Keratin 19 interacts with GSK3β to regulate its nuclear accumulation and degradation of cyclin D3

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Abstract Cyclin D3 regulates the G1/S transition and is frequently overexpressed in several cancer types including breast cancer, where it promotes tumor progression. Here we show that a cytoskeletal protein keratin 19 (K19) physically interacts with a serine/threonine kinase GSK3β and prevents GSK3β-dependent degradation of cyclin D3. The absence of K19 allowed active GSK3β to accumulate in the nucleus and degrade cyclin D3. Specifically, the head (H) domain of K19 was required to sustain inhibitory phosphorylation of GSK3β Ser9, prevent nuclear accumulation of GSK3β, and maintain cyclin D3 levels and cell proliferation. K19 was found to interact with GSK3β and K19–GSK3β interaction was mapped out to require Ser10 and Ser35 residues on the H domain of K19. Unlike wildtype K19, S10A and S35A mutants failed to maintain total and nuclear cyclin D3 levels and induce cell proliferation. Finally, we show that the K19–GSK3β-cyclin D3 pathway affected sensitivity of cells toward inhibitors to cyclin-dependent kinase 4 and 6 (CDK4/6). Overall, these findings establish a role for K19 in the regulation of GSK3β-cyclin D3 pathway and demonstrate a potential strategy for overcoming resistance to CDK4/6 inhibitors.

Introduction Cyclins play a major role in cell cycle progression. During the G1 phase, D-type cyclins dimerize with and activate cyclin-dependent kinase 4 or 6 (CDK4/6) which then phosphorylates tumor suppressor retinoblastoma protein (Rb) (Musgrove et al., 2011). Prior to phos-
autophagy (Alao et al., 2006; Azoulay-Alfaguter et al., 2015; Albrecht et al., 2020). At the molecular level, GSK3β activity is inactivated by phosphorylation at Ser9 by kinases including Akt and Rsk (Jope and Johnson, 2004; Beurel et al., 2015). Inappropriate regulation of GSK3β activity and subcellular localization contributes to the pathogenesis and progression of various diseases including noninsulin-dependent diabetes mellitus, cardiovascular disease, some neurodegenerative diseases, bipolar disorder, and cancer (Beurel et al., 2015; Manning and Toker, 2017).

Here we report a novel regulation of GSK3β activity by keratin 19 (K19). K19 belongs to a keratin family of intermediate filament proteins. Keratins are critical in maintaining structural integrity of epithelial cells and tissues, but they are also involved in other cellular processes such as proliferation and migration, especially in disease settings (Alix-Panabieres et al., 2009; Sharma et al., 2019b). While its normal function has not been studied in detail, K19 is expressed in the developing embryo, mature striated muscles, epithelia, and epithelial stem cells (Moll et al., 1982; Stone et al., 2007; Petersen and Polyk, 2010). Its expression is also observed in pathological conditions including several cancer types where K19 is used as a diagnostic and prognostic marker (Alix-Panabieres et al., 2009; Kabir et al., 2014). Studies using hepatocellular carcinoma (Takano et al., 2016), oral squamous cell carcinoma (Crowe et al., 1999), breast cancer (Sharma et al., 2019a), and lung cancer (Ohtsuka et al., 2016) cell lines have shown that K19 promotes cancer cell proliferation. We previously used KRT19 knockout (KO) of MCF7 breast cancer cells to identify that K19 is required for proper cell cycle progression and maintenance of levels of D-type cyclins (cyclin D1 and cyclin D3) (Sharma et al., 2019a). In particular, K19 was shown to delay degradation of cyclin D3, suggesting that K19 promotes cell proliferation by stabilizing cyclin D3. However, how a cytoskeletal protein can maintain levels of cyclin D3 remained unknown.

In this study, we identify GSK3β as a keratin-interacting protein and find that K19 suppresses GSK3β activity and hinders its nuclear accumulation. GSK3β-binding by K19 required Ser10 and Ser35 residues, and K19 S10A and S35A mutants failed to maintain cyclin D3 levels. Our results reveal a novel regulatory role on GSK3β localization and activity by K19 and provide a mechanism of how a cytoskeletal protein and a signaling molecule coordinate cell proliferation. Clinically, K19-dependent regulation of GSK3β activity may be used to reduce resistance to CDK4/6 inhibitors as KRT19 KO cells became sensitized to CDK4/6 inhibitors on coculturing with a GSK3β inhibitor. Given that K19 expression levels are frequently elevated in various cancers, it may be used to predict the efficacy of CDK4/6 inhibitors and resistant patients may be cotreated with a GSK3β inhibitor.

RESULTS

Cyclin D3 levels are down-regulated by GSK3β in KRT19 KO cells

Since KRT19 KO cells exhibit decreased cell proliferation and express decreased levels of cyclin D3 (Sharma et al., 2019a), colony formation assay was performed to assess the role of cyclin D3 in cell proliferation by K19. Unlike MCF7 parental cells where overexpression of cyclin D3 did not increase colony area, overexpression of cyclin D3 significantly increased colony area compared with vector transfection in KRT19 KO cells (Figure 1A; Supplemental Figure S1). To confirm the requirement of K19 in protein stability of cyclin D3, cyclin D3 levels were examined on inhibition of protein synthesis using cycloheximide. Cyclin D3 levels exhibited a greater decrease in cycloheximide-treated KRT19 KO cells compared with that of the parental control (Supplemental Figure S2) (Sharma et al., 2019a). Indeed, inhibiting proteasome function with MG132 markedly enhanced cyclin D3 protein levels in both of KRT19 KO clones tested (Figure 1B).

