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Cell Cycle Control in Breast Cancer Cells

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Abstract In breast cancer, cyclins D1 and E and the cyclin-dependent kinase inhibitors p21 (Waf1/Cip1)and p27 (Kip1) are important in cell-cycle control and as potential oncogenes or tumor suppressor genes. They are regulated in breast cancer cells following mitogenic stimuli including activation of receptor tyrosine kinases and steroid hormone receptors, and their deregulation frequently impacts on breast cancer outcome, including response to therapy. The cyclin-dependent kinase inhibitor p16 (INK4A) also has a critical role in transformation of mammary epithelial cells. In addition to their roles in cell cycle control, some of these molecules, particularly cyclin D1, have actions that are not mediated through regulation of cyclin-dependent kinase activity but may be important for loss of proliferative control during mammary oncogenesis. J. Cell. Biochem. 97: 261–274, 2006.

Key words: cyclin D1; cyclin E; CDK inhibitors; steroid hormones; receptor tyrosine kinases

The identification of cyclins in the 1980s revolutionized the cell-cycle field and the subsequent functional analysis of cyclins and CDKs provided an unprecedented opportunity to gain a molecular understanding of the cell-cycle machinery, its regulation in normal physiology, and its deregulation during oncogenesis. In the context of breast cancer, cyclins D1 and E were quickly established as early response genes following treatment with known regulators of proliferation including steroids and mitogenic growth factors. Complementary studies showed that these cyclins were deregulated in breast cancer. As the complexity of regulation of CDK activity was revealed, it also became apparent that the cell-cycle machinery was deregulated at multiple levels in breast cancer cells. There is now so much information on the regulation and function of the cell-cycle machinery in breast cancer cells that we have necessarily been selective in the areas covered in this review. We have concentrated here on the cyclins and CDK inhibitors that have been most extensively studied as regulators of the cell cycle in breast cancer cells, as putative mammary oncogenes or tumor suppressor genes, and as potential markers of therapeutic response or outcome: cyclins D1 and E1, and the CDK inhibitors p27 (Kip1), p21 (WAF1/Cip1), and p16 (INK4A). Although this review focuses on cyclins and CDK inhibitors, there are other cell-cycle regulators of known importance in breast cancer, notably the proto-oncogene c-Myc, which has recently been reviewed in this context [Jamerson et al., 2004].

Abbreviations used: CAK, CDK activating kinase; CDK, cyclin-dependent kinase; EGF, epidermal growth factor; ER, estrogen receptor; MMTV, mouse mammary tumor virus; PI3K, phosphatidylinositol-3-OH kinase; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PR, progesterone receptor; RTK, receptor tyrosine kinase.

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CELL-CYCLE CONTROL MECHANISMS

The cell cycle comprises a series of tightly controlled events that drive the replication of DNA and cell division. It is divided into several phases: preparation for (G_1 phase), and execution of, DNA synthesis (S phase), a second gap phase (G_2), and mitosis (M). Quiescence (G_0) is a

biochemically distinct state from which cells can re-enter the cell cycle and go on to DNA replication and mitosis. The transitions between these phases are regulated by changes in the activity of specific CDKs, with Cdk1/Cdk2 and Cdk2/Cdk4/Cdk6 controlling the transitions from G_2 to mitosis and G_1 to S phase, respectively. CDK proteins generally remain at constant levels throughout the cell cycle, while binding partners (such as cyclins) and post-translational modifiers (including kinases and phosphatases) undergo periodic fluctuations to regulate DNA synthesis and cell division. The sequential accumulation of different cyclins allows the formation of specific cyclin–CDK complexes that target substrates involved in transitions between the cell-cycle phases. During G_1 phase the predominant cyclin–CDK complexes are cyclin D–Cdk4/6 and cyclin E–Cdk2, followed by cyclin A–Cdk2 during S phase, then cyclin A–Cdk1 and cyclin B–Cdk1 during G_2 and mitosis.

