with RhoA, not Rac or Cdc42, and demonstrate the residue K112 of cyclin D1 is required for interaction with RhoA.

**Discussion**

The well-characterized nuclear functions of cyclin D1 include firstly, serving as the regulatory subunit of a holoenzyme that phosphorylates the pRB protein, and secondly serving as part of a transcriptional regulatory complex that drives proliferative gene expression48. Consistent with previous studies, that either identified cyclin D1 associated with the cytoplasmic membrane or cytoplasmic membrane proteins24, the current studies identified cyclin D1 colocalized with PACSIN II and paxillin PTyr118 at the cytoplasmic membrane. The current studies extend our understanding of cyclin D1 through characterizing the function and signaling pathways regulated by cyclin D1 at the cytoplasmic membrane vs. the nucleus. Firstly, herein membrane-associated cyclin D1 augmented transwell migration and enhanced the velocity of cellular migration. In contrast, the nuclear-localized form of cyclin D1 neither enhanced cellular migratory velocity nor induced transwell migration in 3T3 cells. These studies are consistent with previous findings that cyclin D1 promotes migration21,26,28,43,44, but extend these findings by demonstrating that it is the membrane-associated form of cyclin D1 that mediates this function. Secondly, these studies show both nuclear and membrane-associated cyclin D1 augment cellular DNA synthesis, cellular proliferation, and contact-independent growth. Thirdly, these studies demonstrate that cyclin D1 tethered to the cytoplasmic membrane induces Akt signaling, characterized by the induction of Akt1 Ser473 phosphorylation. Furthermore, membrane-associated cyclin D1 augmented a physiological function of estrogen, to induce Akt1 Ser473 phosphorylation. Fourthly, as activity of Rho GTPase at the cellular membrane may inhibit cellular adhesion and migration and restrain Akt activity49, we examined and defined a role for cyclin D1 to inhibit Rho activity. Collectively these studies define a novel function for cytoplasmic membrane associated cyclin D1 that may augment aberrant growth control and cellular invasion.

Prior studies had shown the induction of cellular migration by cyclin D121,26,28,43,44. Cyclin D1−/− cells show a more spread morphology than the corresponding wild type and display an increased number of focal adhesions (FAs) with higher levels of tyrosine-phosphorylated paxillin21,26,28,43,44. Herein, using cyclin D1−/− cells, we demonstrated the membrane-associated pool of cyclin D1 is sufficient to augment transwell migration. We identified cyclin D1 at the plasma membrane in inflammatory breast cancer, and cyclin D1 colocalized to the cytoplasmic membrane with PACSIN II and paxillin (Y118) in MRC-5 cells. Cyclin D1 was previously shown by mass spectrometry to bind the membrane-associated proteins PACSIN II23, Filamin A24, Paxillin21, and several additional proteins50. PACSIN II is involved in cell spreading21, as well as endocytosis of cell–surface receptors like the EGF receptor52 and in caveolea-mediated endocytosis53,54. In view of clinical analyses showing a correlation between total cyclin D1 expression and tumor invasiveness and metastasis12–15, our studies suggest further studies assessing membrane-associated cyclin D1 may be warranted.