Since proteasomal degradation of cyclin D3 has been reported to be induced by its phosphorylation by GSK3β (Naderi, 2004) and p38SAPK2 (Casanovas et al., 2004), their roles in cyclin D3 degradation in KRT19 KO MCF7 cells were tested. Inhibiting p38 using two different p38 inhibitors, SB202190 or SB203580, had little to no significant effects in cyclin D3 levels when compared with vehicle controls (Supplemental Figure S3). However, inhibiting GSK3β with lithium chloride (LiCl) increased cyclin D3 levels in both parental and KRT19 KO cells (Figure 1C). This was confirmed with GSK3β siRNA, as cyclin D3 protein levels in KRT19 KO cells were significantly increased when normalized to levels of scrambled siRNA-treated cells (Figure 1D). In addition, GSK3β knockdown using two different siRNA increased proliferation of KRT19 KO cells, whereas slight but statistically significant reductions were observed in parental cells (Figure 1, E and F). Altogether, these data suggest that GSK3β is the culprit for reduced cyclin D3 levels and compromised proliferation rates of KRT19 KO cells.

FIGURE 1: GSK3β down-regulates cyclin D3 in KRT19 KO cells. (A) Colony formation assay was performed in parental (P) and KRT19 KO (KO) cells transiently transfected with vector control or cyclin D3. Colony area normalized to parental cells transfected with vector control is shown as mean ± SEM. N = 3. Bar, 5 mm. (B) Whole cell lysates of parental (P) and KRT19 KO (KO1 and KO2) cells treated with 10 nM MG132 for the indicated time periods were harvested, and immunoblotting was performed with antibodies against the indicated proteins. Signal intensities of cyclin D3 normalized to the α-tubulin loading control and 0 h controls are shown as mean ± SEM. N = 4. (C) Whole cell lysates of parental (P) and KRT19 KO (KO1 and KO2) cells treated with 10 mM LiCl (+) or DMSO vehicle control (−) for 8 h were harvested and immunoblotting was performed with antibodies against the indicated proteins. Signal intensities of cyclin D3 normalized to the α-tubulin loading control and DMSO controls are shown as mean ± SEM. N = 4. (D) Whole cell lysates of parental (P) and KRT19 KO (KO1 and KO2) cells transfected with GSK3β (3β) or scrambled (SCR) siRNA for 48 h were harvested, and immunoblotting was performed with antibodies against the indicated proteins. Signal intensities of cyclin D3 normalized to the α-tubulin loading control and SCR siRNA transfected controls are shown as mean ± SEM. N = 6. (E) Whole cell lysates of parental and KRT19 KO cells transfected with two different GSK3β (A and B) or scrambled (SCR) siRNA for 72 h were harvested, and immunoblotting was performed with antibodies against the indicated proteins. (F) MTT assays were performed on parental and KRT19 KO cells transfected with two different GSK3β (A and B) or scrambled (SCR) siRNA for 72 h. The absorbance at 570 nm of cells with GSK3β knockdown was normalized to that of its scrambled siRNA control to calculate cell viability. Cell viability normalized to SCR siRNA transfected controls are shown as mean ± SEM. N = 5. *P < 0.05, **P < 0.01, ***P < 0.001, and ns, not significant.
Activating GSK3β facilitates cyclin D3 degradation in KRT19 KO cells

To elucidate the role of GSK3β in K19-dependent stability of cyclin D3, forskolin was used. Forskolin is an adenyl cyclase activator, and forskolin-induced elevation of intracellular cAMP activates GSK3β by decreasing phosphorylation at Ser9, leading to degradation of cyclin D proteins and inhibition of cell proliferation in various settings (Musa et al., 1999; Naderi et al., 2000, 2004). Although 8 h treatment of 100 μM forskolin failed to alter cyclin D3 levels in parental cells, it resulted in significantly decreased levels of cyclin D3 in KRT19 KO cells (Figure 2A). The requirement of K19 in inhibiting forskolin-induced decrease of cyclin D3 levels was confirmed when...
expression of GFP-tagged K19 in KRT19 KO cells maintained cyclin D3 levels on forskolin induction (Figure 2B). Next, GSK3β activity was examined by monitoring phospho-GSK3β levels at Ser9 (pGSK3β). Following forskolin treatment, lower pGSK3β in KRT19 KO cells was observed compared with parental cells (Figure 2C), suggesting that K19 suppresses GSK3β activity to protect forskolin-induced decrease in cyclin D3 levels.

Next, the role of K19 in forskolin-induced cyclin D3 degradation was tested by examining cyclin D3 levels following forskolin treatment in the presence of the protein synthesis inhibitor cycloheximide. With inhibition of protein synthesis, forskolin treatment decreased cyclin D3 levels in KRT19 KO cells markedly compared with parental cells (Figure 2D). Also, when cells were pretreated with MG132, forskolin treatment failed to reduce cyclin D3 levels (Figure 2E). A forskolin-induced decrease in cyclin D3 levels was then confirmed to be mediated by GSK3β in KRT19 KO cells, as inhibiting GSK3β activity using LiCl (Figure 2F) or decreasing GSK3β levels with siRNA (Figure 2G) prevented a decrease in cyclin D3 levels on forskolin treatment in KRT19 KO cells (Figure 2, F and G).