Although control of the G_2/M transition is implicated in events in cancer resulting in chromosomal aberrations, the G_1/S transition encompasses many of the important cell-cycle events that may be specifically altered in breast cancer, including the actions of the oncogenes/tumor suppressors cyclin E, cyclin D1, and p27. For this reason the G_1/S transition will be described in some detail, focusing on cyclin E1–Cdk2 and cyclin D1–Cdk4/6. Other D- and E-type cyclins, i.e., cyclins E2 and D3, are also overexpressed in breast cancers, but less commonly than cyclin D1 or cyclin E1 [Sutherland and Musgrove, 2004].

Cyclin D1–Cdk4/6

Cyclin D1 is a critical modulator of the G_1/S transition through Rb phosphorylation and p21/p27 titration (Fig. 1). Its levels and activity are regulated to integrate extracellular signaling with the cell-cycle machinery. During G_1, cyclin D1 is controlled by a number of mitogenic factors, acting on its transcription and translation as well as the stability, localization and CDK complex association of the cyclin D1 protein (see below). Cyclin D1 is excluded from the nucleus during S phase and the protein is rapidly degraded, through phosphorylation by GSK-3β [Sherr and Roberts, 1999]. Cyclin D1–Cdk4/6 can be activated by CAK-mediated phosphorylation [Sherr and Roberts, 1999], although this does not seem to play a major role in physiological regulation of Cdk4 or Cdk6 activity. Cdk4 and Cdk6 display much more restricted substrate preferences than the other CDKs, and the principal known substrates are the product of the retinoblastoma gene, pRb, and other members of the pocket protein family which in their underphosphorylated forms inhibit the E2F-mediated transcription of genes essential for DNA synthesis [Sherr and Roberts, 1999]. The importance of pRb as a Cdk4/6

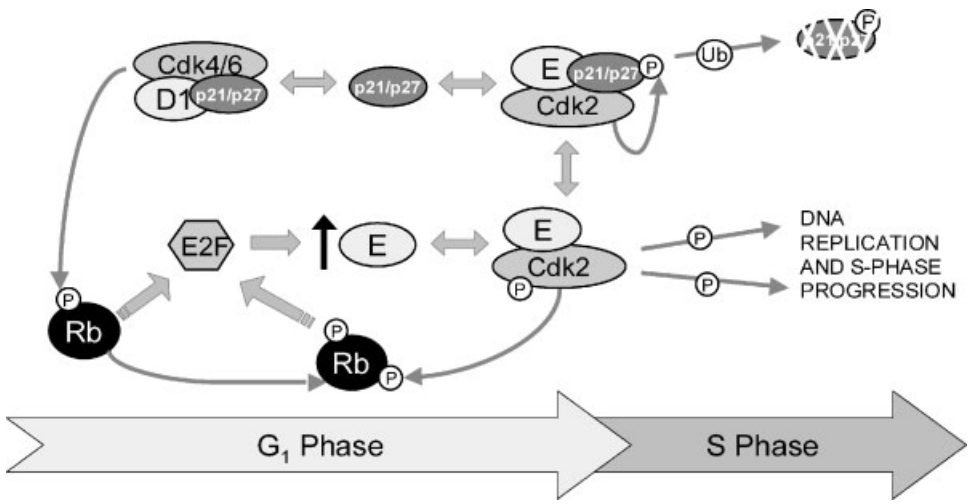

Fig. 1. Control of cell-cycle progression from G_1 into S phase. The main features of G_1 to S phase progression illustrated here are described in detail in the text. Briefly, sequential phosphorylation of Rb by cyclin D1–Cdk4/6 and cyclin E–Cdk2 allows E2F-mediated transcription of target genes including cyclin E and consequent progression into S phase. The distribution of the CDK inhibitors p21 and p27 between these complexes provides an additional level of control over their activity. The levels of these CDK inhibitors are in part regulated by their ubiquitin-mediated degradation. D1: cyclin D1; E: cyclin E; Ub: ubiquitin.

substrate is illustrated by the observation that cyclin D1 is not required for G_1 phase progression in cells lacking pRb [Sherr and Roberts, 1999].

