



Mechanisms of sensitivity and resistance to CDK4/CDK6 inhibitors in hormone receptor-positive breast cancer treatment

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ABSTRACT

Cell cycle dysregulation is a hallmark of cancer that promotes excessive cell division. Cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) are key molecules in the G1-to-S phase cell cycle transition and are crucial for the onset, survival, and progression of breast cancer (BC). Small-molecule CDK4/CDK6 inhibitors

Abbreviations: ADCs, Antibody-drug conjugates; AI, Aromatase inhibitor/s; AP-1, Activator protein-1; AR, Androgen receptor; BC, Breast cancer; CAK, CDK-activating kinase; CBR, Clinical benefit rate; CDKs, Cyclin-dependent kinases; CDK1, Cyclin-dependent kinases 1; CDK2, Cyclin-dependent kinases 2; CDK2i, CDK2 inhibitor/s; CDK4, Cyclin-dependent kinases 4; CDK4/6i, CDK4/CDK6 inhibitor/s; CDK6, Cyclin-dependent kinases 6; CDK7, Cyclin-dependent kinases 7; CDK9, Cyclin-dependent kinases 9; CERAN, Complete ER antagonist; C-MET, Mesenchymal-epithelial transition factor; CtDNA, Circulating tumor DNA; DCR, Disease control rate; DNMT1, DNA methyltransferase 1; DOR, Duration of response; EGFR, Epidermal growth factor receptor; ER, Estrogen receptor; ER⁺, ER-positive; ESR1, Estrogen receptor 1; ET, Endocrine therapy; FAK, Focal adhesion kinase; FDA, Food and Drug Administration; FGFR, Fibroblast growth factor receptor; FOXM1, Forkhead box M1; GAP, GTPase-activating protein; HER, Human epidermal growth factor receptor; HER2⁻, HER2-negative; HER2⁺, HER2-positive; HR⁺, Hormone receptor-positive; IDFS, Invasive disease-free survival; INF, Interferon; MHC, Major histocompatibility complex; MTD, Maximum tolerated dose; MTORC1, mTOR complex 1; MTORC2, mTOR complex 2; NFAT, Nuclear factor of activated T-cells; NF1, Neurofibromin 1; ORR, Overall response rate; OS, Overall survival; PARP, Poly ADP-ribose polymerase; PD-L1, Programmed death-ligand 1; PFS, Progression-free survival; PR, Partial response; PROTAC, Proteolysis targeting chimera; RP2D, Recommended phase 2 dose; RTK, Receptor tyrosine kinase; SARM, Selective androgen receptor modulator; SASP, Senescence-associated secretory phenotype; SD, Stable disease; SERCA, Selective ER covalent antagonists; SERD, Selective estrogen receptor degrader; SERM, Selective ER modulator; ShERPA, Selective human ER partial agonists; SIN1, Stress-activated protein kinase-interacting protein; Tregs, Regulatory T-cells; Trop-2, Trophoblast cell-surface antigen 2; TSC, Tuberous sclerosis complex; TSC1, Tuberous sclerosis complex 1; TSC2, Tuberous sclerosis complex 2.

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CDK4/CDK6 inhibitors (CDK4/6i)
CDK4/6i resistance
hormone receptor-positive (HR⁺) breast cancer
endocrine therapy (ET)

(CDK4/6i) block phosphorylation of tumor suppressor Rb and thus restrain susceptible BC cells in G1 phase. Three CDK4/6i are approved for the first-line treatment of patients with advanced/metastatic hormone receptor-positive (HR⁺)/human epidermal growth factor receptor 2-negative (HER2⁻) BC in combination with endocrine therapy (ET). Though this has improved the clinical outcomes for survival of BC patients, there is no established standard next-line treatment to tackle drug resistance. Recent studies suggest that CDK4/6i can modulate other distinct effects in both BC and breast stromal compartments, which may provide new insights into aspects of their clinical activity. This review describes the biochemistry of the CDK4/6-Rb-E2F pathway in HR⁺ BC, then discusses how CDK4/6i can trigger other effects in BC/breast stromal compartments, and finally outlines the mechanisms of CDK4/6i resistance that have emerged in recent preclinical studies and clinical cohorts, emphasizing the impact of these findings on novel therapeutic opportunities in BC.

1. Introduction

The mammalian cell cycle is precisely controlled to ensure correct genome duplication and its dysregulation often contributes to oncogenesis (Matthews et al., 2022). In recent years, specific small-molecule cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6) inhibitors (CDK4/6i) have revolutionized the standard of care for advanced hormone receptor-positive (HR⁺)/HER2-negative (HER2⁻) breast cancer (BC) patients (Grinshpun et al., 2023) (Stanciu et al., 2023) (Sciocchi et al., 2022). Even though endocrine therapy (ET) has been the main systemic treatment for estrogen-receptor positive (ER⁺) and HER2⁻ BC (Corti et al., 2023), patients still often display innate and acquired drug resistance (Hartkopf et al., 2020) (Rasha et al., 2021). Importantly, addition of CDK4/6i to ET effectively suppresses cell proliferation, reduces tumor progression, and improves patient outcomes (Mittal et al., 2023). Thus, co-treatment of CDK4/6i and ET is considered the current standard of care for first-line therapy in recurrent

unresectable or metastatic advanced ER⁺/HER2⁻ BC patients (Zhou et al., 2023). Accordingly, the small-molecule CDK4/6i palbociclib, ribociclib, and abemaciclib have been Food and Drug Administration (FDA)-approved in combination with an aromatase inhibitor (AI) for HR⁺/HER2⁻ BC (Spring et al., 2020). In addition, combined treatment with fulvestrant, a selective estrogen receptor degrader (SERD), has exhibited positive results for HR⁺/HER2⁻ BC patients after progression on adjuvant AI (Gradishar et al., 2022). Abemaciclib has also been uniquely FDA-approved as monotherapy in refractory advanced HR⁺/HER2⁻ BC (Dickler et al., 2017) (Hamilton et al., 2022). Palbociclib, ribociclib, and abemaciclib (Chen et al., 2016) (Hafner et al., 2019) potently suppress CDK4/6 enzymatic activity at nanomolar levels, but display higher selectivity for CDK4 compared to CDK6 (Fry et al., 2004) (Gelbert et al., 2014) (Klein et al., 2018) (Hafner et al., 2019) (Table 1). Abemaciclib exerts broader CDK suppression and also inhibits cyclin-dependent kinase 1 (CDK1), cyclin-dependent kinase 2 (CDK2) and cyclin-dependent kinase 9 (CDK9) *in vivo* (George et al., 2021).

Table 1

Small-molecule CDK4/6i FDA-approved or currently under clinical development used for advanced ER⁺/HER2⁻ BC patients. BC: breast cancer; FDA: food and drug administration; IC50: half-maximal inhibitory concentration; Ki: inhibitor constant; N/A: not available; nM: nanomolar; R: reference; (*): not applicable.

CDK4/6 inhibitors for HR ⁺ /HER2 ⁻ breast cancer								
Agent	Status	CDK Selectivity (IC50) [Ki]				Clinical development		Company
Palbociclib	Approved	*	*	CDK4 (11 nM)	CDK6 (16 nM)	*	FDA-approved for HR ⁺ /HER2 ⁻ advanced BC in combination with hormonal therapy	Pfizer
Ribociclib	Approved	*	*	CDK4 (10 nM)	CDK6 (39 nM)	*	FDA-approved for HR ⁺ /HER2 ⁻ advanced BC in combination with hormonal therapy	Novartis
Abemaciclib	Approved	CDK1 (N/A)	CDK2 (N/A)	CDK4 (2 nM)	CDK6 (10 nM)	CDK9 (N/A)	FDA-approved for HR ⁺ /HER2 ⁻ advanced BC as monotherapy. FDA-approved for HR ⁺ /HER2 ⁻ advanced BC in combination with hormonal therapy. FDA-approved for HR ⁺ /HER2 ⁻ high-risk, early-stage BC in combination with hormonal therapy	Eli Lilly
Dalpiciclib	Phase 3	*	*	CDK4 (12 nM)	CDK6 (10 nM)	*	Phase 3 clinical trial in combination with hormonal therapy in HR ⁺ /HER2 ⁻ advanced BC (NCT03966898)	Jiangsu Hengrui Medicine
Birociclib	Phase 3	*	*	N/A	N/A	*	Phase 3 clinical trial in combination with fulvestrant in HR ⁺ /HER2 ⁻ advanced BC (NCT05077449)	Xuanzhu Biopharmaceutical
FCN-437 C	Phase 3	*	*	N/A	N/A	*	Phase 3 clinical trial in combination with fluvestrant ± goseraline in HR ⁺ /HER2 ⁻ advanced BC (NCT05438810)	Ahon Pharmaceutical
TQB3616	Phase 3	*	*	N/A	N/A	*	Phase 3 clinical trial in combination with ET (letrozole or anastrozole or tamoxifen) in HR ⁺ /HER2 ⁻ advanced BC (NCT05780567). Phase 3 clinical trial in combination with fulvestrant in HR ⁺ /HER2 ⁻ advanced BC (NCT05375461)	Chia Tai Tianqing Pharmaceutical
PF-06873600	Phase 2	*	CDK2 (0.09 nM) [Ki]	CDK4 (0.13 nM) [Ki]	CDK6 (0.16 nM) [Ki]	*	Phase 2 clinical trial as monotherapy or in combination with hormonal therapy in HR ⁺ /HER2 ⁻ metastatic BC (NCT03519178)	Pfizer
Lerociclib	Phase 2	*	*	CDK4 (1 nM)	CDK6 (2 nM)	CDK9 (28 nM)	Phase 2 clinical trial in combination with fulvestrant in HR ⁺ /HER2 ⁻ advanced or metastatic BC (NCT05085002)	EQRx International
BPI-1178	Phase 2	*	*	N/A	N/A	*	Phase 2 clinical trial in combination with standard ET (fulvestrant or letrozole) in HR ⁺ /HER2 ⁻ advanced BC (NCT04282031)	Beta Pharma
TY-302	Phase 1	*	*	N/A	N/A	*	Phase 1 clinical trial as monotherapy or in combination with tamoxifen in HR ⁺ /HER2 ⁻ advanced BC (NCT04433494)	TYK Medicines

Apart from blocking cancer cells in the G1 phase of the cell cycle, which is common to all three CDK4/6i, abemaciclib can also induce G2 phase arrest, in particular at concentrations of 0.3 μ M and above, perhaps determined by the suppression of CDK1 and CDK2, whose activities are necessary for cell cycle progression through S-phase and mitosis (Hafner et al., 2019). Additionally, abemaciclib long-term treatment also causes increased apoptosis in Rb-proficient BC cells compared to palbociclib or ribociclib (Torres-Guzmán et al., 2017). Generally, the safety profile of these three drugs is favorable (Cejuela et al., 2023) (Becherini et al., 2023). Though there is a high incidence of myelosuppression, particularly neutropenia, there is a low rate of febrile neutropenia (Braul et al., 2021). Abemaciclib provokes more gastrointestinal-related toxicity compared to palbociclib and ribociclib (Kwapisz, 2017) (Braul et al., 2021) possibly due to CDK9 inhibition (Braul et al., 2021). Conversely, abemaciclib is less myelosuppressive. Notably, palbociclib and

ribociclib regimens require a 1-week break at the end of each 4-week treatment cycle to permit recovery of bone marrow; whereas abemaciclib can be administered continuously (Klein et al., 2018) (Hafner et al., 2019) (Zhou et al., 2023). Other newer CDK4/6i are under development, including leroiciclib, which has shown signals of efficacy and favorable safety profile in a phase 1/phase 2 dose escalation/expansion clinical trial (NCT02983071) in combination with fulvestrant (Bulat et al., 2020). Furthermore, dalpiciclib has shown a positive impact on progression free survival (PFS) as a second-line or third-line setting via the DAWNA-1 phase 3 clinical trial (NCT03927456) (Xu et al., 2021), and in combination with either AI letrozole or anastrozole, as a first-line setting, in the DAWNA-2 phase 3 clinical trial (NCT03966898) (Zhang et al., 2023). Patients with metastatic BC eventually progress on CDK4/6i to intrinsic/acquired resistance (O’Leary et al., 2018), for which there is no standard next-line treatment. Thus, improving our

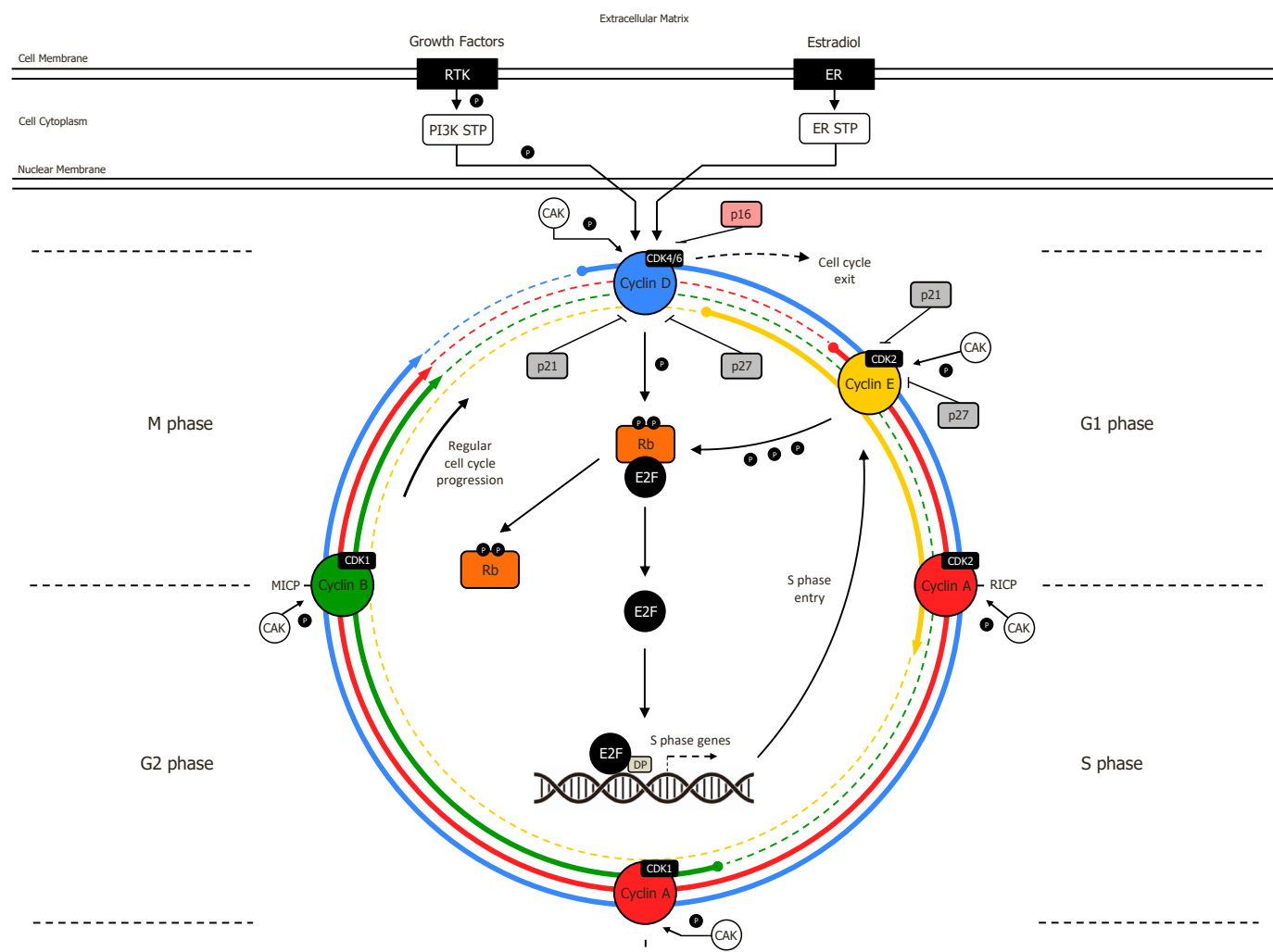


Fig. 1. Regular cyclin D-CDK4/6 activity mediates molecular events governing cell-cycle progression through G1 phase in normal breast cells. In breast cells, cell cycle outset is driven by proteins in the PI3K signaling pathway and estrogen receptor (ER) signaling pathway, leading to activation of cyclin D/CDK4 and cyclin D/CDK6. Cyclin D-CDK4/6 accumulation allows entry into the cell cycle (G1 phase) thereby avoiding cell cycle exit. Accumulation of cyclin E in this phase leads to an increased activity of cyclin E/CDK2, which inactivates Rb by hyperphosphorylation, and in turn activates E2F-dependent transcription. Hence, E2F-dependent transcription determines accumulation of cyclin E and cyclin A in G1 phase, promoting a positive feedback loop that enhances cyclin E/CDK2 activity, accumulates cyclin A/CDK2 activity, and consequently results in replication initiation at the Rb. In G2 phase, accumulation of cyclin A/CDK1 and cyclin B/CDK1 complex promotes mitotic entry at the MICP. In M phase, increase of cyclin A/CDK1 and cyclin B/CDK1 activity complete the cell cycle. Accumulation of cyclin D-CDK4/6 activity then prompts cells to re-enter the cell cycle. Complete cyclin/CDK activation is induced by CAK phosphorylation throughout the cell cycle. Direct activation (phosphorylation) is shown with arrowhead lines, whereas inhibition (phosphorylation) is indicated with blocked lines. Colored circle lines in the cell cycle show CDK activity, whereas colored dash-dotted cycle lines in the cell cycle indicate CDK inactivity. RTK: receptor tyrosine kinases; PI3K STP: PI3K/AKT/mTORC signal transduction pathway; ER STP: estrogen receptor signal transduction pathway. CDK: cyclin-dependent kinase. E2F: activating E2Fs (E2F1-E2F3); CAK: CDK-activating kinase; Rb: replication initiation commitment point; MICP: mitosis initiation commitment point; DP: dimerization partner transcription factor; Circled P (black background with white text): phosphoryl group during direct or indirect phosphorylation.