**K19 interacts with active GSK3β**

To elucidate the mechanism underlying K19-dependent inhibition of GSK3β activity, K19–GSK3β interaction was assessed by co-immunoprecipitation (co-IP) (Figure 3A). Under normal growth conditions, immunoprecipitating K19 pulled down GSK3β and vice versa, demonstrating an interaction between two proteins. Then, forskolin and other small molecules were used to assess how GSK3β activity affects K19–GSK3β interaction. Activating GSK3β using forskolin or Akt inhibitor LY294002 resulted in GSK3β–K19 interaction, while treating cells with GSK3β inhibitor LiCl or phosphatase inhibitor okadaic acid failed to do so (Figure 3B). Consistent with this, constitutively active (S9A) but not wild type (WT) or kinase dead (KD) (K85A) GSK3β co-immunoprecipitated with K19 (Figure 3C), confirming the requirement for GSK3β activity in K19–GSK3β interaction.

To further investigate the interaction between K19–GSK3β and the role of GSK3β activity in K19–GSK3β interaction, lysis buffers with different detergents were used to identify relative amounts of GSK3β in triton-soluble fraction versus keratin filament-rich triton-insoluble fraction. Following forskolin stimulation, cell lysates were first prepared using triton-based lysis buffer. Then, triton-insoluble pellets prepared using high-speed centrifugations were isolated and dissolved in urea-based lysis buffer. Forskolin stimulation did not impact GSK3β levels in triton-soluble fractions of either parental or KRT19 KO cells but specifically increased the triton-insoluble pool of GSK3β in forskolin-treated parental cells (Figure 3D). Consistent with this, quantitation showed that there was an increased ratio of triton insoluble/soluble GSK3β only in parental cells on forskolin stimulation (Figure 3E).
K19 is required for the cytoplasmic localization of GSK3β

GSK3β is generally a cytoplasmic protein, but its localization is dynamic and involves continual shuttling between the nucleus and the cytoplasm (Bechard and Dalton, 2009). Active GSK3β becomes accumulated in the nucleus, where GSK3β phosphorylates its nuclear targets for degradation (Bechard et al., 2012). Since K19 interacts with GSK3β and inhibits GSK3β activity, the ability of K19 to regulate subcellular localization of GSK3β was tested. Immunostaining of GSK3β revealed enhanced nuclear GSK3β localization in KRT19 KO cells on forskolin induction, whereas forskolin-induced GSK3β localization in parental cells remained largely unchanged (Figure 4A). Biochemical subcellular fractionation of parental and KRT19 KO cells confirmed immunostaining results. Whereas GSK3β levels in cytoplasmic fractions remained unaffected by forskolin treatment in parental and KRT19 KO cells, a significant increase of nuclear GSK3β levels was observed specifically in KRT19 KO cells following forskolin induction (Figure 4, B and C).

GSK3β was reported to enhance the cytoplasmic localization of cyclin D protein (Diehl et al., 1998). Immunofluorescence staining of cyclin D3 and quantitating corrected total cell fluorescence (CTCF) showed that nuclear/cytoplasmic cyclin D3 levels were significantly reduced in KRT19 KO cells compared with those of parental cells (Figure 4, D and E). Biochemical subcellular fractionation confirmed the presence of increased nuclear/cytoplasmic cyclin D3 levels in parental cells compared with KRT19 KO cells (Figure 4, F and G). These findings suggest that K19 prevents active GSK3β from shuttling into the nucleus and mediating degradation of nuclear cyclin D3.

Identification of K19 domain required to interact with GSK3β

K19 protein contains an N-terminal head segment, a very short C-terminal tail segment, and a highly conserved alpha-helical central rod (R) domain (Sharma et al., 2019a). To identify the domain required for GSK3β interaction, plasmids encoding GFP-tagged WT or mutant with head-rod (HR), rod-tail (RT), head (H), or R domain of K19 were generated (Figure 5A) and transiently transfected into KRT19 KO cells. GFP-tagged proteins were then pulled down using anti-GFP–conjugated beads, and the extent of co-immunoprecipitation of nuclear cyclin D3 was measured (Figure 5B). While K19 H domain alone was not sufficient to interact with GSK3β, other domains that all included an R domain (WT, HR, RT, and R) successfully interacted with GSK3β (Figure 5B), suggesting that the R domain is required for the GSK3β–K19 interaction. Interestingly, while expression of WT K19 in KRT19 KO cells rescued defects in forskolin-induced enrichment of GSK3β in the filament-rich triton-insoluble pool, the RT mutant failed to do so (Figure 5, C and D). Testing for the effect of GSK3β activity, forskolin-induced pGSK3β levels were
FIGURE 5: Identification of K19 domains required for GSK3β interaction. (A) Schematics of K19 mutants. GFP was tagged at the N-terminus of K19 WT and mutants. HR contains GFP fused to H and R domains of K19; RT, GFP fused to R and tail domains; H, GFP fused to H domain alone; and R, GFP fused to R domain alone. (B) IP was performed with anti-GFP–conjugated beads in KRT19 KO cells transiently transfected with the GFP-K19 chimeras described in A or GFP control. IP and inputs were subjected to SDS–PAGE and immunoblotting was performed with antibodies against the indicated proteins. (C) KRT19 KO cells stably expressing GFP-K19 WT (WT), GFP-K19 RT (RT), or GFP control were treated with 100 µM forskolin (+) or DMSO vehicle control (−) for 10 min. Whole cell lysates were processed for triton solubility. Immunoblotting was performed with antibodies against the indicated proteins. (D) Signal intensities of GSK3β from (C) normalized to the actin loading control. Triton-insoluble/soluble GSK3β levels normalized to DMSO controls are shown as mean ± SEM. N = 4. (E) KRT19 KO cells stably expressing GFP-K19 WT (WT), GFP-K19 RT (RT), or GFP control were treated with 100 µM forskolin for the indicated time periods. Whole cell lysates were harvested, and immunoblotting was performed with the indicated antibodies. (F) Signal intensities of pGSK3β (Ser9) and GSK3β from E were quantitated and normalized to the α-tubulin loading control. Ratios of pGSK3β/GSK3β relative to 0′ controls are shown as mean ± SEM. N = 3. (G) IP was performed with anti-GFP–conjugated beads in KRT19 KO cells transiently transfected with the indicated GFP-K19 chimeras (WT or S10A, S35A, or Y4F mutants) or GFP control. IP and inputs were subjected to SDS–PAGE and immunoblotting was performed with antibodies against the indicated proteins. (H) Signal intensities of GSK3β/GFP IP from (G) normalized to that of WT are shown as mean ± SEM. N = 3. (I) KRT19 KO cells transiently transfected with GFP-K19 chimeras (WT or S10A, S35A, or Y4F mutants) or GFP control were treated with 100 µM forskolin for 8 h. Whole cell lysates were harvested, and immunoblotting was performed with the indicated antibodies. (J) Signal intensities of cyclin D3 from (I) normalized to the α-tubulin loading control and DMSO controls are shown as mean ± SEM. N = 5. *P < 0.05, **P < 0.01, ***P < 0.001, and ns, not significant.
Increased by transfecting WT K19 compared with GFP control in KRT19 KO cells as expected. However, the RT mutant failed to increase forskolin-induced pGSK3β levels compared with GFP-expressing cells (Figure 5, E and F).