Herein, cytoplasmic membrane-associated cyclin D1 augmented phosphorylation of Akt1 at Ser473. Akt, also known as Protein Kinase B, promotes cellular survival, proliferation, growth, and migration55. Akt hyperactivation contributes to human cancer correlating with poor prognosis and therapy resistance and genetic deletion demonstrated Akt1 is required for ErbB2-induced breast cancer progression and tumor metastases in vivo56. Herein the acute nongenomic E2 activation of Akt1, was augmented by the membrane-associated cyclin D1 pool. Estradiol acutely activates Akt57,58 in part through the association of ERα at the plasma membrane associated with the p85 regulatory subunit of PI3-kinase and other proteins including the scaffold protein caveolin-1, G proteins, Src kinase, Ras, and Shc57,59–61. ERα regulates nuclear gene expression via genomic and extranuclear non-genomic signals37,59. Extranuclear pools of ERα reside in the plasma membranes62 and the ability to distinguish nuclear from extranuclear ERα signaling has been enabled through the generation of a 17β-estradiol dendrimer conjugate (EDC) which is localized to the extranuclear compartment41,42. Herein, using nuclear excluded E2 dendrimers, cyclin D1 was shown to participate in the acute non-genomic E2 response. Genetic deletion studies in the mouse demonstrated E2-dependent induction of genes governing growth factors, growth factor receptor and promigratory processes in the mammary gland requires cyclin D163. The biological effects of estrogen, are critically dependent upon cyclin D1 in vivo63,64, with the current studies suggesting an important component is mediated via membrane-associated cyclin D1.
RhoA, Rac1, and Cdc42 are the best characterized members of the Rho GTPase branch of the Ras superfamily and are known to regulate cellular morphology and migration. In the current studies, cyclin D1 restrained RhoA activity, requiring K112. Cyclin D1<sup>−/−</sup> cells have increased RhoA activity, increased ROCK II kinase and increased LIM kinase activation (threonine 505/508). LIM kinase phosphorylation at threonine 505/508 in turn phosphorylates the actin-depolymerizing protein coflin at serine 3 and MLCK at Thr18/Ser19. Herein, FRET analysis evidenced cyclin D1 restrained Rho GTPase activity. In contrast, neither Rac-GTPase nor Cdc42 activity was influenced by cyclin D1. The reduction in RhoA GTPase FRET by cyclin D1 was abolished by mutation of cyclin D1 residue K112. Cyclin D1 participates in multiple functions via K112 including CDK4/6-mutation of cyclin D1 residue K112. Cyclin D1 participate RhoA GTPase FRET by cyclin D1 was abolished by the ~70% of ER<sub>α</sub> augment cellular growth. The major adjuvant therapy for mechanism by which membrane associated cyclin D1 may anti-estrogen therapy. The ER<sub>α</sub> gene c-erbB-2 is a target of Akt1 phosphorylation and the repression of RhoA activity by cyclin D1 may have contributed to the induc- 

**Materials and methods**

A detailed description is provided in the Supplementary Materials.

**Plasmids and tissue culture**

The cyclin D1<sup>WT</sup> and cyclin D1<sup>KE</sup> MEFs<sup>10</sup> were prepared as described previously.<sup>76</sup>

**Transwell migration**

The assessment of transwell migration<sup>77</sup>, migratory velocity, and migratory distance<sup>66</sup> were conducted as previously described.

**Fluorescence resonance energy transfer (FRET) imaging**

HEK293T cells, co-transfected with 3×FLAG vector, cyclin D1 wild-type or cyclin D1<sup>KE</sup> mutant and FRET reporters (pRaichu-RhoA, pRaichu-Cdc42 or pRaichu-Rac1<sup>78,79</sup>), were cultured in a four-well chamber and imaged using a Zeiss laser-scanning microscope, LSM510META, with a 40× oil immersion Doc Plan-Neofluar lens objective (numerical aperture of 1.3). To detect FRET between CFP and YFP, we used time-lapse and lambda stack acquisition linked with the photo-bleaching command.<sup>80</sup>

**Immunostaining**

IF staining and confocal microscopy of cultured cells was conducted as described previously<sup>77</sup>. Chromogen immunostaining of human breast cancer samples was conducted on the breast tissue with the Ventana Benchmark autostainer using deidentified archival tissue which are exempt from review by the Thomas Jefferson University Institutional Review Board. Fluorescence-based immuno-histochemistry for cyclin D1 multiplexed with pan-cytokeratin and DAPI counterstain was performed as previously described<sup>81–83</sup> on a tissue microarray containing cores of 50 de-identified ER-positive breast cancer specimens provided by the Medical College of Wisconsin Tissue Bank under IRB-approved protocol.

**Live cell Akt activity monitoring**

Live cell imaging studies were conducted as described.<sup>46</sup>

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