knowledge of the mechanism of resistance to CDK4/6i is crucial to establish better treatment strategies. Recent preclinical studies have shown that CDK4/6i can also induce a variety of phenotypes beyond cell cycle arrest, emphasizing that deeper insights into the mechanisms of action of these agents is required (Spring et al., 2020). In this review, we first explain the biology and role of the CDK4/6-Rb-E2F pathway in HR⁺ BC, then outline how small-molecule CDK4/6i can trigger other effects in cancer/stromal compartments, and finally describe the notorious mechanisms of CDK4/6i resistance that have emerged in recent preclinical studies and clinical cohorts, highlighting possible therapeutic strategies in the context of CDK4/6i resistance. Our detailed review on CDK4/6i resistance in BC will provide comprehensive insights to clinicians and scientists regarding how to fully exploit the promise of these CDK4/6-inhibiting therapeutic drugs.

2. CDK4/6-mediated regulation of S phase entry in HR⁺ breast cancer

2.1. Cyclin D-CDK4/6 signaling pathway in the cell cycle

The cell cycle (Fig. 1) is characterized by accurate regulation of a myriad of cellular events. A gross departure from this precision can lead to mis-regulation and genomic instability, which often contributes to oncogenesis (Sawant et al., 2023). Cell cycle control and transcription are two essential processes strictly regulated by cyclin-dependent kinases (CDKs). The human cyclin/CDK network includes up to 30 different cyclin proteins and over 20 CDKs (Martínez-Alonso and Malumbres, 2020). Cyclins increase during different phases of the cell cycle, guided by transcription and suppression of protein degradation, to permit cell cycle entry and progression. In turn, cell cycle-mediated transcription depends upon CDK activity (Knudsen et al., 2022). Though Cyclin D1/D2/D3 binding promotes partial activation of CDK4/6, complete induction requires phosphorylation by CDK-activating kinase (CAK), a trimeric protein complex comprised of cyclin H, cyclin-dependent kinase 7 (CDK7), and Mat1 subunit (Peissert et al., 2020). The complexes cyclin D/CDK4 and cyclin D/CDK6 specifically promote a proliferative G1 state, priming cells for a G1-to-S phase transition (Bertoli and de Bruin, 2014) (Rubin et al., 2020), induced by E2F transcription factors whose activity determines DNA replication-related gene expression (Hume et al., 2020). Tumor suppressor Rb forms complexes with E2Fs and downregulates numerous genes coding for key cell cycle regulators. Besides, Rb-recruited epigenetic modifiers place repressive chromatin marks at the promoters of E2F target genes (Engeland, 2022). Commitment to replication initiation is tightly associated with induction of E2F-dependent transcription, which is activated in G1 phase and successively suppressed during S phase (Pennycook et al., 2020) (Fouad et al., 2020). In G0 phase, the quiescent cellular state outside of the cell cycle, Rb is unphosphorylated (Oki et al., 2014). Mitogenic growth factors activate and increase the levels of cyclin D, which thereupon binds to CDK4/6. The holoenzyme complex cyclin D-CDK4/6 then interacts with p21 or p27, resulting in phosphorylation of Rb (Narasimha et al., 2014) (Kim et al., 2022). Recent studies suggest that cyclin D-CDK4 and cyclin D-CDK6 are not directly related to the initial inactivation of Rb, but instead, prime cells for a new cell cycle entry (from mitosis into G1 phase) by either hindering its exit from G1 phase, or promoting its entry into G1 phase (Narasimha et al., 2014), probably by regulating the metabolic state of cells (Sanidas et al., 2019) (Caillot et al., 2020). In both cases, the complex cyclin D-CDK4/6-p21/p27 endorses mono-phosphorylation of Rb, which does not inactivate Rb, and consequently is inadequate to stimulate E2F-dependent transcription (Goodrich et al., 1991) (Harbour et al., 1999). Instead, this CDK4/6-mediated mono-phosphorylation of Rb generates a temporary state between a new cell cycle entry (from mitosis into G1 phase) and cell cycle exit (Bertoli and de Bruin, 2014). Concurrently, in this state, accumulation of cyclin E results in the formation of the complex cyclin E/CDK2, which then inactivates Rb via

hyperphosphorylation leading to activation of E2F-dependent transcription (Topacio et al., 2019) (Kaulich et al., 2021). Since both cyclin E and cyclin A are E2F targets, this causes their hyper-accumulation, initiating a positive feedback loop with cyclin E/CDK2 further activating E2F-dependent transcription via Rb inactivation and accumulation of cyclin A/CDK2 activity, thereby driving S phase entry (Bertoli et al., 2013). However, considering potential redundancy between CDK2 and CDK4/6 is important in regard to irregular responses of Rb-proficient tumors to CDK4/6 inhibition. In this respect, CDK2 or CDK4/6 singly are also capable of driving proliferation. For instance, distinct Rb-proficient tumor cells can proliferate without the presence of CDK2 (Tetsu and McCormick, 2003), and cells absent of CDK4/6 can continue proliferating by forming peculiar cyclin D-CDK2 complexes, thereby maintaining their ability to phosphorylate Rb (Malumbres et al., 2004) (Goel et al., 2022). Once S phase entry occurs, cell cycle proceeds toward S phase, G2 phase, and M phase (Matthews et al., 2022). The onset of DNA replication, and thus commitment to begin S phase mainly depends on cyclin A/CDK2 activity. As cells traverse through S phase, cyclin E/E2F association gradually decreases, and conversely, cyclin A/E2F association increases (Lees et al., 1992). This triggers progression through the replication initiation commitment point (RICP), DNA replication, and a new cell cycle (Arias and Walter, 2007). Once DNA replication is attained, there is a post-replicative state, during which, CDK1 activity undergoes meticulous regulation to allow prompt and speedy mitotic entry. CDK1 then associates with cyclin A and cyclin B, which progressively increase in G2 phase (Otto and Sicinski, 2017). This translates into a prompt increase in CDK1 activity which drives mitotic entry (Ha and Ferrell, 2016). Upon attaining a proper level of CDK1 activity, and reaching the mitosis initiation commitment point (MICP), entry into mitosis is generated by phosphorylation of indefinite CDK1 substrates (Blethrow et al., 2008) (Dephoure et al., 2008). Finally, mitosis-associated events are mechanistically controlled predominantly by the cyclin B/CDK1 complex. Activation of the anaphase-promoting complexes then directs degradation of cyclins, which abrogates CDK activity when the two daughter cells return to interphase (Gavet and Pines, 2010) (Fig. 1).

2.2. Altered cyclin D-CDK4/6 signaling pathway in the cell cycle

2.2.1. Hyperactivity of cyclin D-CDK4/6 caused by upstream signaling pathways

Ubiquitous mechanisms without genetic alterations in the cyclin D-CDK4/6 signaling pathway can also drive CDK4/6 hyperactivity in BC, leading to upregulation of cyclin D. This is caused by high-levels of mitosis-inducing signals, disrupting the rise and fall counteract of cyclin D levels normally observed in G1 phase (Choi and Anders, 2014) (Bertoli and de Bruin, 2014) (Emond et al., 2023). Mutations deregulating the PI3K/AKT pathway augment cyclin D expression through multiple mechanisms, such as enhanced mRNA translation (Muisse-Helmericks et al., 1998), decreased nuclear export/protein degradation (Diehl et al., 1998), and derepression of cyclin D gene transcription (Schmidt et al., 2002); whereas the MAPK signalling pathway-mediated mutations can directly drive *CCND1* transcription (Peepers et al., 1997). In line with this, downstream of both the PI3K/AKT and MAPK axes, mTOR complex 1 (mTORC1) can principally augment mRNA translation of cyclin D (Averous et al., 2008). Moreover, mutations in other signalling pathways can also enhance the activity of CDK4/6 in tumor cells, through a variety of biochemical mechanisms. For instance, ER augments *CCND1* transcription in estrogen-sensitive tumors such as BC, and β -catenin can induce transcription from the *CCND1* promoter in BC cells (Goel et al., 2022). Besides, mutations in estrogen receptor 1 (*ESR1*) are common in ER⁺ BC patients who progress on ET. Most of these mutations involve three residues (Leu-536, Tyr-537, and Asp-538) at the C-terminal helix 12 of the hormone binding domain (Harrod et al., 2022), resulting in constitutive, ligand-independent ER signaling (Fig. 2).

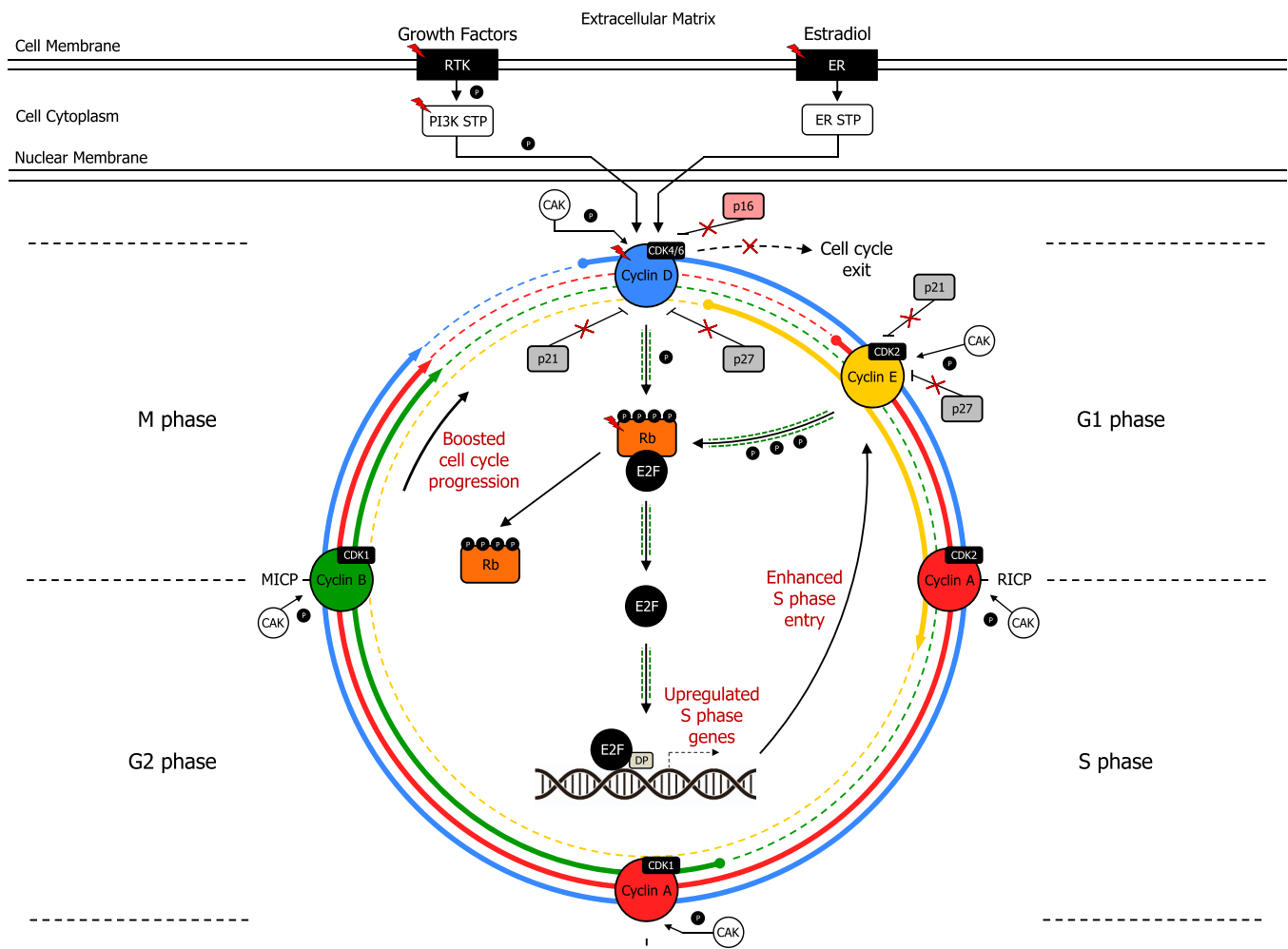


Fig. 2. Abnormal cyclin D-CDK4/6 activity mediates molecular events regulating cell-cycle progression through G1 phase in breast cancer cells. In breast cancer cells persistent cell cycle progression is mainly driven by mutations of proteins in PI3K signaling pathway and estrogen receptor (ER), as well as in cyclin D/CDK4 and cyclin D/CDK6. Mutations in pocket protein family (Rb, p107, and p130) enhance E2F dependent transcriptional regulation, thereby endorsing cyclin E/CDK2, as well as downstream cyclin A/CDK2 activity in cancer cells. Mutation-induced CDK overactivity results in augmented phosphorylation and inactivation of Rb, allowing expression of E2F-dependent genes (e.g. cyclin E1-encoding *CCNE1* and cyclin E2-encoding *CCNE2*). Successively, cyclin E binds and activates CDK2, determining further hyperphosphorylation of Rb and phosphorylation of several other molecules, preventing cell cycle exit, and consequently leading to a permanent commitment to S phase entry. Red lightning symbols show common mutations normally found in signaling pathways upstream cell cycle or related to cell cycle regulation in breast cancer. Direct activation (phosphorylation) is shown with arrowhead lines, whereas inhibition (phosphorylation) is indicated with blocked lines. Colored circle lines in the cell cycle show CDK activity, whereas colored dash-dotted circle lines in the cell cycle indicate CDK inactivity. Red crosses emphasize signaling blockage in breast cancer, whereas green dash-dotted lines (adjacent to arrowhead lines) highlight signaling enhancement in breast cancer. RTK: receptor tyrosine kinases; PI3K STP: PI3K/AKT/mTORC signal transduction pathway; ER STP: estrogen receptor signal transduction pathway. CDK: cyclin-dependent kinase. E2F: activating E2Fs (E2F1-E2F3); CAK: CDK-activating kinase; Rb: replication initiation commitment point; MICP: mitosis initiation commitment point; DP: dimerization partner transcription factor; Circled P (black background with white text): phosphoryl group during direct or indirect phosphorylation.