Both p21 and p27 are able to bind and stabilize cyclin D1–Cdk4/6 complexes, but do not necessarily inhibit their kinase activity at physiological levels [Sherr and Roberts, 1999]. The significance of this sequestration of p21 and p27 is demonstrated by the ability of p27 ablation to restore normal development in tissues that display defects in cyclin D1-null mice, including the mammary gland [Sherr and Roberts, 1999]. A further family of CDK inhibitors, of which $p16^{INK4a}$ is the prototypic member, binds specifically to Cdk4 and Cdk6, preventing Cdk activity and disrupting cyclin D association [Pei and Xiong, 2005]. In addition to directly inhibiting cyclin D-associated CDKs, the disruption of cyclin D–Cdk4/6 complexes by INK4 family CDK inhibitors indirectly inhibits cyclin E–Cdk2 by releasing sequestered p21 and p27. Thus the cyclins and CDK inhibitors form an interdependent network that allows precise regulation of CDK activity and, ultimately, cell-cycle progression (Fig. 1).

Cyclin E–Cdk2

The two E-type cyclins, cyclins E1 and E2 (collectively referred to as cyclin E), are closely related and often co-expressed. During G_1 phase, CDK2 is activated through binding cyclin E, and then, via phosphorylation of target proteins, facilitates the first steps of S phase. One critical target for cyclin E–Cdk2 is pRb, but in addition to promoting S phase entry through pRb phosphorylation, cyclin E–Cdk2 also phosphorylates a number of proteins with a more direct role in DNA replication, including NPAT, which facilitates histone synthesis, and components of the pre-replication complex [Hwang and Clurman, 2005].

The activity of cyclin E–Cdk2 is finely regulated through protein-protein interactions, phosphorylation events and degradation, but a primary level of control is through the periodic expression of cyclin E. Cyclin E1 is a well-established E2F target gene and cyclin E2 is also likely to be E2F-regulated. During G_1 phase, pRb phosphorylation by cyclin D1–Cdk4/6 allows the release of E2F, which in turn drives cyclin E expression to reach a maximum near the G_1/S phase boundary [Sherr and Roberts, 2004; Hwang and Clurman, 2005], see Figure 1. As the cyclin E–Cdk2 complex is activated it phosphorylates pRb, providing a positive feedback loop for cyclin E transcription [Sherr and Roberts, 2004; Hwang and Clurman, 2005]. Cyclin E availability is also regulated by ubiquitin-mediated degradation. Unbound cyclin E monomers are degraded through the Cul3 ubiquitin ligase, although this may be more important during embryogenesis than in adult cells, while Cdk2-bound cyclin E is degraded through one of the SCF (Skp1/Cullin/F box) family of ubiquitin ligases, Cdc4/Fbw7, in a process that requires phosphorylation of cyclin E by kinases including Cdk2 [Hwang and Clurman, 2005] (Fig. 2A). Thus the positive feedback loop activating cyclin E transcription is counterbalanced by the rapid degradation of cyclin E protein following activation of cyclin E–Cdk2 (Fig. 2A).

A secondary level of control of cyclin E–Cdk2 is through the CDK inhibitors p21 and p27. These proteins bind to cyclin E–Cdk2, profoundly inhibiting kinase activity and hence stabilizing cyclin E [Hwang and Clurman, 2005]. The availability of p21 and p27 to inhibit cyclin E–Cdk2 can be modulated not only through alterations in their overall abundance but also through their sequestration by cyclin D1–Cdk4/6 and by cytoplasmic relocalisation [Sherr and Roberts, 1999; Coqueret, 2002]. The cyclin E–Cdk2 complex also positively regulates its own activity by phosphorylating p27, which is then targeted for degradation [Sherr and Roberts, 2004].