Since the H domain of K19 was required to inhibit GSK3β activity (Figure 5, E and F), we further explored to locate essential amino acids. On the H domain, there are several phosphorylation sites including Ser35, which is a major phosphorylation site on K19 (Zhou et al., 1999). Keratin phosphorylation reorganizes keratin filaments and can affect their interactions with associated proteins (Eckert and Yeagle, 1990; Liao and Omary, 1996; Chung et al., 2015). Therefore, we tested K19-GSK3β interaction using K19 with Y4F, S10A, or S35A mutation on the H domain. Plasmids encoding GFP-tagged WT, Y4F, S10A, or S35A K19 were transfected into KRT19 KO cells. Immunoprecipitates of exogenous K19 were then assessed for the interaction using K19 with Y4F, S10A, or S35A K19 were transfected into KO cells stably expressing GFP-K19 WT (WT), GFP-K19 HR (HR), GFP-K19 RT (RT), or GFP control. Immunoblotting was performed with antibodies against the indicated proteins. PARP was used as a control for the nuclear fraction, whereas α-tubulin was used for the cytoplasmic fraction. (D) Signal intensities of cyclin D3 from C were quantitated. Nuclear/cytoplasmic cyclin D3 levels normalized to the GFP control are shown as mean ± SEM. N = 8. (E) Subcellular fractionation of KRT19 KO cells stably expressing GFP-K19 WT (WT), GFP-K19 RT (RT), or GFP control treated with 100 µM forskolin (+) or DMSO vehicle control (−). Immunoblotting was performed with antibodies against the indicated proteins. PARP was used as a control for the nuclear fraction, whereas α-tubulin was used for the cytoplasmic fraction. (F) Signal intensities of GSK3β from (E) normalized to the PARP loading control and DMSO controls are shown as mean ± SEM. N = 6. (G) KRT19 KO cells transiently transfected with GFP-K19 WT (WT), GFP-K19 S10A (S10A), GFP-K19 S35A (S35A), or GFP control were immunostained with anti-cyclin D3 and anti-GFP antibodies. Images were obtained using an epifluorescence microscope. Nuclei are shown with DAPI. Bar, 20 µm. (H) CTCF of cyclin D3 from G was quantitated and normalized to the background CTCF. Nuclear/cytoplasmic cyclin D3 levels normalized to the GFP control are shown as scatter box plots with median maxima and minima; n = 19 cells for each condition. *P < 0.05, **P < 0.01, ***P < 0.001, and ns, not significant.

**K19 domains regulating subcellular localization of cyclin D3 and GSK3β**

Since K19 H domain was required for forskolin-induced phosphorylation of GSK3β Ser9 (Figure 5, E and F), the role of K19 H domain on cyclin D3 localization was examined. For this, immunostaining of cyclin D3 was done in KRT19 KO cells stably expressing GFP control, WT, HR, or RT K19. Increased localization of nuclear cyclin D3 was observed in WT- or HR-expressing cells compared with GFP control- or RT-expressing cells (Figure 6A). Quantitating CTCF of nuclear and cytoplasmic cyclin D3 in cells revealed higher nuclear/cytoplasmic ratios of cyclin D3 in WT- or HR-expressing cells as compared with GFP control- or RT-expressing cells (Figure 6B). Biochemical subcellular fractionation also showed higher nuclear/cytoplasmic cyclin D3 levels in cells expressing WT K19 compared with those expressing
GFP control or RT mutant (Figure 6, C and D). Consistent with increased nuclear cyclin D3 levels, forskolin-induced nuclear accumulation of GSK3β in KRT19 KO cells was absent in cells expressing WT K19 (Figure 6, E and F). In contrast, nuclear GSK3β levels in RT K19-expressing cells mirrored those of GFP-expressing cells, suggesting that the H domain of K19 is required to prevent the nuclear localization of GSK3β. Last, immunostaining of cyclin D3 was performed in cells expressing GSK3β in KRT19 KO cells with WT, S10A, or S35A K19, and CTCF/cyclin D3 were quantitated to assess how mutations affect nuclear/cytoplasmic ratios of cyclin D3. Compared with GSK3β-expressing cells, nuclear/cytoplasmic cyclin D3 levels were significantly increased in cells expressing WT K19 and to a lesser extent for those expressing S35A mutant, but not for cells with S10A mutant (Figure 6, G and H).