2.2.2. Intrinsic cell cycle mutation-induced hyperactivity of cyclin D-CDK4/6

Abnormal functioning of cyclin D/CDK4 and cyclin D/CDK6 are common in various human cancers, including BC (Fig. 2), priming cells for S phase entry, and thus contributing to activation of E2F family of transcription factors (Xie et al., 2021), to guide cyclin E/CDK2- and downstream cyclin A/CDK2-induced cell cycle advancement from G1 phase to S phase (Rubin et al., 2020). Additionally, mutations in pocket protein family (Rb, p107, and p130) (Giacinti and Giordano, 2006) augment E2F-dependent transcriptional regulation, thereby endorsing cyclin E/CDK2, as well as downstream cyclin A/CDK2 activity in cancer cells. Thus, mutation-mediated CDK overactivity determines increased phosphorylation and inactivation of Rb, allowing expression of E2F-dependent genes, such as cyclin E1-encoding *CCNE1* and cyclin E2-encoding *CCNE2*. Subsequently, cyclin E binds and activates CDK2,

resulting in further hyperphosphorylation of Rb and phosphorylation of several other molecules, leading to a permanent commitment to S phase entry (Bertoli et al., 2013). Molecular studies strongly suggest that cyclin D-CDK4/6 signaling pathway is frequently hyperactivated in HR⁺ BC (Rampioni Vinciguerra et al., 2022). Indeed, cyclin D1-encoding *CCND1* oncogene amplification (Jeffreys et al., 2022), and either CDK4 amplification or loss of p16^{INK4a} and p14^{ARF}-encoding tumor suppressor *CDKN2A* (Cheng et al., 2022), have been reported in BC. Transcriptional factor estrogen receptor, the main driver of tumor growth and survival in HR⁺ BC, also directly targets *CCND1* (Shagisultanova et al., 2022) (Fig. 2).

3. Mechanisms of action of CDK4/6i in HR⁺ breast cancer

The exact molecular mechanisms whereby FDA-approved small-

molecule CDK4/6i inhibit cellular proliferation is still uncertain. These compounds interact with the ATP-binding pocket of CDK4/6, leading to competitive suppression of active CDK4/6 kinases. Nevertheless, though active CDK4/6 is commonly present in a trimer with cyclin D and p21 or cyclin D and p27 (LaBaer et al., 1997), *in vitro* efficacy of these compounds has been demonstrated using cyclin D-CDK4/6 dimers (Fry et al., 2004) (Gelbert et al., 2014) (Bisi et al., 2016). Interestingly, palbociclib, ribociclib, and abemaciclib, exhibit no inhibition of active trimeric complexes *in vitro*, and consequently, it has been hypothesized that instead they may directly suppress active CDK4/6 in cells (Goel et al., 2022). Alternatively, CDK4/6i may act by binding and sequestering monomeric inoperative CDK4/6, precluding formation of cyclin D-CDK4/6-p21/p27 holoenzyme trimers, thereby freeing p21 to bind and suppress CDK2 (Guiley et al., 2019). However, the hypothesis that CDK4/6i mainly act through indirect CDK2 suppression has not been tested (Pennycook and Barr, 2021), and ought to be demonstrated by showing that CDK4/6i-sensitive tumor cells are more susceptible to genetic disruption of the CDK2 kinase function than CDK4/6 kinase

function (Goel et al., 2022). In addition, CDK6 exists in both thermo-stable and thermounstable forms; and the former exhibits resistance to current pharmacological CDK4/6i (Wu et al., 2021a).

3.1. Inhibiting G1 phase/S phase CDKs

Palbociclib, ribociclib, and abemaciclib target the ATP-binding domains of CDK4 and CDK6 (Wander et al., 2022a). These drugs are cytostatic and their activity leads to a G1-phase arrest in Rb-proficient luminal HR⁺ BC cells (George et al., 2021) (Fig. 3A). Interestingly, unlike palbociclib and ribociclib, treatment with abemaciclib at higher concentrations blocks cancer cells in both G1 and G2 phases (Hafner et al., 2019) and activates apoptosis (Torres-Guzmán et al., 2017) in Rb-proficient BC cells. This characteristic may be partially due to differences in secondary targets of abemaciclib such as proto-oncogene PIM kinases (Litchfield et al., 2020). In addition, abemaciclib-specific targets can also include CDK1, CDK2, and CDK9, although current data does not provide significance that these kinases are fully

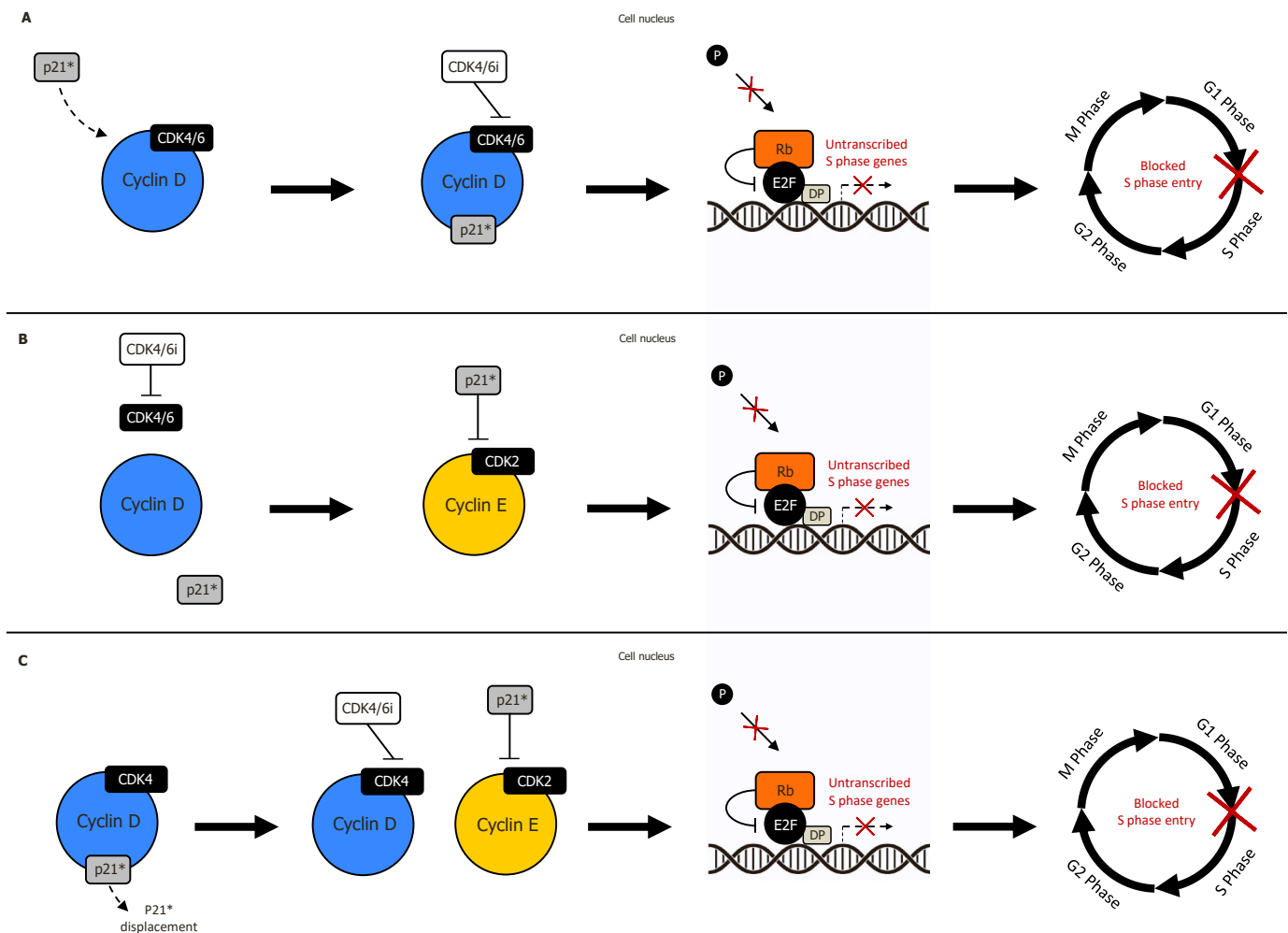


Fig. 3. CDK4/6i-mediated inhibition of G1 phase/S phase CDKs. **A.** The classical model indicates that CDK4/6i suppress active cyclin D-CDK4/6i-p21/p27 trimer holoenzymes, averting CDK4/6-mediated Rb phosphorylation, and blocking S phase entry in the cell cycle. **B.** The Guiley et al. model suggests that CDK4/6i bind to monomeric CDK4/6, precluding the establishment of cyclin D-CDK4/6-p21/p27 trimer holoenzymes. The resulting freed p21 protein then binds to and suppresses cyclin E/CDK2 complex, preventing Rb phosphorylation, and blocking S phase entry in the cell cycle. This model indicates that CDK4/6i determine cell cycle arrest through indirect inhibition of CDK2, instead of direct inhibition of CDK4/6 activity. **C.** The Pack et al. model proposes that CDK4/6i directly inhibit CDK4/6 catalytic activity, but also sequester p21 from already-formed cyclin D-CDK4-p21 trimer holoenzymes, thereby leading to indirect suppression of cyclin E/CDK2 complex, and blocking S phase entry in the cell cycle. Inhibition is indicated with blocked lines, and G1 phase/S phase arrest of cell cycle in breast cancer. CDK4/6: cyclin-dependent kinase 4 and cyclin-dependent kinase 6; CDK4/6i: cyclin-dependent kinase 4 and cyclin-dependent kinase 6 inhibitors; CDK4: cyclin-dependent kinase 4; CDK2: cyclin-dependent kinase 2; p21*: p21 protein or p27 protein; E2F: activating E2Fs (E2F1-E2F3); DP: dimerization partner transcription factor; circled P (black background with white text): phosphoryl group during phosphorylation.

hypophosphorylated after treatment of HR⁺ BC cells (George et al., 2021) (Watt and Goel, 2022). Importantly, all these three aforementioned CDK4/6i display their strongest potency in the presence of functional Rb (Loibl and Furlanetto, 2022). There is still uncertainty regarding the mechanisms by which CDK4/6i exert their cytostatic activity toward BC cells. Guiley et al. have proposed a model whereby CDK4/6-mediated arrest of cell cycle is mainly determined by indirect inhibition of CDK2. In this case, CDK4/6i primarily bind to inactive monomeric CDK4 or CDK6 instead of binding and suppressing the active cyclin D-CDK4/6-p21/27 complexes. As a result, CDK4/6i exert no blockage of endogenous CDK4 activity, but rather preclude the establishment of steady cyclin D-CDK4/6-p21/27 trimers. Consequently, this allows p21-mediated inhibition of CDK2 activity, resulting in G1-phase cell cycle arrest (Guiley et al., 2019) (Fig. 3B). However, Pennycook and Barr (2021) have shown that palbociclib-mediated cell cycle arrest can occur in the absence of the CDK inhibitors p21 and p27 (Pennycook and Barr, 2021). The Pack et al. model has recently suggested that treatment with CDK4/6i promptly dissociates p21 selectively from CDK4 complexes, but unexpectedly, not from CDK6 complexes; and that the indirect inhibition of CDK2 activity is achieved by p21 but not by p27 redistribution (Pack et al., 2021). Overall, this study indicates that CDK4/6i could mediate two concurrent major roles to repress cell cycle advancement: 1) a direct catalytic suppression of CDK4/6-induced Rb phosphorylation, independent of p21; and 2) an indirect non-catalytic inhibition of CDK2 by displacing p21 from cyclin D/CDK4/p21 trimeric complex, which is unique to CDK4-p21, and not CDK6-p27 (Pack et al., 2021) (Fig. 3C). Interestingly, recent studies have identified a role for the INK4 tumor suppressor proteins in mediating resistance to CDK4/6i. Chandarlapaty and colleagues have demonstrated that CDK6 causes resistance by inducing and binding INK4 proteins such as p18^{INK4C}. Notably, suppression of INK4 expression or its binding to CDK6 restores the sensitivity to CDK4/6i (Li et al., 2022).

3.2. Inducing a senescence-like state

Inhibition of CDK4/6 has also been reported to activate a senescence-like state, normally featured by cellular enlargement and enhanced β -galactosidase activity (Wagner and Gil, 2020) (Wang et al., 2022). The main phenotypic hallmarks of classical cellular senescence are irreversible cell cycle exit, apoptosis resistance, remodelling of chromatin, metabolic dysregulation, and secretion of cytokines/growth factors, known as senescence-associated secretory phenotype (SASP) (Gorgoulis et al., 2019). Watt et al. (2021) have detected chromatin remodelling in CDK4/6i-treated BC cell-based models, mouse models, and clinical specimens, defined by widespread enhancer induction (Watt et al., 2021). Newly activated enhancer-mediated transcriptional activity is directly involved in regulating apoptotic evasion, increased cellular immunogenicity, and is mainly guided by activator protein 1 (AP-1) transcription factors (Martínez-Zamudio et al., 2020). Particularly, these new enhancers also determine a stronger differentiation of cellular phenotype, emphasizing an association between Rb induction and cellular differentiation in tumors (Walter et al., 2019). However, this senescence-like state is in some cases reversible when CDK4/6 inhibition is removed, since cells re-enter cell cycle and restart cell proliferation, indicating that CDK4/6i do not, in this particular case, cause an irreversible cell cycle exit and a senescence state (Vijayaraghavan et al., 2017). CDK4/6i have shown induction of *in vitro/vivo* senescence in several tumor types, including HR⁺ BC (Wagner and Gil, 2020) (Klapp et al., 2023). This senescent phenotype mostly depends on Rb (Kumari and Jat, 2021), but may also be associated with decreased CDK4/6-mediated phosphorylation on DNA methyltransferase 1 (DNMT1) and forkhead box M1 (FOXO1) (Schmitt et al., 2022). Interestingly, suppression of mTOR complex 1 (mTORC1) during palbociclib treatment prevents the activation of senescence, whereas genetic depletion of mTORC1 negative regulator tuberous sclerosis complex 2 (TSC2) leads to sustained mTORC1 activity and induction of senescence

(Maskey et al., 2021). This is in line with recent publications showing a major role for active cellular growth signalling in CDK4/6i sensitivity during palbociclib treatment (Manohar et al., 2023) (Crozier et al., 2023) (Foy et al., 2023). Interestingly, treatment with palbociclib or abemaciclib can downregulate mTOR signaling in BC, supporting the notion that CDK4/6i can also prevent senescence through the downregulation of mTOR signaling in BC cells (Maskey et al., 2021) (Romero-Pozuelo et al., 2020). Notwithstanding p53 is a crucial player in classical senescence, its role in CDK4/6i-induced senescence has not been fully elucidated. Indeed, TP53 mutations have recently arisen as the most valuable genomic predictor of CDK4/6i resistance in a panel of 560 tumor cell lines (Gong et al., 2017) (Goel et al., 2022), indicating an active contribution of functional p53 to drug-mediated proliferative repression. On the contrary, CDK4/6i inhibition has also been found to activate phenotypic aspects of senescence in cancer cells with unfunctional p53 (Zou et al., 2002) (Michaud et al., 2010) (Yoshida et al., 2016). Since both p53 and Rb may overlap their diverse actions in the induction of typical senescence, p53 wild-type, as well p53 mutant BC cells, may possibly show aspects of senescence in response to CDK4/6i suppression; but qualitatively these two senescent phenotypes are distinct (Goel et al., 2022). Further studies are required to fully understand how CDK4/6i generates senescent phenotypes, such as SASP state, in HR⁺ BC; and, better clarify what roles TP53 plays in regulating CDK4/6i resistance.