Phosphorylation and dephosphorylation of the cyclin E–Cdk2 complex provides a third level of control. CAK activates cyclin-bound CDKs including Cdk2 by phosphorylating a conserved threonine residue that optimizes substrate binding and alignment for phosphorylation [Pavletich, 1999]. In addition, inhibitory phosphorylation of the ATP-binding domain by the Wee1/Myt1 protein kinases is reversed by dephosphorylation by the Cdc25A phosphatase [Donzelli and Draetta, 2003].

CDK Inhibitors

CIP/KIP family. The CDK inhibitors p21 (WAF1/Cip1), p27 (Kip1), and p57 (Kip2) exert negative effects on the activity of Cdk2 complexes during G_1 phase and Cdk1 (Cdc2) complexes during G_2 phase. Both p21 and p27 have been widely studied and will be reviewed here. Regulation of p27 expression occurs via

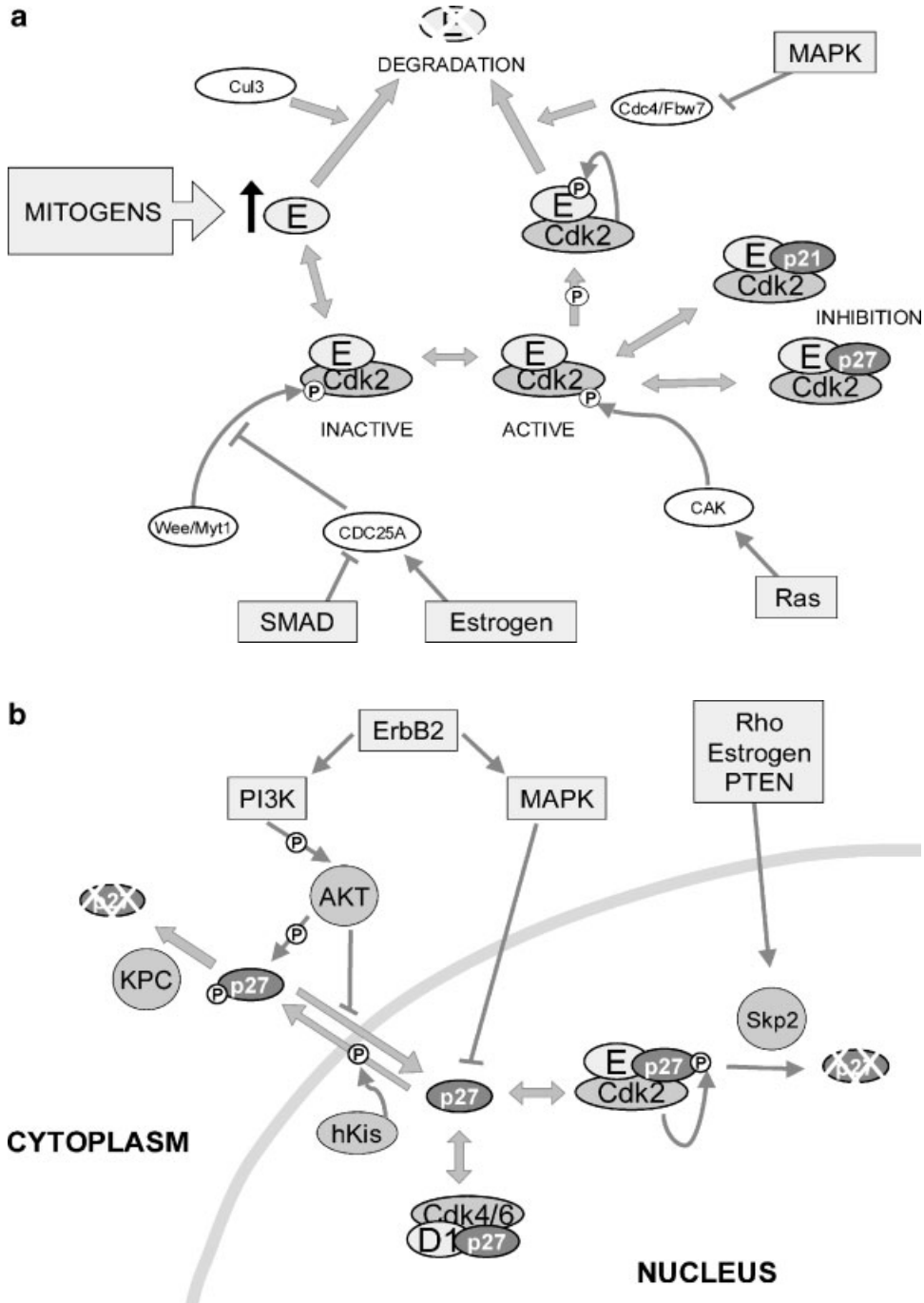

Fig. 2. **A**: Control of cyclin E–Cdk2 activity. The activity of cyclin E–Cdk2 is controlled by the presence of p21 and p27 and regulatory phosphorylation as well as by modulation of cyclin E abundance, both transcriptionally and post-transcriptionally. Further details are provided in the text. **B**: Post-transcriptional regulation of p27. Several pathways regulating the phosphorylation, localization, and degradation of p27 have been identified and are illustrated here. Further details are provided in the text.

transcriptional and translational control mechanisms, through modulation of protein stability, and by changes in subcellular localization. Although transcription of the *p27* gene is regulated by factors including AFX-like Forkhead transcription factors [Medema et al., 2000], regulation of p27 abundance throughout the cell cycle is largely mediated post-transcriptionally. As cells exit quiescence and progress into S phase, p27 protein levels fall due to decreased translation of p27 mRNA and targeted proteolysis [Sherr and Roberts, 1999]. Degradation of