**Impact of K19–GSK3β interaction on cell proliferation**

Next, we asked whether inhibition of GSK3β activity by K19 contributes to cell proliferation. To this end, KRT19 KO cells stably expressing various K19 mutants were tested for cell proliferation. Overexpression of WT or HR K19 increased proliferation of KRT19 KO cells as assessed by counting cell numbers (Figure 7A), performing MTT assays (Figure 7B), and measuring cell confluence (Figure 7C) every 24 h following cell passaging. However, the RT mutant lacking the H domain that was required to inhibit GSK3β activity failed to induce increased cell proliferation. This result was confirmed by performing colony formation assays (Figure 7, D and E). Overexpression of WT or HR K19 showed increased colony area compared with GFP control, but RT mutant resulted in a significant decrease of colony area. To examine the effect of K19–GSK3β interaction more specifically, effects of mutations on Ser10 and Ser35 of K19 on cell proliferation were tested. While overexpression of WT K19 in KRT19 KO cells resulted in increased colony area similar to Figure 7D, overexpressing S10A or S35A K19 was unable to induce an increase in colony area (Figure 7, F and G). In fact, cells overexpressing S10A K19 showed a significantly reduced area of colony.

**Inhibition of GSK3β increased sensitivity of KRT19 KO cells to CDK4/6 inhibitors**

We previously found that KRT19 KO cells showed increased resistance to CDK4/6 inhibitors ribociclib and palbociclib (Sharma et al., 2019a). Since K19 inhibited GSK3β to stabilize cyclin D3, we examined the role of GSK3β in mediating resistance to ribociclib and palbociclib in KRT19 KO cells. Cells were cultured for 3 d in the presence of each drug alone or in combination with GSK3β inhibitor CHIR 99021, and colony formation assay was performed to assess cell viability. The colony area of cells treated with drugs was normalized against that from cells treated with a vehicle control to calculate cell viability. As previously identified, KRT19 KO cells were more resistant toward ribociclib and palbociclib compared with the parental control (Figure 8, A and B). However, cotreatment with CHIR 99021 increased the sensitivity of KRT19 KO cells toward CDK4/6 inhibitors, although cotreatment of CHIR 99021 with CDK4/6 inhibitors had no significant effects on parental cells compared with CDK4/6 inhibitors alone. Re-expressing GSK3β-tagged K19 in KRT19 KO cells resensitized KRT19 KO cells to CDK4/6 inhibitors (Figure 8, C and D) but abrogated the effect of CHIR 99021 on how cells respond to CDK4/6 inhibitors, mimicking phenotypes of parental MCF7 cells.

**DISCUSSION**

Overexpression of cyclin D proteins drives cancer cell proliferation (Biliran et al., 2005; Zhang et al., 2011), and high cyclin D levels are detected in approximately 50% of breast cancers (Barnes and Gillett, 1998; Arnold and Papanikolaou, 2005; Chi et al., 2015).
However, the fact that only 15–20% of breast cancers have amplification of cyclin D genes (Bartkova et al., 1994; Gillett et al., 1994; Dickson et al., 1995; Chi et al., 2015) suggests that cyclin D protein stability plays a key role in breast cancer cases. Previous evidence showed that GSK3β phosphorylation of cyclin D3 led to cyclin D3 degradation (Naderi et al., 2004) and expression of kinase inactive GSK3β increased cyclin D levels (Farago et al., 2005), establishing GSK3β as a negative regulator of cyclin D3. However, how GSKβ is regulated to phosphorylate cyclin D3 remained unclear.

We had previously shown that K19 is required for proliferation of MCF7 breast cancer cells (Sharma et al., 2019a). This study indicates that maintenance of cyclin D3 levels underlies promotion of cell proliferation by K19, and K19–GSK3β interaction plays a key role in the process (Figure 8E). In settings where K19–GSK3β interaction was absent, GSK3β became more active, as assessed by Ser9 phosphorylation and cyclin D3 levels. Interestingly, instead of showing a preference for inactive GSK3β, K19 interacted better with active GSK3β. Therefore, K19 is likely to interact with activated GSK3β to suppress GSK3β activity rather than interacting with inactive GSK3β to keep it in an inactive state. However, it is still unclear how K19–GSK3β interaction impacts GSK3β activity at the molecular level. Since K19 was required to maintain phosphorylation at Ser9 when GSK3β activity was stimulated by forskolin, physical association with K19 may expose Ser9 residue of GSK3β for phosphorylation or protect phosphorylated Ser9 from phosphatases.

Instead of inhibiting GSK3β directly, K19 may be regulating GSK3β upstream regulators such as Akt to inhibit GSK3β activity. It has been reported that K19 maintains the stability of HER2, which regulates Akt (Ju et al., 2015). However, the expression of HER2 is absent in MCF7 cells used in this study (Subik et al., 2010). Nevertheless, other upstream regulators of GSK3β may be regulated by K19 to inhibit GSK3β activity. Because of the increasing number of keratin-interacting proteins reported so far (Sharma et al., 2019a), it is likely that K19 not only binds to GSK3β but also forms larger complexes with additional proteins, allowing multiple signaling proteins to communicate with one another.