3.3. Evading apoptosis

Even though CDK4/6i can activate a senescent-like state there are still doubts as to whether these agents are able to exert a direct killing of luminal HR⁺ BC cells. Indeed, like senescent cells, BC cells that enter CDK4/6i-triggered senescence exhibit resistance to apoptotic insults (Goel et al., 2017) (Watt et al., 2021). Several studies have suggested that CDK4/6i inhibit apoptosis; simply by inducing senescence, which represents an anti-apoptotic state (Watt and Goel, 2022). For instance, co-treatment of palbociclib and ET reduces apoptosis in HR⁺ BC cells (Johnston et al., 2019a). Thus, further clinical studies are required to consider using senolytic agents that can selectively kill BC senescent cells. Apoptosis resistance in BC partly derives from the induction of a super-enhancer spanning the *BCL2L1* gene, whose activity augments intra-tumoral levels of Bcl-xL, a notorious anti-apoptotic Bcl-2 family protein (Kervadec and Priault, 2023) (Valentini et al., 2023). Accordingly, this apoptosis-resistance state can be reversed by Bcl-xL inhibitors (Alcon et al., 2021), which re-establish susceptibility to apoptosis in CDK4/6i-pre-treated HR⁺ BC cells (Watt et al., 2021). At present, anti-apoptotic BH3-family proteins are considered the principal molecules involved in CDK4/6i-mediated apoptotic evasion in HR⁺ BC cells. Pre-clinical studies report that Bcl-2 inhibitor BH3-mimetic venetoclax (Roberts and Huang, 2017) increases response to CDK4/6i palbociclib by triggering apoptosis in BC cells, including in senescent cells. In line with this, the primary objective of the ongoing PALVEN phase 1b clinical trial (NCT03900884), exploring the combination of CDK4/6i palbociclib, aromatase inhibitor (AI) letrozole, and venetoclax, is to determine the maximum tolerated dose (MTD) and the recommended phase 2 dose (RP2D) of this co-treatment therapy in HR⁺ BC patients (Muttiah et al., 2022). Interestingly, the recent VERONICA phase 2 clinical trial (NCT03584009) did not indicate clinical utility of combining venetoclax and fulvestrant in ET-resistant, CDK4/6i-refractory metastatic HR⁺/HER2⁻ BC patients; but does suggest enhanced dependence on Bcl-xL in this setting. Indeed, there was prolonged PFS and a marginal improvement in clinical benefit rate (CBR) with venetoclax in cancers exhibiting strong Bcl-2 expression (IHC 3+) and a Bcl-2/Bcl-xL histoscore ratio ≥ 1 , indicating dependence of cancer survival on Bcl-xL in the post-CDK4/6i setting (Lindeman et al., 2022). Further insight is required to fully clarify how CDK4/6i-mediated cancer regression occurs, and establish new strategic therapeutic combinations using CDK4/6i and newer anti-apoptotic inhibitors, such as venetoclax.

Interestingly, despite these aforementioned insights, a certain number of studies address the possibility that CDK4/6i can directly determine apoptosis in ER⁺ BC cells (Torres-Guzmán et al., 2017) (Hafner et al., 2019) (Watt and Goel, 2022) (Qi and Ouyang, 2022). Indeed, prolonged abemaciclib treatment can also trigger apoptosis in Rb-proficient BC cell lines, as indicated by TUNEL staining and annexin V staining (Torres-Guzmán et al., 2017). Accordingly, CDK4/6i-induced endorsement of apoptosis has also been detected in leukemic cells (Choi et al., 2012). Hence, abemaciclib treatment-mediated inhibition of BC proliferation can possibly occur through the promotion of long-term modifications in BC cells including apoptosis. These *in vitro* results suggest that short-term abemaciclib treatment inhibits BC cell progression, whereas long-term abemaciclib exposure can result in sustained anticancer effects through the activation of apoptosis, the induction of senescence, and the alteration of cellular metabolism (Torres-Guzmán et al., 2017).

3.4. Enhancing Autophagy

Recent studies suggest that further exploration is warranted focused upon how autophagy may decrease the efficacy of targeted therapies, ET, chemotherapy, radiotherapy, and immunotherapy *via* modulation of intermediate proteins. In fact, the potential use of autophagy inhibitors to improve the antitumor effects of agents by avoiding cyto-protective autophagy is an active focus area in cancer biology (Xiao et al., 2021) (Bestion et al., 2023) (Kim and Koo, 2023). Interestingly, regulation of senescence and autophagy often depends on the same signaling pathways (Pawlowska et al., 2018) (Cassidy and Narita, 2022). Activity of the cyclin D-CDK4 axis is crucial for the suppression of autophagy in mammary epithelial cells (Wu and Sharma, 2023). In fact, studies have demonstrated that pharmacological suppression of CDK4/6 signaling axis determines autophagy in both normal breast cells and BC cells (Brown et al., 2012) (Vijayaraghavan et al., 2017). In line with this, CDK4/6i enhance several markers of autophagy in HR⁺ BC cell lines and xenograft models. A combination of autophagy inhibitors, such as hydroxychloroquine and chloroquine, and CDK4/6i, therefore displays a synergistic effect. This combinatorial treatment cannot kill CDK4/6i-treated BC cells, but instead, induces a permanent cell cycle exit *via* senescence *in vitro/in vivo* (Vijayaraghavan et al., 2017). Suppression of CDK4/6 and autophagy is also synergistic in other solid tumors with normal G1/S checkpoint, which may indicate promising new combination therapies (Rampioni Vinciguerra et al., 2022).

3.5. Epigenetic regulation

Using cell-based and mouse models of BC, Watt et al. have recently shown that CDK4/6i can reprogram the active enhancer landscape by stimulating the transcriptional activity of AP-1 in an Rb-dependent manner (Song et al., 2023) (Wu et al., 2021b) in luminal BC cells *in vitro/vivo* (Watt et al., 2021). Even though chromatin at the promoters of cell cycle genes displays CDK4/6i-induced suppressive modifications, numerous intergenic and intronic regions exhibit augmented accessibility to H3K27ac, a histone H3 epigenetic modification (Sinha et al., 2023) associated with higher activation of transcription (Watt et al., 2021). Importantly, these CDK4/6i-induced enhancers, which are regulated by AP-1 transcription factor family members, play critical roles in resistance to apoptosis, luminal differentiation, and tumor immunogenicity. Therefore, inhibition of CDK4/6 increases the levels AP-1 transcription factor family members (Watt et al., 2021), which are in turn involved in the activity of these new enhancers (Watt and Goel, 2022). In line with this, AP-1 also guides enhancer activation and chromatin accessibility in benign senescent cells (Martínez-Zamudio et al., 2020). More studies are required to interrogate whether the estrogen receptors itself, or its cofactor, the pioneer transcription factor FOXA1, is/are involved in CDK4/CDK6i-mediated induction of enhancers, and whether concurrent treatment of CDK4/6i with ET can reshape these chromatin-based mechanisms.

3.6. Interaction with oncogenic kinase signaling pathway

Combination of CDK4/6i and growth factor receptor, e.g. receptor tyrosine kinase (RTK) (Sobhani et al., 2021), or PI3K/AKT/mTOR pathway (Clark et al., 2021) (Zhao et al., 2021) inhibitors, has shown synergistic or strengthened effects (Watt and Goel, 2022). Triple combination of CDK4/6i, PI3Ki, and ET leads to strong efficacy *in vivo*, in term of rapid tumor growth reduction, in HR⁺ BC models. In addition, continuous exposure of this triplet *in vitro* determines significant decrease of colonies, compared to the palbociclib + fulvestrant doublet, in MCF-7 and T47D cell lines. Besides this triplet combination displays higher reduction of pRb S807/811 phosphorylation, decreased cyclin E2 and CDK2 expression, and augmented poly (ADP-ribose) polymerase (PARP) cleavage, in comparison to the palbociclib + fulvestrant doublet, in both cell lines (Herrera-Abreu et al., 2016). The molecular mechanisms underlying the additive or synergistic effects of these beneficial preclinical combinations have not been fully elucidated. This synergism may be explained by the fact that these signaling pathways affect cell-cycle machinery through cyclin D-CDK4/6 (Klein and Assoian, 2008). A typical hallmark is the augmented activity of upstream pathways, especially the PI3K/AKT/mTOR pathway (Li et al., 2020), in CDK4/6i-treated luminal BC cells (Zhao et al., 2021). In addition, CDK4/6i also enhance the phosphorylation of human epidermal growth factor receptor (HER) family and AKT in luminal HER2-positive (HER2⁺) BC cell lines (Goel et al., 2016). These phenomena may be partially explained by cyclin D/CDK4 and cyclin D/CDK6 phosphorylation activities. In fact, without inhibition of CDK4/6, cyclin D/CDK4 and cyclin D/CDK6 can phosphorylate TSC2, which negatively regulates mTOR (Romero-Pozuelo et al., 2020). Conversely, CDK4/6i-mediated suppression of CDK4/6 decreases TSC2 phosphorylation, thereby reducing the action of mTORC1, and rebounding upstream RTK activity (Chandarlapaty et al., 2011) (Romero-Pozuelo et al., 2020). A direct effect of enhanced RTK signaling is a continued induction of mTORC1 activity, which can lead to progression of S phase (He et al., 2021). Alternatively, augmented levels of cyclin D may contribute to forming anomalous cyclin D/CDK2 complexes that increase phosphorylation on Rb (Herrera-Abreu et al., 2016). As a result, new combinations of CDK4/6i and growth factor pathway inhibitors have reached clinical trials. In line with this, studies exploring the combination of CDK4/6i and HER2 inhibitors, as well as CDK4/6i and PI3K inhibitors, have been initiated (Rampioni Vinciguerra et al., 2022). Regarding RTKs, combination of CDK4/6i abemaciclib, SERD fulvestrant, and monoclonal antibody trastuzumab has demonstrated improved PFS and safety profile when compared to standard-of-care (SOC) chemotherapy and trastuzumab in pretreated HR⁺, HER2⁺ advanced BC patients. Importantly, this clinical study indicates that a chemotherapy-free regimen can be a potential alternative therapy for these patients (Tolaney et al., 2020). In relation to PI3K/AKT/mTOR pathway (Glaviano et al., 2023), triple combination of CDK4/6i palbociclib with PI3Ki taselelisib and fulvestrant has exhibited an overall response rate (ORR) of 37.5 %, and a favourable safety profile, in *PIK3CA*-mutant ER⁺/HER2 advanced BC patients (Pascual et al., 2021).

3.7. Augmenting immunogenicity

CDK4/6i have been shown to improve anticancer immune responses in several preclinical studies of BC. Indeed, it has been demonstrated that CDK4/6i promote tumor microenvironment inflammation, and CD4⁺ T-cells and/or CD8⁺ T-cells can in part mediate therapeutic responses (Goel et al., 2017) (Deng et al., 2018) (Jerby-Arnon et al., 2018). This phenomenon is detected in all FDA-approved CDK4/6i. Inhibition of CDK4/6i augments antigen presentation on major histocompatibility complex (MHC) class I molecules, *via* Rb, in cancer cells (Lelliott et al., 2021). Interestingly, CDK4/6i decrease the expression of (the E2F target gene) DNA methyltransferase 1-encoding *DNMT1*, leading to hypomethylation, and thence transcription of endogenous retroviral

elements (Haggerty et al., 2021). Consequently, double-stranded RNA in the cell activates a viral mimicry response, defined by generation of interferon, as well as expression of interferon-induced genes. In addition, chromatin remodeling, mediated by CDK4/6i, triggers activity of enhancers that overlie endogenous retroviral element sequences possibly involved in driving interferon-stimulated gene expression (Watt and Goel, 2022) (Watt et al., 2021). CDK4/6i also promote metabolic stress in BC cells, resulting in chemokine expression including CXCL10 and CCL5, which can further augment the anticancer immune response (Uzhachenko et al., 2021). Importantly, CDK4/6i exert direct effects on T-cells. Indeed, various CDK4/6i can effectively repress, perhaps via Rb, regulatory T-cells (Tregs) proliferation in the tumor microenvironment (Lai et al., 2020) (Whittle et al., 2020) (Sciocchi et al., 2022). Conversely, CDK4/6i can enhance effector T-cell function, highlighted by decreased expression of T-cell exhaustion markers, and by increased production of effector cytokines. This is partially due to suppression of CDK6-induced phosphorylation of nuclear factor of activated T-cells (NFAT) transcription factor members (Schaer et al., 2018) (Deng et al., 2018). In addition, CDK4/6i can also favour CD8⁺ T-cell differentiation toward a memory cell fate, thereby contributing to increasing anticancer efficacy (Heckler et al., 2021) (Lelliott et al., 2021). These phenomena lead to a T-cell-inflamed tumor microenvironment and augmentation of effector T-cell activity (Deng et al., 2018), resulting in enhanced anticancer effects of CDK4/6i (Lelliott et al., 2021). Accordingly, combination of different CDK4/6i and several immunotherapies have demonstrated more significant reduction in cancer growth, as well as improvement of T-cell memory, compared to each agent alone, in pre-clinical studies not including ER⁺ BC animal models (Watt and Goel, 2022). In the recent RIBECCA phase 3 clinical trial (NCT03096847), ribociclib treatment has demonstrated the induction of an already-existing immune response instead of *de novo* immune activation in ER⁺ BC patients (Peuker et al., 2022). Gene expression analyses of biopsies suggests that this immune effect occurs in luminal BC patients. Indeed, in the neoMONARCH clinical trial, combination of CDK4/6i abemaciclib and aromatase-inhibitor anastrozole have shown significant efficacy via potent cell-cycle arrest and augmented immune activation, with manageable toxicity, in HR⁺/HER2⁻ early BC patients (Hurvitz et al., 2020). However, leveraging these CDK4/6i-mediated immune effects to improve patient outcomes have encountered challenges related to: 1) observations that combinations of CDK4/6i and immuno-oncology therapy have been hindered by unfavourable safety profiles; and 2) ER⁺ metastatic BC has not displayed satisfactory responses to immune-based strategies (Watt and Goel, 2022). Interestingly, in two neoadjuvant trials of CDK4/6i plus ET, highly-enriched interferon (IFN)-related signatures have been observed in ER⁺ BC patients with intrinsic and acquired resistance to CDK4/6i, suggesting that aberrant IFN signaling is an important driver of resistance (De Angelis et al., 2021).

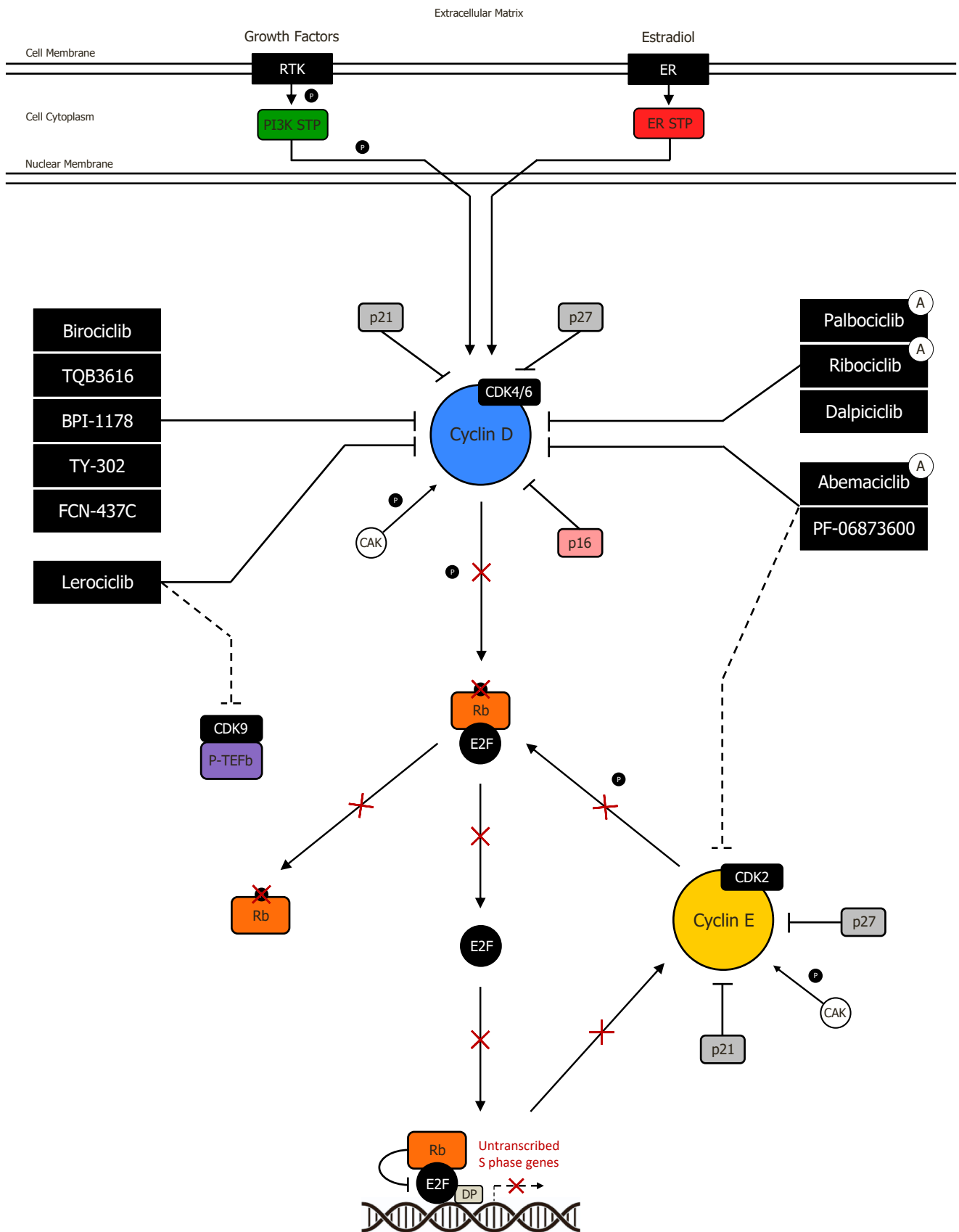
4. Available and emerging CDK4/6i in ER⁺ breast cancer