p27 in G_1 and S phase is regulated by two RING-finger E3 ubiquitin ligase-containing complexes with contrasting subcellular locations, that ubiquitylate p27 and target it for proteosomal degradation [Hengst, 2004] (Fig. 2B). The first is the Kip ubiquitin-promoting complex, KPC, consisting of two subunits, KPC1 and KPC2, that function together with two E2 enzymes, Ubc4 and UbcH5A [Kamura et al., 2004]. This complex functions in the cytoplasm during G_1. In contrast, the second complex acts in a nuclear location during S phase and is triggered by phosphorylation of p27 on a threonine residue, T187, by cyclin E–Cdk2. This allows binding of an SCF E3 ubiquitin ligase through the F-box protein Skp2 [Hengst, 2004].

In addition to promoting p27 degradation, phosphorylation also regulates p27 subcellular localization (Fig. 2B). Upon mitogen stimulation, this CDK inhibitor is subject to phosphorylation on serine 10 by human kinase-interacting stathmin (hKis), which promotes nuclear export [Boehm et al., 2002]. Furthermore, it is also phosphorylated on two threonine residues, T157 and T198, by Akt, which results in cytoplasmic retention of p27 [Liang and Slingerland, 2003]. In the case of T157 phosphorylation, cytoplasmic localization is due to inhibition of nuclear import [Liang and Slingerland, 2003]. Overall, this suggests a model in which mitogenic signaling pathways promote cytoplasmic localization of p27 and its subsequent degradation by the KPC. This will increase the activity of nuclear cyclin E–Cdk2 resulting in p27 T187 phosphorylation and degradation of p27, resulting in a positive feedback loop for enhancement of cyclin E-Cdk2 activity.