Once activated by forskolin, GSK3β levels were increased in a K19 filament-rich pool of the cell. Keratin filaments have been proposed to serve as cytoplasmic scaffolds for various proteins. In particular, keratin filaments are enriched in the perinuclear region, regulating nucleocytoplasmic shuttling of proteins including 14-3-3 (Liao and Omary, 1996), hnRNP K (Chung et al., 2015), β-catenin/RAC1 (Saha et al., 2016), and Egr1 (Ju et al., 2013). Indeed, an increase in nuclear GSK3β levels on forskolin treatment in KRT19 KO cells suggests that physical association with K19 filaments prevents Akt and other upstream regulators of GSK3β activity.
seems that the H domain harbors the ability to regulate the interaction and subsequent downstream events.

Our data point to an interesting possibility that Ser10 and Ser35 residues on the H domain of K19 may be regulated differently. While both residues were required for K19 to bind to GSK3β and maintain total cyclin D3 levels and cell proliferation, S35A mutation had milder effects compared with S10A mutation in general. At this point, the cause for the difference is unclear. Although the kinase responsible for phosphorylating Ser10 is unknown, Ser35 has been shown to be phosphorylated by Akt (Ju et al., 2015). However, Akt inhibitor LY294002 increased K19–GSK3β interaction. Therefore, it may be that K19 Ser35 regulates K19–GSK3β interaction either in a mechanism independent of its phosphorylation or through phosphorylation by a kinase other than Akt. Also, S35A mutation resulted in altered filament dynamics of K19 unlike S10A mutation, but the significance of altered filament dynamics to cell proliferation is unclear (Zhou et al., 1999).

There is a possibility that K19 Ser10 is phosphorylated by GSK3β. On the N-terminus of K19, MTSSYRQSSSATSS at Ser10 matches the consensus GSK3β substrate sequence Ser/Thr-X-X-pSer/Thr where pSer/Thr is called a priming site that is Ser/Thr prephosphorylated by another kinase 4 or 5 amino acids C-terminal to the GSK3β target site (Sutherland, 2011). Also, GSK3β interaction with its substrate typically occurs when the N-terminal domain of GSK3β recognizes the phosphorylated priming site (Beurel et al., 2015). Therefore, Ser14 may serve as a priming site to induce phosphorylation of Ser10 by GSK3β and thus could be involved in K19–GSK3β interaction as well. In addition, K19 Ser10 itself is 5 amino acids C-terminal to another serine, Ser5, suggesting that Ser10 could be a priming site for Ser5. This would explain why the K19 S10A mutant failed to interact with GSK3β. Related to this, GSK3β was shown to phosphorylate the H domain of another intermediate filament protein desmin in hearts (Agnetti et al., 2014) and muscles (Aweida et al., 2018). Also, keratin filament disassembly was regulated by a GSK3β priming kinase casein kinase-1 in colon cancer cells (Kuga et al., 2013). These previous reports further support the idea that K19 may be phosphorylated by GSK3β.

Our previous study showed that decreased levels of cyclin D3 in KRT19 KO cells correlated with resistance to CDK4/6 inhibitors (Sharma et al., 2019a). In this present study, GSK3β was found to be responsible for facilitating cyclin D3 degradation in the absence of K19, and the treatment of GSK3β inhibitor increased the sensitivity of KRT19 KO cells but not parental cells to CDK4/6 inhibitors. These results suggest the high cyclin D3 levels might be responsible for making cells more sensitive to the CDK4/6 inhibitors. Indeed, cells with “D-Cyclin Activating Features (DCAF)” including elements regulating cyclin D3 expression were found to be sensitive to CDK4/6 inhibition (Gong et al., 2017). Our study shows that GSK3β inhibition can be used to sensitize tumors resistant to CDK4/6 inhibitors to improve the therapeutic efficacy of CDK4/6 inhibitors. In summary, the novel mechanism presented here offers an opportunity to improve therapeutic strategy to combat cancer based on K19 expression.

**MATERIAL AND METHODS**

Request a protocol through Bio-protocol.

**Plasmids, siRNA**

Plasmids Tag5Amyc-GSK3β WT (GSK3β with C-terminal myc tag in the CMV promoter-containing pCMV-Tag 5A vector backbone, plasmid #16260), Tag5Amyc-GSK3β 5A (constitutively active GSK3β), plasmid #16261), and Tag5Amyc-GSK3β K85A (KD GSK3β, plasmid #16262) were from Addgene (Watertown, MA). Cyclin D3 cDNA was cloned out of Rc/CMV cyclin D3 (CMV promoter; #10912 from Addgene) with PCR using the primers listed in Table 1 and cloned into pClone (Novagen; #17484. Addgene) with plasmid pMRB10-K19 WT, pMRB10-C3 K19 S10A, and pEGFP-C3 K19 S35A were generated using plasmids pMRB10-K19 WT, pMRB10-K19 S10A, and pMRB10-K19 S35A (courtesy of Bishr Omayry [Zhou et al., 1999]) along with the primers listed in Table 1 and the In-Fusion HD cloning system (Takara Bio, Mountain View, CA) following the manufacturer’s instructions. For pEGFP-C3 K19 Y4F, the forward primer with a mutation shown in Table 1 was used to generate KRT19 Y4F cDNA from pMRB10-K19 WT. pEGFP-C3 K19 mutants (HR, RT, H, and R) were generated from pEGFP-C3 K19 WT using the primers listed in Table 1 with the In-Fusion HD cloning system (Takara Bio). GFP and GFP-tagged K19 WT and mutant constructs were cloned from pEGFP-C3 K19 constructs into pLenti CMV Hygro (plasmid #17484. Addgene) using the primers listed in Table 1 with the In-Fusion HD cloning system (Takara Bio). To silence GSK3β expression, siRNA oligo duplex of Locus ID 2932 (SR301799) and scrambled negative control siRNA Duplex siRNA (SR30004) were purchased from Origene (Rockville, MD).