Palbociclib (Walker et al., 2016) (Finn et al., 2016) (Cristofanilli et al., 2016), ribociclib (Shah et al., 2018) (Slamon et al., 2018) (Hortobagyi et al., 2019), and abemaciclib (Sledge et al., 2017) (Johnston et al., 2019b) (Takahashi et al., 2022) (Raheem et al., 2022) have shown a statistically significant PFS benefit when used in combination with ET in first/second-line advanced ER⁺/HER2⁻ BC clinical settings. Despite similar improvements in PFS benefit, important differences have emerged with regard to overall survival in the first-line setting (Cristofanilli et al., 2022) (Slamon et al., 2021) (Sledge et al., 2020). Only clinical studies using ribociclib in co-administration with AI have demonstrated a significantly prolonged overall survival (OS) (Lu et al., 2022) (Hortobagyi et al., 2022). In fact, OS benefit has not been observed for first-line palbociclib in combination with letrozole (Finn et al., 2022), and results regarding OS for first-line abemaciclib in combination with AI (NCT02246621) have not been reported yet (Goetz

et al., 2022b). Having demonstrated beneficial results in metastatic setting, CDK4/6i have also been tested in the adjuvant setting for early-stage HR⁺/HER2⁻ BC patients. The PALLAS (NCT02513394) and the Penelope-B (NCT01864746) clinical trials failed to display an invasive disease-free survival (IDFS) benefit by adding 1 year (Mayer et al., 2021) and 2 years (Loibl et al., 2021) of palbociclib, respectively, to adjuvant ET. Nevertheless, the phase 3 monarchE clinical trial (NCT03155997), demonstrated that adding 2 years of abemaciclib to adjuvant ET significantly improves IDFS in high-risk early-stage ER⁺/HER2⁻ BC patients. Indeed, abemaciclib decreases the risk of recurrence, and its benefit appears to extend beyond the completion of treatment, with an absolute increase at 4 years (Johnston et al., 2023). Besides, data from the ongoing phase 3 NATALEE clinical trial (NCT03701334), which is evaluating the broadest population to date of HR⁺/HER2⁻ early non-metastatic BC patients treated with 3 years of ribociclib and ET, are still pending. Notably, the reduced starting dose of ribociclib in this trial is expected to further minimize dose-dependent toxicities without compromising the efficacy of the co-treatment. Thus, the NATALEE clinical trial will plausibly provide important answers that at present are still unmet (Slamon et al., 2023). The contrasting results between the PALLAS/Penelope-B clinical studies and the monarchE trial are possibly due to duration of the CDK4/6i treatment, pharmacological distinctions between the agents, and differences in study population. Additional CDK4/6i are being investigated in clinical trials. In the DAWNA-2 phase 3 clinical trial (NCT03966898), combination of CDK4/6i dalciclib and anastrozole or letrozole, has shown a significantly longer PFS (30.6 months) compared to placebo plus letrozole or anastrozole (18.2 months), suggesting that these co-treatments could be a novel alternative first-line treatment alternative option to the current treatment landscape, in HR⁺/HER2⁻ advanced BC patients. Grade 3 or grade 4 adverse events were seen in 90 % of patients with dalciclib and 12 % with placebo, whereas serious adverse events were 12 % in dalciclib-treated patients and 7 % in placebo-exposed patients (Zhang et al., 2023). Other clinical trials are testing the anticancer activity and safety of novel CDK4/6i in HR⁺/HER2⁻ advanced BC patients. Indeed, a phase 3 clinical trial (NCT05077449) is investigating biraciclib in combination with fulvestrant; a phase 3 clinical trial (NCT05438810) is evaluating FCN-437c in combination with fulvestrant ± goseraline; phase 3 clinical trials are using TQB3616 combined with ET (letrozole or anastrozole or tamoxifen) (NCT05780567) and fulvestrant (NCT05375461); a phase 2 clinical trial (NCT03519178) is investigating CDK2/CDK4/CDK6 inhibitor PF-06873600 either alone or in combination with hormone therapy; a phase 2 clinical trial (NCT05085002) is evaluating CDK4/CDK6/CDK9 inhibitor lerociclib in combination with standard ET (fulvestrant or letrozole); a phase 2 clinical trial (NCT04282031) is using BPI-1178 in combination with fulvestrant or letrozole; and finally, a phase 1 clinical trial (NCT04433494) is testing TY-302 both singly and in combination with tamoxifen. The clinical trials outlined above, including those that are ongoing, have established the following points, and may provide further insights related to: 1) the role CDK4/6i monotherapy treatment can play in mediating antiproliferative and clinical anticancer activity in both pre- and post-menopausal HR⁺ BC patients; 2) how co-treatment with CDK4/6i and ET synergistically improves clinical outcomes, suggesting that CDK4/6i inhibition can delay or overcome ET resistance; and 3) how different toxicity profiles of CDK4/6i impact their treatment schedule, leading in some cases to intermittent dose interruptions as opposed to continuous administration, for example as it necessary with regard to palbociclib/ribociclib versus abemaciclib (Goel et al., 2022) (Fig. 4) (SUPPLEMENTARY FIGURE 1) (Table 1) (SUPPLEMENTARY TABLE 1)

5. Mechanisms of resistance to CDK4/6i in ER⁺ breast cancer

Though small-molecule CDK4/6i have shown significant clinical benefit, treatment failure and eventual resistance still represent major obstacles for HR⁺ BC patients (Finn et al., 2016) (Goetz et al., 2017)



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Fig. 4. CDK4/6i suppress cell cycle progression to S phase in ER⁺ breast cancer. FDA-approved CDK4/6i palbociclib, ribociclib and abemaciclib, as well as other CDK4/6i currently being tested such as dalpiciclib (phase 3), biroiciclib (phase 3), FCN-437 C (phase 3), TQB3616 (phase 3), PF-06873600 (phase 2), lerociclib (phase 2), BPI-1178 (phase 2), and TY-302 (phase 1), suppress cell cycle progression to S phase in ER⁺ breast cancer. Activation is shown with arrowhead lines, whereas inhibition is indicated with blocked lines. Dotted lines indicate lower relative inhibition activity. Red crosses emphasize signaling blockage in breast cancer. PI3K STP: PI3K/AKT/mTOR signal transduction pathway; ER STP: estrogen receptor signal transduction pathway; CDK4/6: cyclin-dependent kinase 4 and cyclin-dependent kinase 6; CDK4/6i: cyclin-dependent kinase 4 and cyclin-dependent kinase 6 inhibitors; CDK2: cyclin-dependent kinase 2; CDK9: cyclin-dependent kinase 9; E2F: activating E2Fs (E2F1-E2F3); CAK: CDK-activating kinase; DP: dimerization partner transcription factor; Circled P (black background with white text): phosphoryl group during direct or indirect phosphorylation.

(Goetz et al., 2020a). In the PALOMA-3 clinical trial, analysis of circulating tumor DNA (ctDNA) from HR⁺ BC patients demonstrated that two different types of resistance can occur due to co-treatment of CDK4/6i and anti-estrogens. In this study, mutation-induced resistance depended on the type of treatment and appeared at definite times. This suggested that mutations causing initial resistance are different compared to mutations needed for resistance arising after a period of efficacious treatment (Scheidemann and Shajahan-Haq, 2021). Hence, resistance occurring immediately after treatment that cause no response to BC patients is referred as intrinsic resistance (Kong et al., 2019), whereas resistance occurring in BC patients who initially respond but progress later on treatment is defined as acquired resistance (O’Leary et al., 2018). Estrogen receptor (ER) is the only routinely utilized clinical biomarker to select ER⁺ BC patients for co-treatment of CDK4/6i and ET

(Griffiths et al., 2021) (Scheidemann and Shajahan-Haq, 2021). Numerous preclinical studies have implicated a wide array of potential resistance mechanisms (Fig. 5), such as enhanced activity through oncogenic signaling pathways, modifications of cell cycle machinery components, metabolic variations within tumor cells, and drug-induced alterations in stromal function (Zhou et al., 2023). In line with this, several mechanisms of CDK4/6i resistance highlight new targets, such as signaling mediators AKT1, fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), and RAS, as well as cell cycle elements, including cyclin E, CDK2, CDK6, Rb1, AURKA, and c-Myc (Wander et al., 2020) (Asghar et al., 2022). It is possible that suppression of CDK4/6 can delay initiation of endocrine resistance, and resistance to combination therapy is mainly caused by ET backbone-associated resistance (O’Leary et al., 2018). In favor of this, biomarker analysis

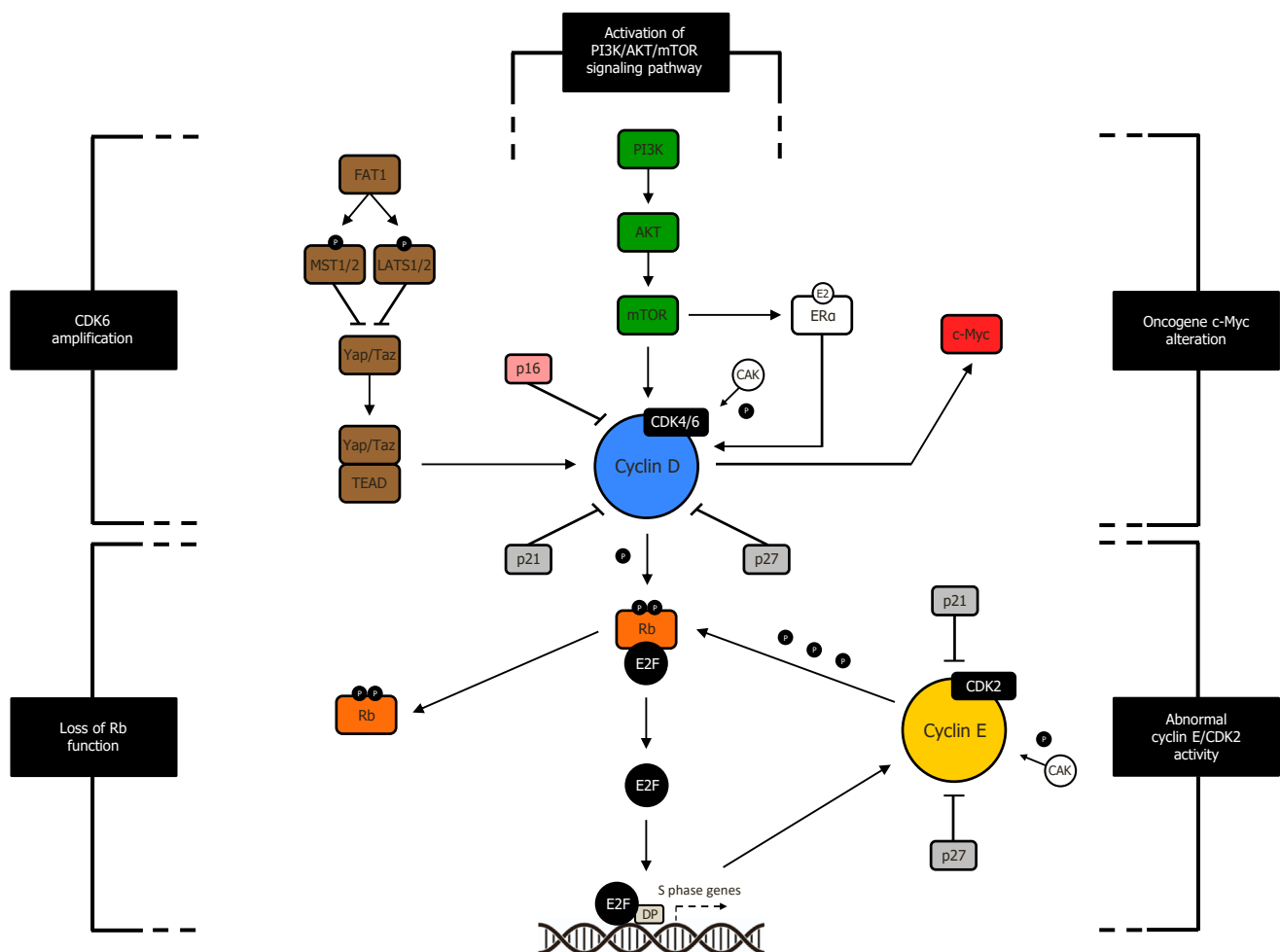


Fig. 5. Mechanisms of CDK4/6i and ET resistance. Mechanisms of CDK4/6i and ET resistance presently reported in literature comprise loss of Rb function, acquired CDK6 amplification, abnormal cyclin E/CDK2 activity, oncogene c-Myc alteration, and activation of PI3K/AKT/mTOR signaling pathway. Activation is shown with arrowhead lines, whereas inhibition is indicated with blocked lines. CDK4/6: cyclin-dependent kinase 4 and cyclin-dependent kinase 6; CDK2: cyclin-dependent kinase 2; ERα: estrogen receptor alpha; E2: estradiol; E2F: activating E2Fs (E2F1-E2F3); CAK: CDK-activating kinase; DP: dimerization partner transcription factor; circled P (black background with white text): phosphoryl group during direct or indirect phosphorylation.

of ctDNA in MONARCH-3 clinical trial has demonstrated reduced frequency of *ESR1* mutations with the combination of abemaciclib and AI (17 %) in comparison to AI singly (31 %) (Goetz et al., 2020b). In this section, the genomic aberrations identified to date, and the major mechanisms of resistance presently sustained by preclinical studies and clinical trials are discussed in detail (Fig. 5).

5.1. Loss of Rb function

CDK4/6i act to preclude CDK4/6-mediated phosphorylation-induced Rb protein inactivation (Fassl et al., 2022) (Watt and Goel, 2022). Thus, mutations leading to *RB1* biallelic loss of function drive resistance to CDK4/6i (Herrera-Abreu et al., 2016). Accordingly, cells harboring a dysfunctional Rb protein display continuing proliferation, and their cell cycle remains unchecked even during exposure to CDK4/6i. Therefore, loss of functional Rb is a well-established mechanism of resistance to CDK4/6i, though it appears to occur only rarely. Indeed, combinations of palbociclib and fulvestrant, or ribociclib and letrozole, have been shown to provoke *RB1* mutations in metastatic ER⁺ BC patients (Conadorelli et al., 2018). In the PALOMA-3 phase 3 clinical trial (NCT01942135), using whole-exome sequencing of paired ctDNA samples, acquired *RB1* mutations were seen in BC patients who progressed on the combination of palbociclib and fulvestrant (O'Leary et al., 2018). Moreover, combination of ribociclib and ET displayed shorter PFS in cancer patients harboring *RB1* mutations compared to wildtype (Kumarasamy et al., 2022). Notoriously, *RB1* loss of heterozygosity is also related to intrinsic CDK4/6i resistance. In addition, *RB1* mutations are commonly acquired in CDK4/6i-treated ER⁺ BC patient-derived xenografts and ER⁺ BC patients with pre-existing *RB1* loss of heterozygosity (Palafox et al., 2022). Other clinical studies have also identified loss of functional *RB1* as a principal feature of resistance to CDK4/6i in HR⁺ metastatic BC (Wander et al., 2020) (Fig. 5).