In contrast with the largely post-transcriptional regulation of p27, the closely related CDK inhibitor, p21, is transcriptionally regulated through the cell cycle, following DNA damage, and during differentiation and senescence [Gartel and Radhakrishnan, 2005]. However, the stability of the p21 protein is also regulated by proteosomal degradation. The Skp2 ubiquitin ligase has been implicated in this process, but there is evidence that other ubiquitin ligases also target p21 [Bloom and Pagano, 2004]. Unusually, p21 is ubiquitinated through its N-terminus rather than the lysine residues that are almost universally the site of ubiquitin binding [Bloom and Pagano, 2004]. In addition, it does not strictly require phosphorylation for Skp2 association, although a characteristic feature of F-Box proteins like Skp2 is that substrate binding requires phosphorylation [Bloom and Pagano, 2004]. Like p27, phosphorylation of p21 by Akt leads to its cytoplasmic localization [Liang and Slingerland, 2003].

INK4 family. The four members of this family of CDK inhibitors are closely related and have very similar functions in in vitro assays, suggesting that apparent differences in their physiological roles are largely the result of differences in expression or regulation [Pei and Xiong, 2005]. Only p19 is regulated in concert with cell-cycle progression, and regulation of INK4 expression appears to make little contribution to cell-cycle progression in unperturbed cell cycles in normal cells, although the best-studied INK4A protein, p16, is a tumor suppressor with an important role in senescence.

Functions of Cyclins and CDK Inhibitors Independent of Cell-Cycle Regulation

The cyclins and CDK inhibitors reviewed above are best understood as regulators of CDK activation and hence cell-cycle progression. However, it is becoming increasingly apparent that many of these molecules also have CDK-independent functions. For example, cyclin D1 interacts with a number of transcription factors or transcriptional coregulators, some of which alter chromatin structure via histone acetylation or other functions [Fu et al., 2004]. Recent evidence indicates that CDK-independent functions of cyclin D1 likely contribute to its oncogenic actions (reviewed in Arnold and Papanikolaou [2005]). Similarly, p21 and p27 have been implicated in regulation of nuclear import, apoptosis, and cell motility through interactions with proteins other than cyclins and CDKs [Coqueret, 2002].

SIGNALLING TO THE CELL-CYCLE MACHINERY

Signaling pathways transduce multiple extracellular inputs during G_1 phase that influence cell commitment to DNA replication and division. Breast epithelial cells receive signals from growth factors, steroid hormones such as estrogen and progesterone, cytokines, and the cell matrix. These signals generally converge on pRb inactivation by affecting the levels and activity of cyclins and CDK inhibitors, and

thus control the G_1/S transition. We briefly summarize here the effects of some key extracellular regulators of breast cancer cells: ligands for receptor tyrosine kinases, steroid and polypeptide hormones, and integrins, together with the antiproliferative cytokine TGFβ.

Receptor Tyrosine Kinase Signaling

Deregulated signaling by specific RTKs, particularly members of the erbB family, is strongly implicated in breast cancer progression [Salomon et al., 1995]. Two key signaling pathways activated by RTKs are those leading to activation of the low molecular weight G protein Ras and PI3K. Together, these pathways regulate cell survival, proliferation, growth, and motility. Ras signaling is often enhanced in breast cancers, due to increased expression of particular erbB receptors, signaling pathway intermediates, and/or Ras proteins themselves [Malaney and Daly, 2001]. Following growth factor binding, receptor autophosphorylation promotes binding of adaptor proteins. In the case of the Ras pathway, the Grb2 adaptor mediates plasma membrane recruitment of the GDP/GTP exchange factor Sos and its juxtaposition with membrane-anchored Ras, thus stimulating Ras activation [Schlessinger, 2000]. An important Ras effector pathway resulting in mitogenic signaling is the Raf/MEK/Erk cascade, which influences multiple endpoints including increased transcription of cyclin D1 [Coleman et al., 2004]. A variety of transcription factors have been implicated in this response, including Fos and Jun family transcription factors and Ets family members [Pestell et al., 1999; Coleman et al., 2004]. Expression of activated Ras or MEK results in p27 degradation, presumably due to altered p27 phosphorylation [Coleman et al., 2004]. In addition, the residual p27 is impaired in its ability to inhibit cyclin E–Cdk2. Consistent with Ras/Erk acting downstream of erbB2, enhanced signaling by this receptor in fibroblasts or breast cancer cells decreases the protein half-life of p27 [Hynes and Lane, 2005]. Ras activation of cyclin E–Cdk2 occurs through mechanisms that include the ability of Erk to regulate the activating phosphorylation of Cdk2 at Thr 160 [Lents et al., 2002], and MAPK-dependent regulation of the Cdc4/Fbw7 ubiquitin ligase, which targets Cdk2-bound cyclin E for degradation [Minella et al., 2005]. In addition to these direct effects on cyclin E-Cdk2, Ras/Erk signaling also indirectly activates this complex by increasing cyclin D1 gene transcription, resulting in sequestration of p21 and p27 in cyclin D1–Cdk4/6 complexes.