**Antibodies and other reagents**

The following antibodies K19 (A53-B/A2), GSK3β (E-11), pGSK3β (F-2), PARP-1 (F-2), β-actin (C4), and GAPDH (0411) were from Santa Cruz Biotechnology (Santa Cruz, CA); cyclin D (DCS22) and GSK3β (D5CS2) were from Cell Signaling Technology (Danvers, MA); GFP (3H9) and RFP (5F8) were from Chromotek (Islandia, NY); cyclin D (26755-1-AP) was from Proteintech (Rosemont, IL); and anti-tubulin (12G1), anti-GFP (1D2), and c-Myc (9E10) were from Developmental Studies Hybridoma Bank (Iowa City, IA). Forskolin was from ApexBio (#B1421) (Houston, TX), LiCl (# EM-LX0331) and DMSO were from Developmental Studies Hybridoma Bank (Iowa City, IA). A53 cells were grown in DMEM (VWR Life Sciences) supplemented with 100 μg/ml hygromycin (#H-270-5; Bio-protocol). For transfections, cells were seeded in 96-well plates at 50% confluence and incubated with transfection reagent (Bio-protocol) for 24 h. After transfection, cells were treated with 1 μg/ml puromycin for 2 days. For serum-starved cells, cells (ATCC, Manassas VA) were grown in DMEM (VWR Life Sciences) with 1% FBS for 24 h. For forskolin treatment, cells were serum-starved for 48 h and then treated with either 1 μM forskolin. For ribociclib, palbociclib, or CHIR 99021 treatment, cells were treated for 72 h with indicated concentrations of drugs. For pretreatment of inhibitors (LiCl, LY294002, SB 203580, SB 202190, okadaic acid, cycloheximide, or MG132), cells were incubated with inhibitor or vehicle control (DMSO) for indicated time periods. For forskolin treatment, cells were treated with 10 μM forskolin for 30 min or 8 h.
Cells stably expressing K19 mutants
Lentiviral supernatants were generated using the pLenti plasmids as described previously (Sharma et al., 2019a). Lentiviral supernatants, collected 48 hours after transfection, were used to infect subconfluent MCF7 KRT19 KO cells in three sequential 4-hour incubations in the presence of 4 μg/ml polybrene (Sigma-Aldrich). Transductants were selected in hygromycin (100 μg/ml), beginning 60 hours after infection.

Transfection
Overexpression plasmids were transiently transfected using Continuous transfection reagent (Gemini Bio-Products, West Sacramento, CA) according to the manufacturer’s protocol. siRNAs were transiently transfected using Lipofectamine RNAiMAX transfection reagent (Life Technologies) using the manufacturer’s protocol.

Biochemical subcellular fractionation
Subcellular fractionation was performed as described previously (Chung et al., 2015). Cells were washed in 1x phosphate-buffered saline (PBS) and lysed at 4°C in ice-cold cytoplasmic extract buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 1.5 mM MgCl₂; 0.1 mM EDTA; 0.5 mM dithiothreitol; 0.4% NP-40; 1 mM phenylmethylsulfonyl fluoride (PMSF); 10 mM sodium pyrophosphate; 1 μg/ml each of chymostatin, leupeptin, and pepstatin; 10 μg/ml each of aprotinin and benzamidine; 2 μg/ml antipain; 1 mM sodium orthovanadate; and 50 mM sodium fluoride). Supernatants were collected as nuclear fractions and 4°C, and supernatants were collected as cytosolic fractions.

MTT assay
MTT assay was performed as described previously (Sharma et al., 2019a); 1000 cells were plated into each well of 96 well plate and grown in 37°C with 5% of CO₂ and 95% of relative humidity condition. On the day of the experiment, cells were incubated with 0.5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Alfa Aesar, Haverhill, MA) containing media for 3.5 hours, and formed formazan crystals were dissolved with 150 μl of isopropyl alcohol at 4 mM HCl, 0.1% NP40. The absorbance of plate was then measured at 570 nm on a SpectraMax microplate reader (Molecular Devices, San Jose, CA) and the data results were processed on a SoftMax Pro software (Molecular Devices). Each experiment was performed at least in triplicate.

Colony formation assay
Colony formation assay was performed as described previously (Franken et al., 2006); 10,000 cells per well were plated in a 6-well plate. Cells were then grown for 72 hours and at the end of the experiments, cells were fixed with 3.7% formaldehyde and stained using 0.1% crystal violet for 30 minutes. The colony area was calculated using ImageJ (National Institutes of Health [NIH]). To determine the effect of ribociclib, palbociclib, and CHIR 99021 on cell viability, cells were treated with DMSO vehicle control, 1 μM ribociclib, 700 nM palbociclib, or with no 5 mM CHIR 99021, 24 hours after plating. Cells