5.2. Acquired CDK6 amplification

Interestingly, CDK6 amplification can also provoke BC resistance to CDK4/6i (Yang et al., 2017). It is unclear whether this phenomenon is due to incomplete drug-mediated CDK6 inhibition, or alternatively, to other kinase-independent effects of CDK6 (Wu et al., 2021a). CDK6 upregulation can occur in response to continued exposure to CDK4/6i, and sensitivity can be re-established via subsequent CDK6 knockdown in preclinical studies. Accordingly, a recent clinical study has shown an inverse correlation between CDK6 amplification and shorter PFS with CDK4/6i treatment in ER⁺ BC patients. Besides, combined score of cyclin E1, p-CDK2, and CDK6 predict a worsen outcome in co-treated ER⁺ BC patients, as well as in only ET-treated ER⁺ BC patients (Al-Qasem et al., 2022). Cadherin superfamily member FAT1 (Chen et al., 2022) loss of function mutations, though rare, are related to CDK4/6i resistance, possibly by enhancing the expression of CDK6 in ER⁺ BC patients (Li et al., 2018). Notably, FAT1 inactivation/deletion leads to induction of Hippo signal transduction pathway (Martin et al., 2018), known to regulate apoptosis and cell growth (Steinhardt et al., 2008). Consequently, loss of FAT1 promotes augmentation of Yap/Taz transcription factors, whose activity endorses the overexpression of CDK6. Patients with biallelic FAT1 inactivation demonstrate a 2.4-month PFS, compared to ER⁺ BC patients harboring FAT1 missense mutations, that show only a slightly shorter PFS (10.1 months) when compared to patients with wildtype FAT1 (11.3 months) (Li et al., 2018). The exosomal miRNA-432-5p, which is a target of TGFBR3 and SMAD4, also confers acquired resistance to CDK4/6i by augmenting CDK6 expression, downregulating SMAD4, and ultimately reducing G1/S cell cycle arrest (Cornell et al., 2019). Interestingly, removal of palbociclib downregulates CDK6, cyclin D1-encoding *CCND1*, and miRNA-432-5p; and conversely, upregulates Rb. Thus, resistance of ER⁺ BC cells to palbociclib can be modulated both *in vitro* and preclinical models (Cornell et al., 2019), which may suggest why some ER⁺ BC patients progressing

on a particular CDK4/CDK6i may subsequently respond to a distinct CDK4/CDK6i (Fig. 5).

5.3. Abnormal cyclin E/CDK2 activity

Evasion from CDK4/6i may also occur via Rb phosphorylation through upregulation of cyclin E/CDK2. Indeed, cyclin E1 and cyclin E2 amplification leads to augmented CDK2 activity and decreased expression of p27 (Goel et al., 2018) (Fassl et al., 2022) (Watt and Goel, 2022). Cyclin E2-encoding *CCNE2* gene amplification is often detected in HR⁺ metastatic BC treatment-resistant tumor specimens (Wander et al., 2020), and overexpression of G1/S-specific cyclin-E1-encoding *CCNE1* mRNA is associated with poor response to palbociclib in tumor tissue taken from HR⁺ metastatic BC patients (Turner et al., 2019). This may be due to the fact that elevated levels of cyclin E results in CDK2-induced phosphorylation of Rb, overcoming CDK4/6i-generated G1-phase arrest (Herrera-Abreu et al., 2016). CDK2 mRNA expression is also upregulated in ER⁺ cell lines resistant to palbociclib; and susceptibility to palbociclib can be successfully re-established by siRNA-mediated CDK2 knockdown, leading to enhanced apoptosis and senescence (Pandey et al., 2020) (Zhou et al., 2023). Interestingly, mesenchymal-epithelial transition factor (c-MET) (Su et al., 2023) signaling, and its downstream effector focal adhesion kinase (FAK) (Li et al., 2023), may also contribute to abnormal CDK2 activation independently of CDK4/6 (Zhang et al., 2019). Accordingly, ctDNA analysis of abemaciclib monotherapy has shown 8 % acquired genomic aberrations in *MET* (Goetz et al., 2020b). Moreover, PI3K/AKT/mTOR pathway upregulation can also promote non-canonical CDK2 activity by binding to cyclin D, leading to consequent progression of cell cycle and acquired resistance to CDK4/6i (Herrera-Abreu et al., 2016) (Fig. 5).

5.4. Oncogene c-Myc alteration

Members of the MYC oncogenic family are transcription factors responsible for the regulation of several genes. c-Myc gene, which belongs to the MYC family, is often persistently expressed in cancer (Karakhelkar et al., 2023), leading to enhanced expression of various genes involved in cell proliferation (Gao et al., 2023). In addition, c-Myc has been found to be highly expressed in BC (Fallah et al., 2017) (Gao et al., 2023). c-Myc is activated by CDK2, CDK4 and CDK6, and is upregulated in preclinical models of CDK4/6i resistance (Pandey et al., 2020) (Freeman-Cook et al., 2021a). In line with this, the nextMONARCH-1 clinical trial (NCT02747004) has shown an enhancement of acquired *MYC* genomic aberrations after treatment of abemaciclib monotherapy or in combination with a nonsteroidal AI, in HR⁺ BC patients (Goetz et al., 2020b). Moreover, S6K1 kinase activation has been detected in more than 10 % of BC patients, and several studies have shown that increased S6K1 can drive palbociclib resistance by inducing c-Myc signal transduction in preclinical models and clinical ER⁺ BC samples (Mo et al., 2022) (Fig. 5).

5.5. Activation of growth factor signaling pathways

Analysis of genomic alterations in clinical samples has implicated growth factor signal transduction pathways as a mechanism of CDK4/6i resistance. Determining the unilateral impact of growth factor pathway activation on CDK4/6i resistance is complex, as most patients are treated with the combination of these agents and ET. Many of these pathways have been involved in ET resistance. Collecting tumor biopsies at progression on CDK4/6i in several cohorts have provided further insights related to acquired and intrinsic resistance. These studies have demonstrated significant enrichment of hyperactivating alterations in *ERBB2*, *P TEN*, *AKT1*, *FGFR*, *KRAS*, *HRAS*, and *NRAS* genes, in CDK4/6i resistant cancers (Watt and Goel, 2022) (Wander et al., 2020). Additionally, similar findings have been discovered in ctDNA biomarker analysis performed in the MONARCH-3 clinical trial (NCT02246621),

by which acquired mutations in *FGFR1* and *EGFR* and are detected in the abemaciclib group (Goetz et al., 2020b). Elevated phospho-AKT level in BC metastases is also biomarker for poor prognosis and is associated with reduced PFS in ER⁺ BC patients who have been administered CDK4/6i and ET (Alves et al., 2021). Nonetheless, these analyses are limited by multiple factors, including the absence of control group consistent of an ET-only treated cohort, administration of other lines of therapy after CDK4/6i before acquiring cancer tissue for assessment, and a modest number of BC patients. These studies suggest that alterations in growth factor signaling pathways, especially the PI3K/AKT/mTOR axis (Glaviano et al., 2023), can contribute to CDK4/6i resistance. However, the mechanism of action whereby these mutations confer CDK4/6i resistance, and their relative contribution to concurrent ET resistance, is still uncertain (O'Brien et al., 2020) (Fig. 5). Considering recent evidence that active cellular growth signalling promotes CDK4/6i sensitivity (Manohar et al., 2023) (Crozier et al., 2023) (Foy et al., 2023) these mechanisms of resistance might be specific for the combinatorial treatment of CDK4/6i with ET. The activation of the MAPK signaling pathway and its key effectors, RAS, RAF, MEK, and ERK, has also been observed in BC preclinical models that are resistant to CDK4/6i. Notably, the triple combination of MEK inhibitors, CDK4/6i and ET has shown promising anticancer activity in CDK4/6i-resistant models (de Leeuw et al., 2018) (Gomes et al., 2023). These findings suggest that MEK inhibition may be a novel valuable therapeutic option to treat or prevent CDK4/6i resistance. Besides, the loss of neurofibromin 1 (NF1), a gene that downregulates RAS signaling and cellular proliferation, is an established mechanisms of resistant to ET (Hanker et al., 2020). However, its role in CDK4/6 resistance remains to be fully clarified.

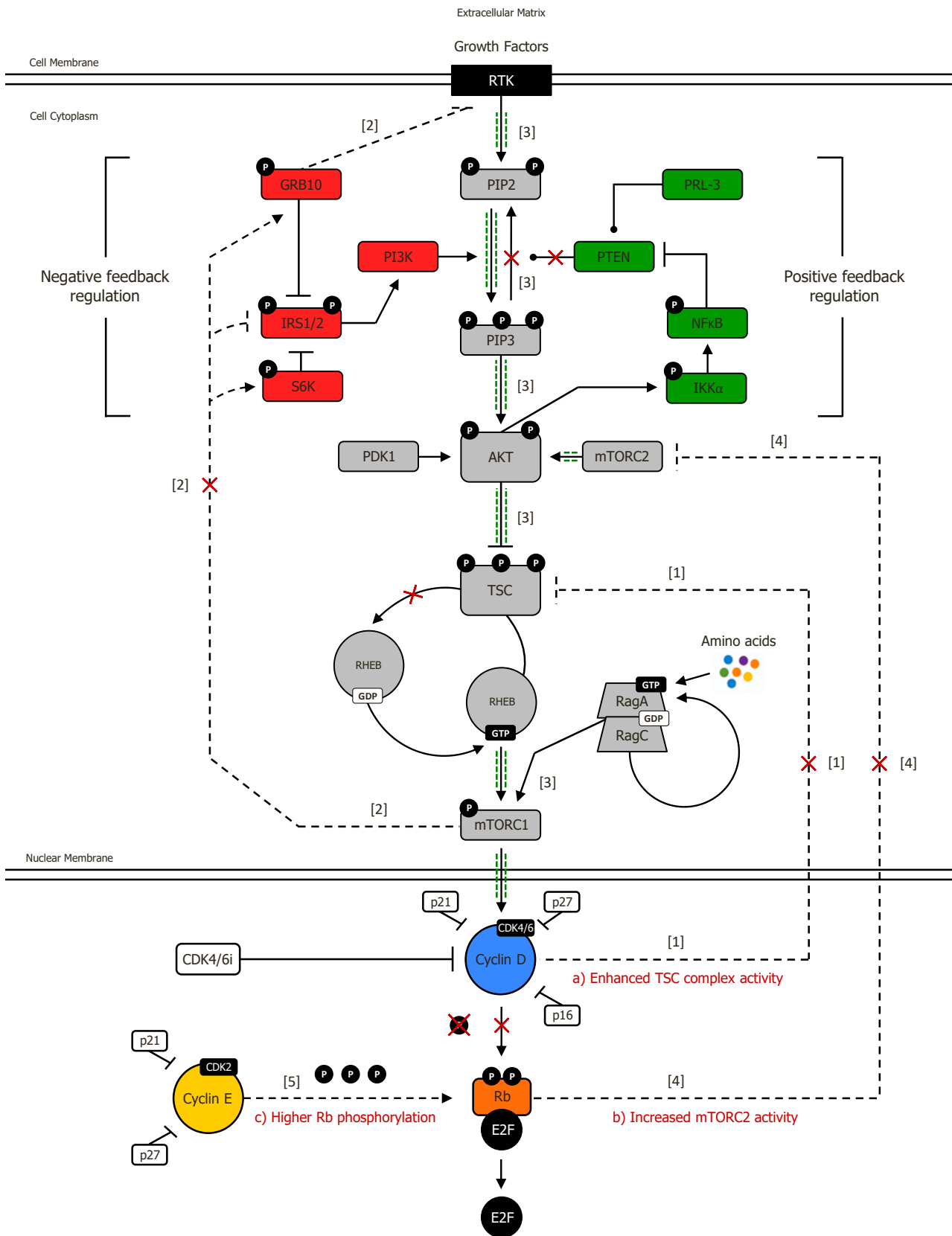
5.6. CDK4/6i-induced rewiring of the PI3K/AKT/mTOR pathway

In the PI3K/AKT/mTOR signaling pathway, activation of PI3K mainly occurs by growth factor-induced receptors, such as receptor tyrosine kinases (RTKs). PI3K is then recruited to its substrate PIP2, endorsing the production of PIP3. Inactive AKT in the cytoplasm binds to PIP3 on the cell membrane and allows phosphorylation by PDK1 and mTORC2, resulting in complete activation of AKT, which successively phosphorylates multiple sites on the tuberous sclerosis complex 2 (TSC2). The TSC2 protein forms a functional complex with tuberous sclerosis complex (TSC1) protein called TSC1-TSC2 complex or simply tuberous sclerosis complex (TSC). AKT-mediated phosphorylation on TSC2 hinders the ability of TSC to act as a GTPase-activating protein (GAP) toward the small GTPase Rheb, endorsing Rheb-GTP accumulation (Manning and Toker, 2017). Importantly, the PI3K/AKT/mTOR axis is regulated by negative feedback, via S6K/IRS/GRB10, and positive feedback, via IKK α /NF κ B/PTEN, to ensure that signal transductions are captured and conveyed transiently (Glaviano et al., 2023). Importantly, suppression of CDK4/6 can promote adaptive rewiring of the PI3K/AKT/mTOR signaling pathway in BC cells, thereby augmenting their dependence on this axis. Indeed, inhibition of CDK4/6 has been found to enhance phosphorylation and activity of HER receptors and PI3K/AKT/mTOR pathway in various HR⁺ BC models (Goel et al., 2022). This adaptive rewiring is driven by three diverse mechanisms: 1) CDK4/6 directly activates mTORC1 through phosphorylation of the tumour suppressor TSC, and partial inhibition of mTORC1 mediated by CDK4/6i can relieve feedback suppression on upstream RTKs (Romero-Pozuelo et al., 2020) (Goel et al., 2016); 2) CDK4/6i-mediated hypo-phosphorylation of Rb directly promotes access of AKT to stress-activated protein kinase-interacting protein (SIN1), a crucial component of mTOR complex 2 (mTORC2), leading to increased mTORC2-induced phosphorylation of AKT (Zhang et al., 2016); and 3) CDK4/6i activate secretion of cancer cell-derived growth factors, perhaps as part of SASP, which trigger signaling through RTKs in an autocrine manner (Olmez et al., 2018). These adaptive changes may dictate acquired CDK4/6i resistance, and cause failure of CDK4/6i

monotherapy, emphasizing the importance of combinatorial studies targeting CDK4/6 and PI3K/AKT/mTOR pathway or other mitogenic signaling axes. Notably, upstream mitogenic pathways augment CDK2 activity, endorsing Rb phosphorylation and S phase entry, in spite of ongoing CDK4/6 inhibition (Herrera-Abreu et al., 2016) (Knudsen et al., 2019) (Kumarasamy et al., 2021). Mitogenic signaling-mediated enhancement of CDK2 activity may occur through p21/p27 sequestration in cyclin D1-CDK4/6 complexes, direct downregulation of p27, and increased formation of atypical cyclin D1/CDK2 complexes caused by higher levels of cyclin D1 (Goel et al., 2022). Besides, enhanced mitogenic signalling pathways augments mTORC1 activity, which regulates S phase entry and drives proliferation despite continued blockage of CDK4/6 (Fingar et al., 2004) (Goel et al., 2022). Accordingly, numerous studies have shown synergistic efficacy by co-inhibiting CDK4/6 and mTOR (Michaloglou et al., 2018). Additionally, in the phase 1b TAKTIC clinical trial (NCT03959891), combination therapy with CDK4/6i, AKT inhibitor ipatasertib, and ET, has shown significant clinical activity, emphasizing the importance of PI3K/AKT/mTOR pathway inhibitor-based combinations in HR⁺/HER⁻ metastatic BC patients (Wander et al., 2022b). BC cell responses to co-treatment of CDK4/6i and PI3K/AKT/mTOR inhibitor include cytostasis and/or apoptosis. Normally, combination-induced cytostasis is characterized by increased Rb hypo-phosphorylation compared to either inhibitor alone, followed by enhanced downregulation of E2F targets and augmented senescence (Vora et al., 2014) (Goel et al., 2016) (Herrera-Abreu et al., 2016) (Jansen et al., 2017) (Goel et al., 2022) (Fig. 6).

5.7. Proliferation mechanisms despite CDK suppression

Due to the intrinsic plasticity of cell cycle machinery, BC cells may develop peculiar mechanisms whereby they can still divide in spite of co-inhibition of all CDKs acting in the interphase. It was believed that mammalian cells required the subsequent induction of CDK2, CDK3, CDK4 and CDK6, to guide cells through interphase, and CDK1 activation to advance during mitosis (Malumbres and Barbacid, 2005). Nonetheless, recent genetic data have shown survival of mice in the absence of different CDKs. Indeed, mammalian cells absent of CDK2, CDK3, CDK4, and CDK6 are still able to proliferate via CDK1. Thus, it would be important to investigate whether BC cells exposed to combinations of CDK2 inhibitor (CDK2i) and CDK4/6i may also adopt this particular mechanism (Santamaría et al., 2007). Interestingly, while cells treated with the specific CDK2 inhibitor PF3600 (Freeman-Cook et al., 2021a) (Freeman-Cook et al., 2021b) can proliferate by potentially using CDK1, combinatorial treatment with CDK2i and CDK4/6i prevents this phenomenon (Arora et al., 2023). Overall, this plausible mechanism could be overcome by suppressing CDK7, which exerts two essential roles: 1) as CDK-activating kinase, exerting phosphorylation on CDK1, CDK2, CDK4 and CDK6; and 2) as an intermediary molecule of RNA polymerase II-induced transcription (Larochelle et al., 1998) (Glover-Cutter et al., 2009). Accordingly, specific CDK7 inhibitors such as samuraciclib have been evaluated in clinical development (NCT03363893) and have displayed promising antitumor activity in CDK4/6i-resistant HR⁺/HER⁻ advanced BC patients (Coombes et al., 2023). Furthermore, recent work from Wilson et al. (2023) has shown that cellular growth also has a central role in CDK7 inhibitor samuraciclib sensitivity. Indeed, increased growth alone determines sensitivity to a CDK7 inhibitor, contributing to explaining why some tumors are more susceptible to CDK inhibition than normally growing cells (Wilson et al., 2023). Since enhanced growth signalling is common in cancer cells, these aforesaid studies help explain the anti-cancer efficacy of CDK inhibitors, but also suggests that decreased growth signalling might allow proliferation despite suppression of all CDKs.



(caption on next page)

Fig. 6. CDK4/6 inhibition induces adaptive rewiring of PI3K/AKT/mTOR signaling pathway. Suppression of CDK4/6 inhibits Rb phosphorylation, thereby impeding E2F-induced cell cycle progression. However, other mechanisms may counterbalance these cell cycle-promoting effects. Indeed, a) reduced CDK4/6 activity can downregulate mTORC1 signalling via increased TSC activity [1], which may result to loss of negative feedback regulation of RTK signalling [2], leading to up-regulated upstream PI3K/AKT signaling pathway activity and cell proliferation [3]. Similarly, b) decreased phosphorylated-Rb levels can also result in augmented AKT phosphorylation by mTORC2 [4], which stimulates cell survival mechanisms. Besides, c) compensatory CDK2-mediated Rb phosphorylation [5] can induce cell cycle progression in the absence of CDK4/6 signalling. In PI3K/AKT/mTORC signaling pathway, proteins involved in negative feedback regulation are figured in red rectangles, whereas proteins involved in positive feedback regulation are figured in green rectangles. Activation (phosphorylation or non-phosphorylation) is shown with arrowhead lines, dephosphorylation is indicated with roundhead lines, and inhibition is displayed with blocked lines. CDK4/6i-mediated adaptive rewiring of PI3K/AKT/mTOR signaling pathway and cyclin E/CDK2 is shown with blocked dotted lines. Red crosses emphasise signaling blockage, whereas green dash-dotted lines (adjacent to arrowhead lines or blocked lines) highlight signaling enhancement. RTK: receptor tyrosine kinase; PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: Phosphatidylinositol (3,4,5)-trisphosphate; TSC: Tuberous sclerosis complex; RHEB: Ras homolog enriched in brain; GDP: Guanosine diphosphate; GTP: Guanosine triphosphate; Rag A and Rag C: Rag heterodimers; CDK4/6: cyclin-dependent kinase 4 and cyclin-dependent kinase 6; CDK4/6i: cyclin-dependent kinase 4 and cyclin-dependent kinase 6 inhibitors; CDK2: cyclin-dependent kinase 2; E2F: activating E2Fs (E2F1-E2F3); circled P (black background with white text): phosphoryl group.

6. Therapeutic strategies to address drug resistance following CDK4/6i treatment in ER+ breast cancer

There are a variety of available and emerging therapeutic approaches that may be utilized after progression on combination of small-molecule CDK4/6i and ET, including: 1) prolonging CDK4/6i after progression; 2) targeting CDK2 concurrently with or following CDK4/6i resistance; 3) co-targeting CDK4/6 and the PI3K/AKT/mTOR pathway; 4) using new endocrine therapies; 5) using non-endocrine therapies. Results from completed and current clinical trials in ER+/HER2- BC patients following progression on CDK4/6i, are summarized in [SUPPLEMENTARY TABLE 2](#); whereas ongoing clinical trials in ER+/HER2- BC patients following progression on CDK4/6i, whose results are still being awaited, are summarized in [SUPPLEMENTARY TABLE 3](#).

6.1. Continuation of CDK4/6i blockade after progression

The potential utility of CDK4/6i after prior progression on one of these agents remains an important clinical question and an active area of research. In the phase 2 PACE trial (NCT03147287), the combination of fulvestrant plus palbociclib demonstrated no significant improvement in PFS (4.6 months) compared to fulvestrant alone (4.8 months), in advanced ER+/HER2- BC patients who received prior CDK4/6i (91 % of whom received palbociclib) and an AI ([Mayer et al., 2023](#)). The triplet combination therapy with abemaciclib, programmed death-ligand 1 (PD-L1)-neutralizing antibody atezolizumab, and fulvestrant is being evaluated in the MORPHEUS HR+ BC platform trial (NCT03280563) ([Yardley et al., 2019](#)). In contrast to the results from PACE, in the phase 2 MAINTAIN trial (NCT05207709), the combination of altered ET backbone (fulvestrant or exemestane) and ribociclib exhibited significant PFS benefit (5.2 months) compared to a change in the ET backbone (fulvestrant or exemestane) plus placebo (2.7 months), in advanced ER+/HER2- BC patients who progressed on a CDK4/6i (84 % of whom received palbociclib). In this population the PFS rate was 41.2 % at 6 months and 24.6 % at 12 months with ribociclib, compared to 23.9 % at 6 months and 7.4 % at 12 months with placebo ([Kalinsky et al., 2023](#)). In the phase 2 BioPER trial (NCT03184090), a change in the ET and continued palbociclib displayed a PFS of 2.6 months and a CBR of 34 %, in advanced ER+/HER2- BC patients who demonstrated prior clinical benefit with palbociclib plus ET. Notably, a biomarker signature of high cyclin E1 (≥ 10 % BC cells with positive nuclear staining), low Rb score (< 1 % BC cells with positive nuclear staining), and *ESR1* mutation was independently related to shorter PFS in this study, suggesting that there may be practical strategies to identify a subset of patients who may not obtain benefit from palbociclib treatment continuation ([Albanell et al., 2023](#)). Moreover, in the phase 1b TAKTIC clinical trial (NCT03959891), the co-administration of palbociclib, AKT inhibitor ipatasertib, and ET determined a CBR of 48 %, a PFS of 5.5 months, and an OS of 24.5 months, after prior CDK4/6i progression, in HR+/HER- metastatic BC patients ([Wander et al., 2022b](#)).

6.2. Targeting CDK2 concurrently with or after CDK4/6i resistance

Due to the important role of cyclin E/CDK2 in CDK4/6i resistance ([Pandey et al., 2020](#)) ([Al-Qasem et al., 2022](#)), there has been increasing interest in targeting CDK2 either concurrently with or after occurrence of CDK4/6i resistance. Interestingly, the triple combination of non-selective CDK2i dinaciclib, CDK4/6i palbociclib and AI letrozole, demonstrated enhanced therapeutic efficacy compared to palbociclib and/or letrozole singly in preclinical studies ([Al-Qasem et al., 2022](#)). A variety of CDK2i are being investigated in phase 1/phase 2 clinical trials in ER+/HER2- BC patients (NCT04553133 and NCT05252416), as well as advanced solid tumor patients (NCT05867251, NCT04553133 and NCT05252416). Indeed, in the recent VELA phase 1 clinical trial (NCT05252416), CDK2i BLU-222 monotherapy has shown preliminary evidence of cell cycle pathway inhibition, clinical anticancer activity, and an acceptable safety profile, in HR+/HER2- BC and other advanced solid tumor patients. Dose escalation of BLU-222 is ongoing to reach the RP2D ([Patel et al., 2023](#)). In another recent phase 1/phase 2 clinical trial (NCT04553133), the novel CDK2i PF-07104091 has exhibited anticancer activity as a single agent [partial response (PR): 18.8 %, duration of response (DOR) > 6 months: 12.5 %, stable disease (SD): 37.5 %, and disease control rate (DCR): 61.5 %], and a favorable safety profile in heavily-pretreated HR+/HER2- advanced/metastatic BC patients who progressed on prior ET plus CDK4/6i. Dose expansions of PF-07104091 are ongoing in combination with fulvestrant in HR+/HER2- BC patients ([Yap et al., 2023](#)). In addition, an ongoing phase 1/phase 2 clinical trial (NCT03519178) is evaluating the safety, tolerability, as well as pharmacokinetic and pharmacodynamic properties of the CDK2/CDK4/CDK6 inhibitor PF-06873600 both singly and in combination with ET (letrozole or fulvestrant) in heavily pretreated advanced/metastatic HR+/HER2- BC patients after progression on previous CDK4/6i, ET, and ≤ 2 prior lines of chemotherapy. Early results suggest that the combination of PF-06873600 and ET exerts promising preliminary anticancer activity, modulation of cell cycle pharmacodynamic biomarkers (Ki-67 and pRb) in paired cancer biopsies, and a manageable safety profile ([Yap et al., 2022](#)).

6.3. Co-targeting PI3K/AKT/mTOR pathway

FDA-approved small molecule inhibitors targeting the PI3K/AKT/mTOR axis are available and under clinical development. Due to the clinical advantage of prolonged CDK4/6 inhibition and the complex crosstalk between CDK4/6 and the PI3K/AKT/mTOR pathway, various combinations of PI3K/AKT/mTOR inhibitors are being investigated in ER+ BC patients who have displayed progression on CDK4/6i. Anticancer potency of PI3K/AKT/mTOR pathway inhibitors, as well as potential toxicity has prompted careful ongoing evaluation before establishing feasible multidrug regimens.

6.3.1. PI3K co-inhibition

Induction of PI3K/AKT/mTOR pathway activation via *PIK3CA*

mutations frequently occurs in ER⁺/HER2⁻ advanced BC patients and is associated with poor prognosis. In the phase 3 SOLAR-1 trial (NCT02437318), the combination of isoform-specific PI3K α inhibitor alpelisib and fulvestrant demonstrated improvement in OS (by 7.9 months) compared to fulvestrant alone in *PIK3CA*-mutated advanced ER⁺/HER2⁻ BC patients who had relapsed after previous AI. Even though the analysis of this trial did not reach statistical significance, the 7.9-month improvement in median OS upon alpelisib addition to fulvestrant treatment further supported the statistically significant prolongation of PFS detected with alpelisib + fulvestrant in this ER⁺/HER2⁻ BC patient population, which presents a poor prognosis due to a *PIK3CA* mutation. Hence, this study established a new standard of care for patients with *PIK3CA* mutations following progression on first-line therapy, and alpelisib is now in widespread clinical use based upon these results. The safety profile was manageable, with relatively high rates of hyperglycemia, and ER⁺/HER2⁻ BC patients who discontinued alpelisib and placebo due to adverse events were 25 % and 4.2 %, respectively (André et al., 2021). In the phase 2 BYLieve trial (NCT03056755), the co-treatment of the isoform-specific PI3K α inhibitor alpelisib and fulvestrant improved PFS and OS with manageable toxicity in ER⁺ BC patients following progression on CDK4/6i with detectable *PIK3CA* mutations, as compared to SOLAR1 trial, which included relatively few patients with prior CDK4/6 exposure. Notably, the most frequent \geq grade 3 adverse events were hyperglycaemia (28 %) and rash (9 %). Serious adverse events occurred in 26 % of ER⁺ BC patients, and no treatment-related deaths were reported (Rugo et al., 2021). In addition, there are a number of ongoing clinical studies evaluating co-administration of alpelisib, along with other novel next-generation PI3K inhibitors and antiestrogen therapy in ER⁺/HER2⁻ BC patients following progression on CDK4/6i, including the SEQUEL-Breast phase 2 (NCT05392608) (Almekinders et al., 2023), and the EPIK-B5 phase 3 (NCT05038735) (De Laurentiis et al., 2022).

6.3.2. AKT co-inhibition

Analysis of clinical samples following CDK4/6 progression have implicated enhanced AKT signaling as a potential resistance mechanism (Wander et al., 2020). As a result, there has been increasing interest in the use of AKT-specific kinase inhibitors following CDK4/6 progression. Co-treatment with CDK4/6i palbociclib, AKT1/AKT2/AKT3 inhibitor capivasertib, and fulvestrant, has demonstrated effectiveness in inhibiting cancer growth in preclinical models resistant to CDK4/6i and ET (Alves et al., 2021). Indeed, in the phase 1b TAKTIC clinical trial (NCT03959891), the combination of palbociclib, AKT inhibitor ipatasertib, and ET showed 48 % CBR, 5.5 months PFS, and 24.5 months OS, after prior CDK4/6i progression, in HR⁺/HER⁻ metastatic BC patients (Wander et al., 2022b). In line with this, the FAKTION phase 2 clinical trial (NCT01992952) interrogated the combination of capivasertib and fulvestrant and demonstrated superior PFS and OS compared to fulvestrant and placebo, in (CDK4/6i naïve) endocrine-resistant advanced ER⁺/HER2⁻ BC patients. Of note, clinical antitumor activity was more pronounced in PI3K/AKT/PTEN pathway-altered ER⁺/HER2⁻ BC patients (Howell et al., 2022). The phase 3 CAPItello-291 trial (NCT04305496) also explored the co-administration of capivasertib and fulvestrant and demonstrated significant improvement in PFS (7.2 months) compared to fulvestrant and placebo (3.6 months) in ER⁺/HER2⁻ BC patients, among whom 69 % had received prior CDK4/6i therapy. The most frequent \geq grade 3 adverse events after treatment with capivasertib plus fulvestrant were rash (12.1 %) and diarrhea (9.3 %) compared to patients receiving placebo (0.3 % rash and 0.3 % diarrhea, respectively). Adverse events resulting in discontinuation were observed in 13 % of capivasertib-receiving patients and in 2.3 % of placebo-receiving patients (Turner et al., 2023). Finally, the ongoing FINER phase 3 clinical trial (NCT04650581) is investigating the combination of AKT inhibitor ipatasertib and fulvestrant, in ER⁺/HER2⁻ BC patients who had progression on CDK4/CDK6i and ET.

6.3.3. mTOR co-inhibition

At present, co-treatment with exemestane and the allosteric non-competitive mTOR inhibitor everolimus has only shown limited clinical benefit in ER⁺ BC patients after progression on CDK4/6i (Cook et al., 2021). In the TRINITY-1 phase 1/phase 2 clinical trial (NCT02732119), the triplet combination of ribociclib, exemestane, and everolimus, exhibited a PFS of 5.7 months, a CBR (at week 24) of 41 %, and no detection of new safety signals, in heavily-pretreated advanced ER⁺/HER2⁻ BC patients after progression on CDK4/6i (Bardia et al., 2021). In the successive B2151009 phase 1b clinical trial (NCT02684032), the combination of CDK4/6i palbociclib, ET, and the pan-PI3K/mTOR inhibitor gedatolisib displayed a 1-year PFS of 53 % in metastatic ER⁺/HER2⁻ BC patients who had received prior CDK4/6i therapy (Wesolowski et al., 2023). In addition, two ongoing clinical trials of triplet co-treatment with CDK4/6i, ET, and PI3K/AKT/mTOR pathway inhibitors include the CAPItello-292 phase 1b/phase 3 (NCT04862663) (Rugo et al., 2022a), and the VIKTORIA-1 phase 3 (NCT05501886) (Hurvitz et al., 2023) trials.