| Plasmid | Oligonucleotide primer sequence |
|---------|--------------------------------|
| pmCherry-C1 | Forward CCGACTCAGATGCTCAGAGGCTAGTGTGTTT |
| Cyclin D3 | Forward TATATGGTTGATCTCGAGAGAGGAGGAGGAGG |
| pEGFP-C3 K19 WT | Forward TAGATCTCGAGAGGCTAGTGTGTTT |
| pEGFP-C3 HR | Forward TATATGGTTGATCTCGAGAGAGGAGGAGGAGG |
| pEGFP-C3 RT | Forward TAGATCTCGAGAGGCTAGTGTGTTT |
| pEGFP-C3 H | Forward TATATGGTTGATCTCGAGAGAGGAGGAGGAGG |
| pEGFP-C3 R | Forward TAGATCTCGAGAGGCTAGTGTGTTT |
| pEGFP-C3 K19-Y4F | Forward TATATGGTTGATCTCGAGAGAGGAGGAGGAGG |
| pLenti CMV/TO hygro GFP-K19 WT | Forward GAAAGCTGGGTCTAGTCAGTAGTGATCTTCCTGTCCCTCG |
| pLenti CMV/TO hygro GFP-HR | Forward TAGATCTCGAGAGGCTAGTGTGTTT |
| pLenti CMV/TO hygro GFP-RT | Forward TATATGGTTGATCTCGAGAGAGGAGGAGGAGG |
| pLenti CMV/TO hygro GFP-H | Forward TAGATCTCGAGAGGCTAGTGTGTTT |
| pLenti CMV/TO hygro GFP-R | Forward TATATGGTTGATCTCGAGAGAGGAGGAGGAGG |

TABLE 1: List of primers used to construct plasmids described in experimental procedures.
were then grown for 96 h, and colony formation assay was performed. The colony area from drug-treated cells was normalized to that from DMSO-treated cells to calculate cell viability.

**Cell counting for proliferation**

Cell counting was performed as described previously (Sharma et al., 2019a). To measure cell proliferation, 50,000 cells were initially plated on each well of 6-well plates. Trypsinized cells were counted using a hemacytometer after every 24 h following cell passaging.

**Preparation of cell lysates, protein gel electrophoresis, and immunoblotting**

Cell lysates were prepared as described previously (Chung et al., 2012; Sharma et al., 2019a). Cells grown on tissue culture plates were washed with 1x PBS and lysed in cold triton lysis buffer solution (1% Triton X-100, 40 mM HEPES (pH 7.5), 120 mM sodium chloride, 1 mM EDTA, 1 mM phenyl methylsulfonyl fluoride, 10 mM sodium pyrophosphate, 1 μg/mL each of cymostatin, leupeptin and pepstatin, 10 μg/mL each of aprotonin and benzamidine, 2 μg/mL antipain, 1 mM sodium orthovanadate, 50 mM sodium fluoride). To isolate the filament-rich pool of proteins, triton-soluble versus insoluble proteins were analyzed as described previously (Blikstad and Lazarides, 1983; Chou et al., 1993; Chung et al., 2012; Wang et al., 2016). The insoluble material following triton lysis buffer incubation was pelleted, washed thrice with triton lysis buffer, and dissolved in urea lysis buffer (6.5 M urea; 50 mM Tris (pH 7.5); 1 M ethylene glycol tetraacetic acid; 2 mM dithiothreitol; 1 mM PMSF; 1 μg/mL each of cymostatin, leupeptin, and pepstatin; 10 μg/mL each of aprotonin and benzamidine; 2 μg/mL antipain; 50 mM sodium fluoride). For immunoblotting, cell lysates were centrifuged to remove cell debris. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin (BSA) as standard. Aliquots of cell lysate were then incubated with the indicated antibody or IgG control, and immune complexes were captured using either Protein G Sepharose (GE Healthcare) or GFP-Trap beads from Chromotek (Islandia, NY).

**Immunofluorescence staining**

Immunofluorescence staining was performed as described previously (Lam et al., 2020). For immunostaining of cells in culture, cells grown on glass coverslips (WWR Life Science) were washed in PBS, fixed in 4% paraformaldehyde in PBS, and permeabilized in 0.1% Triton X-100. Samples were blocked in 5% normal goat serum (NGS) in 1x PBS overnight before staining with primary antibodies diluted in blocking buffer for 1 h followed by Alexa Fluor 488- or Alexa Fluor 594-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA) for 1 h; 1 μg/mL Hoechst 33342 was used to stain nuclei, and coverslips were mounted on microscope slides with mounting media containing 1,4-diazabicyclo[2.2.2]octane (Electron Microscopy Sciences, Hatfield, PA). Labeled cells were visualized by epifluorescence with an Olympus BX60 fluorescence microscope (OPELCO, Dulles, VA) using an Olympus Fluo 60× NA 1.3, phase 1, oil immersion objective (Olympus). Images were taken with an HQ2 CoolSnap digital camera (Roper Scientific, Germany) and Metamorph Imaging software (Molecular Devices, Sunny Vale, CA). ImageJ software version 1.51J (NIH, Bethesda, MD) was used to process images. For confocal images, immunostained cells were observed and their images were captured under Zeiss LSM 710 confocal microscopy (Carl Zeiss). The captured images were processed using Zen Blue software (Carl Zeiss). CTCF was calculated using the ImageJ software, according to previously described protocols to control for local background fluorescence and cell size (82). The following formula was used: CTCF = integrated density – (area of selected cell × mean fluorescence of background readings). Calculated cytoplasmic and nuclear CTCF scores indicate fluorescence relative to their local backgrounds.

**Graphs and statistics**

All graphs in the manuscript are shown as mean ± SEM. For comparisons between the two data sets, Student’s t test (tails = 2, type = 1) was used, and statistically significant p values are indicated in figures and figure legends with asterisks (*P < 0.05, **P < 0.01, ***P < 0.001).

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