6.4. Using new endocrine therapies

The development of novel antiestrogen therapies that have the potential to restore sensitivity after progression on standard hormonal agents remains an active and exciting area of research and clinical development (Manohar and Davidson, 2021). Four completed randomized clinical trials of next-generation oral SERDs have shown conflicting results in advanced ER⁺/HER2⁻ BC patients who had progression on previous ET with/without CDK4/6i. In both the phase 2 SERENA-2 trial (NCT04214288) of camizestrant versus fulvestrant (Oliveira et al., 2023), and the phase 3 EMERALD trial (NCT03778931) of elacestrant versus physician's choice ET (Bidard et al., 2022), SERD administration demonstrated meaningful PFS benefit. Conversely, the accelERA phase 2 clinical trial (NCT04576455) of giredestrant (Martin Jimenez et al., 2022), and the AMEERA-3 phase 2 clinical trial (NCT04059484) of amcenestrant (Tolaney et al., 2023), failed to demonstrate any difference in PFS between SERD treatment and physician's choice ET. Notably, among these SERD monotherapy-based clinical studies, the EMERALD trial is the only study to mandate prior CDK4/6i. Importantly, there was also heterogeneity amongst these studies in the proportion of ER⁺/HER2⁻ patients with *ESR1* mutant disease. Across these clinical studies, the *ESR1* mutant subgroup demonstrated more clinical benefit in comparison to *ESR1* wildtype, further implicating *ESR1* as a valuable biomarker for ER dependence in these patients (Hoy, 2023). Based upon data from the EMERALD study, elacestrant was FDA approved for patient with metastatic HR⁺ BC and *ESR1* mutations. For further reading, SERDs have been extensively reviewed by Downton et al. (2022) (Downton et al., 2022). In addition to oral SERDs, there are a variety of additional antiestrogen agents under active clinical development, such as selective human ER partial agonists (ShERPA) TTC-352 (Dudek et al., 2020), selective ER covalent antagonists (SERCA) H3B 6545 (Johnston et al., 2022), complete ER antagonist (CERAN) OP-1250 (Patel et al., 2022), proteolysis targeting chimera (PROTAC) ARV-471 (Schott et al., 2023), and the third-generation selective ER modulator (SERM) lasofoxifene (Damodaran et al., 2022). Interestingly, preclinical results also support androgen receptor (AR) activation as a potential therapeutic approach for CDK4/6i and ET resistant ER⁺ BC models (Hickey et al., 2021). Indeed, induction of AR leads to abnormal ER chromatin binding distribution and alters crucial co-activators, such as p300 and SRC-3, thereby suppressing ER-transcribed cell cycle genes (Panet-Raymond et al., 2000). Consequently, clinical trials have been investigating the selective androgen receptor modulator (SARM) enobosarm either singly (NCT04869943), and in co-treatment with abemaciclib (NCT05065411), in ER⁺/HER2⁻ metastatic BC patients who have progressed on CDK4/6i and ET.

6.5. Using non-endocrine therapies including antibody-drug conjugates

Chemotherapy can be a valuable option for ER⁺ BC patients who have progressed on combination of CDK4/6i and ET. Novel classes of ETs can successfully delay the need for chemotherapy; however this approach is only possible in ER⁺ BC patients who maintain dependence on ER signaling. Antibody drug conjugates (ADCs), which have immune-mediated and cytotoxic properties (Fu et al., 2022) (Dumontet et al., 2023), are a prominent class of therapies that have recently been deployed in ER⁺ BC patients (Grinda et al., 2023). Indeed, ADCs span the gap between cytotoxic drugs and monoclonal antibodies employed to increase the therapeutic efficacy of BC treatments (Abbas et al., 2021) (Subhan and Torchilin, 2023). Biochemically, ADCs are composed of tumor antigen-targeting antibodies linked to robust chemotherapy payloads. After directing cytotoxicity agents towards BC cells, the cleavable linker determines the extent of bystander killing of nearby cancer cells that may not express the target antigen (Staudacher and Brown, 2017). The HER2-directed ADC trastuzumab deruxtecan is formed by humanized anti-HER2 monoclonal antibody bound to a topoisomerase I inhibitor payload through a tetrapeptide cleavable linker. Interestingly, in the DESTINY-Breast03 phase 3 clinical trial (NCT03529110), trastuzumab deruxtecan demonstrated reduced risk of disease progression or death with an ORR of 79.7 % compared to trastuzumab emtansine (34.2 %), in advanced HER2⁺ metastatic BC patients previously treated with trastuzumab and a taxane. Grade 3–4 drug-related adverse events were 45.1 % with trastuzumab deruxtecan and 39.8 % with trastuzumab emtansine. Based on these positive results in HER2⁺ metastatic BC patients treated with a prior anti-HER2-based regimen, trastuzumab deruxtecan has been FDA-approved for use in this cohort (Cortés et al., 2022). In the DESTINY-Breast04 phase 3 clinical study (NCT03734029), trastuzumab deruxtecan resulted in significantly longer PFS (10.1 months) compared to treatment of physician's choice chemotherapy (5.4 months), and increased OS (23.9 months) in comparison to treatment of physician's choice chemotherapy (17.5 months), in HR⁺ HER2-low (defined as score of 1+ on immunohistochemistry or as score of 2+ on IHC and negative results on *in situ* hybridization) unresectable or metastatic BC patients who had been administered one/two previous lines of chemotherapy. Notably, BC patients with previous CDK4/6i treatment displayed a PFS benefit similar to those without previous CDK4/6i treatment (Modi et al., 2022). The safety profile of trastuzumab deruxtecan is consistent with that observed in previous HER2⁺ BC patient-directed clinical trials (Cortés et al., 2022), with no new toxicity-related concerns. Based on these data on HR⁺ HER2-low BC, trastuzumab deruxtecan has also been FDA-approved for use in this cohort (Modi et al., 2022). The ADC sacituzumab govitecan acts toward transmembrane glycoprotein trophoblast cell-surface antigen 2 (Trop-2) and is connected to a topoisomerase I inhibitor SN-38 cytotoxic payload (Syed, 2020). Notoriously, Trop-2 is overexpressed in several epithelial cancers, and particularly, in more than 90 % of ER⁺/HER2⁻ BC (Vidula et al., 2022). In the TROPiCS-02 phase 3 clinical trial (NCT03901339), sacituzumab govitecan determined significantly longer PFS (5.5 months) compared to treatment of physician's choice chemotherapy (4 months), in three-median-line systemic therapy-pretreated ER⁺/HER2⁻ BC patients who were previously administered CDK4/6i. In addition, the PFS at 6 months and 12 months after sacituzumab govitecan or physician's choice chemotherapy were 46 % versus 30 %, and 21 % versus 7 %, respectively. Notably, clinical benefit in these BC patients were independent of Trop-2 expression status. Based upon these data, sacituzumab govitecan has been FDA-approved for use in this ER⁺ cohort (Rugo et al., 2023) (Rugo et al., 2022b) (Rugo et al., 2022c). Datopotamab deruxtecan is an ADC directed against Trop-2 using the same payload as trastuzumab deruxtecan. In the recent TROPION-PanTumor01 phase 1 clinical trial (NCT03401385), datopotamab deruxtecan monotherapy demonstrated promising anticancer activity (29 % ORR, 85 % DCR, and 41 % CBR), and a manageable safety profile in heavily-pretreated ER⁺/HER2⁻ BC

patients, the majority of whom had received previous CDK4/6i (Meric-Bernstam et al., 2023). The efficacy and safety profile of datopotamab deruxtecan, compared to investigator's choice of standard-of-care (SOC) chemotherapy, is being further assessed in the TROPION-Breast-01 phase 3 clinical study (NCT05104866) (Bardia et al., 2023). Based upon these studies, and other recently completed/ongoing studies, ADCs will continue to emerge as an important and expanding option in our therapeutic arsenal. The optimal sequence of treatment, and the role that ADCs play in the setting of specific ET and CDK4/6i resistance scenarios, remains an active area of ongoing research.

7. Conclusions

Even though cyclin D and CDK4/6 have been studied for almost three decades, many facets of their biology and biochemistry have only been recognized recently and the complete functional spectrum of these crucial cell cycle-regulating kinases in BC cells remains elusive. The co-administration of small-molecule CDK4/6i and ET is the first-line treatment for advanced ER⁺/HER2⁻ BC patients. Despite the clear and significant clinical benefits of this combination, patients can still develop intrinsic or acquired drug resistance. Ongoing and future studies must be able to address these four main objectives: 1) elucidating the full spectrum of CDK4/6i-mediated mechanisms of action; 2) clarifying mechanisms underlying synergy between CDK4/6i and various endocrine therapies; 3) improving current targeted treatment options post-progression; 4) understanding mechanisms of resistance to dual CDK4/6i-ET treatment with suitable clinical validations:

- 1) Despite our deep understanding of basic mechanisms related to cyclin-CDK activity, additional work is required to decipher more complex mechanisms of action outside of this canonical pathway. CDK4/6i directly suppress the enzymatic activity of CDK4/6; however, they may also act as indirect CDK2 inhibitors and impact other signal transduction pathways within the cancer cell. Clarifying these important factors may allow an improvement in the clinical activity of these agents, and a better understanding of potential resistance mechanisms. The hypothesis related to the importance of CDK4/6i-induced CDK2 inhibition is supported by the fact that CDK4/6 activity depends upon establishment of trimers comprising cyclin D and p21 or p27.
- 2) Clarification is still needed in relation to the mechanisms underlying synergy between CDK4/6i and various ETs in HR⁺ BC. Particularly, a more in-depth investigation of the role of ET in augmenting and/or changing the therapy-mediated senescence phenotype, versus promoting apoptosis, is required. Insights like this will permit developing novel co-treatments with CDK4/6i plus ET and provide important information to improve our understanding of resistance in clinic trials. Further investigation is required to better understand the CDK4/6i-induced cellular senescence phenotype by clarifying a) to what extent CDK4/6i determine a SASP; b) how loss of p53 function can modify the senescence phenotype; and c) whether CDK4/CDK6i can also determine senescence in other proliferative cells (e.g., fibroblasts) in BC. Feasibility of single cell profiling, accurate genetic modeling *in vitro/in vivo*, and multi-omic profiling of CDK4/6i-treated cells will provide important insights related to these processes and new combinatorial treatments.
- 3) Though there are numerous treatment options post-CDK4/6i progression, there are still doubts regarding the optimum treatment-sequence and delineating a suitable biomarker strategy in this clinical setting is essential. ER⁺ BC patients whose cancer remains dependent on ER signaling benefit more from switching ET backbone. Understanding the mechanisms of CDK4/6i resistance on a personalized individual level may allow better therapeutic planning for each patient and delay the need for more toxic conventional chemotherapy. New targeted options exist or are in development that allow targeting of PI3K, AKT, and PARP, along with novel ADC

therapies. However, the use of these targeted therapies is hampered by limited genomic testing and treatment-induced side effect profiles, which requires further investigation.

- 4) Finally, and most importantly, CDK4/6i resistance still represents a major challenge in clinical practice, and there is a wide array of potential resistance mechanisms. Indeed, clinical studies have been based on DNA sequencing of resistant tumors, and preclinical data have implicated disparate non-genomic resistance mechanisms such as stromal cell senescence, abnormal function of chromatin modifiers, and altered kinase signaling. Further investigation of CDK4/6i-ET treatment-resistant samples is needed, for instance through transcriptomic and/or epigenomic profiling at single cell resolution. A better understanding of these complex resistance pathways can have a tremendous potential to inform regarding new therapeutic approaches at a personalized level. Frequency estimation related to new mutations in CDK4/6i-resistant BC differ remarkably, and thus clarifying this topic is crucial as we work to elucidating all the pathways leading to drug resistance.

These four unanswered questions highlight some of the key areas in which our understanding of cyclin D-CDK4/6 biology and biochemistry will evolve in the coming years, with a therapeutic focus on development and testing of novel CDK4/6i in future BC clinical trials.

Ethical approval

Not applicable

Consent for publication

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all the authors.

Author contributions

A.G. and A.P.K. conceptualized the manuscript topic; A.G., S.A.W., R. D.B., P.D., E.T., J.S., K.V., R.A.M.dB., U.S., A.B., and A.P.K. collated literature for the manuscript; A.G. and A.P.K. wrote the manuscript; A. G., S.A.W., R.D.B., K.CH.Y., H.Y.L., M.T., D.C., B.G., V.S., R.H.J., J.N., P. D., E.T., J.S., K.C., R.S.F., K.V., R.A.M.dB., U.S., A.B., and A.P.K. edited the manuscript; A.G. designed the figures. All authors have read and agreed to the submitted version of the manuscript.

Supplementary Material

Fig. S1 Timeline of FDA approvals for small-molecule CDK4/6i palbociclib, ribociclib and abemaciclib in advanced ER+/HER2- BC patients. Small-molecule CDK4/6i palbociclib, ribociclib and abemaciclib are indicated with blue, green and red rectangles, respectively. Black arrows below CDK4/6i: years in which FDA approval of CDK4/6i occurred for a particular type of treatment. Grey squares at the top-left of each CDK4/6i: type/s of treatment related to the FDA approval of the corresponding inhibitor (A: abemaciclib monotherapy; A + F: abemaciclib plus fulvestrant; A + AI: abemaciclib plus aromatase inhibitor; A + ET: abemaciclib plus endocrine therapy; P + F: palbociclib plus fulvestrant; A + AI: palbociclib plus aromatase inhibitor; R + F: ribociclib plus fulvestrant; R + AI: ribociclib plus aromatase inhibitor). Circled capital letters at the top-right of each CDK4/6i: biopharmaceutical companies related to the FDA approval for a particular type of treatment. ©: Pfizer; ©: Novartis; ©: Eli Lilly.

Supplementary Table 1. Clinical use of FDA-approved small-molecule CDK4/6i palbociclib, ribociclib and abemaciclib in advanced ER+/HER2- BC patients. MRA: medication routes of administration; AI:

aromatase inhibitor; BC: breast cancer; ET: endocrine therapy.

Supplementary Table 2. Completed and ongoing clinical trials with favorable, acceptable or manageable safety profile, which have already shown results in ER+/HER2- BC patients after progression on small-molecule CDK4/6i in advanced ER+/HER2- BC patients. ADCs: antibody drug conjugates; AKTi: AKT inhibitor/s; AI: aromatase inhibitor; allosteric non-competitive mTORi: allosteric non-competitive mTOR inhibitor/s; A/NR: active, not recruiting; A/R: active, recruiting; C: completed; CA: comparator arm; CBR: clinical benefit rate; CDK4/6i: CDK4/CDK6 inhibitor/s; CERAN: complete estrogen receptor antagonist; dual PI3K/mTORi: dual PI3K/mTOR inhibitor/s; ET: endocrine therapy; FP/LUP: first posted/last update posted; IA: investigational arm; I/D: intermittent dosing; N/A: not applicable; NCT: national clinical trial; N^o/P: number of ER+/HER2- BC patients; N/R: not reported; ORR: overall response rate; OS: overall survival; PCI: Prior CDK4/6i (%); PFS: Progression-free survival; PROTAC: proteolysis targeting chimera; SERCA: selective estrogen receptor covalent antagonist; SERD: selective estrogen receptor degrader; SERM: selective estrogen receptor modulator; ShERPA: selective human estrogen receptor (ER) partial agonist; W/D: weekly dosing; (*): N^o of patients in the ER+/HER2- BC cohort.

Supplementary Table 3. Ongoing clinical trials which have not yet displayed results in ER+/HER2- BC patients following progression on CDK4/6i. ADCs: antibody drug conjugates; AI: aromatase inhibitor; Allosteric non-competitive mTORi: allosteric non-competitive mTOR inhibitor/s; ASTs: advanced solid tumors; BC: breast cancer; CDK2i: CDK2 inhibitor/s; CDK4/6i: CDK4/CDK6 inhibitor/s; ET: endocrine therapy; ICIs: immune checkpoint inhibitors; MTD: maximum tolerated dose; N/A: not applicable; NCT: national clinical trial; N^o/P: number of ER+/HER2- BC patients; ORR: overall response rate; OS: overall survival; PFS: Progression-free survival; PROTAC: proteolysis targeting chimera; RP2D: recommended phase 2 dose; RP3D: recommended phase 3 dose; SCE: Study Completion (Estimated); SERD: selective estrogen receptor degrader. (*): Anticipated total study enrolment of clinical trial still recruiting ER+/HER2- BC patients.

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Appendix A. Supporting information

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