In an alternative Ras effector pathway, the Ral GTPase is activated and reinforces particular regulatory events mediated by the Ras/Erk cascade. Ral activates NF-κB which subsequently binds to response elements in the cyclin D1 promoter to stimulate transcription of this gene [Henry et al., 2000]. Ral also induces phosphorylation of Forkhead family transcription factors (FOXO) to prevent their nuclear entry, which in turn prevents their transcriptional regulation of p21 and p27 [Coleman et al., 2004].

Ligand-bound RTKs stimulate PI3K activity by binding to the p85 subunit of this enzyme or indirectly via Ras, which in its GTP-bound form binds to the p110 catalytic subunit [Schlessinger, 2000]. Once activated, PI3K generates PIP3 at the plasma membrane, in a process that can be inhibited by the lipid phosphatases PTEN and SHIP. PIP3 accumulation leads to activation of the serine/threonine kinase Akt/PKB that positively regulates G_1/S cell-cycle progression through phosphorylation of a variety of substrates [Vivanco and Sawyers, 2002]. First, Akt phosphorylates and inhibits GSK3β. Since phosphorylation of cyclin D1 and E by GSK3β promotes their degradation [Sherr and Roberts, 2004], regulation of GSK3β by Akt stabilizes these cyclins. Second, FOXO proteins are phosphorylated to decrease p21 and p27 transcription, as described above for the Ral GTPase. In addition, Akt directly phosphorylates both p21 and p27 on residues within their nuclear localization motifs. This leads to their relocalization in the cytoplasm, away from the nuclear cyclin E/Cdk2 complex [Liang and Slingerland, 2003]. Evidence that erbB2 overexpression regulates p27 localization via this mechanism is that treatment of BT-474 breast cancer cells, which exhibit erbB2 amplification, with a PI3K inhibitor abolishes cytosolic staining for phosphorylated p27 and results in nuclear translocation of p27 [Shin et al., 2002]. Also, there is some evidence that altered expression of the PI3K-inhibitory phosphatase PTEN may regulate p27 stability via alterations in Skp2 abundance [Alkarain et al., 2004]. Finally, Akt phosphorylation may also indirectly regulate p21 and p27 abundance via activation of mTOR, a protein serine/threonine kinase that

promotes translation of proteins important for cell-cycle progression, including the pro-proliferative transcription factor c-Myc. Enhanced expression of c-Myc results in repression of p21 and p27 [Massague, 2004].

Integrin Regulation

Signaling from integrins, which convey information about the extracellular matrix and cytoskeletal structure, is integrated with growth factor receptor signaling to control the activity of cyclins and CDK inhibitors. Integrins cooperate with RTKs to transmit pro-proliferative signals through the Ras/Erk and PI3K pathways, as well as concurrently altering cell-cycle progression through the Rho family of GTPases (Rho, Rac, and Cdc42). Rac and Cdc42 are able to upregulate cyclin D1 expression through NF-κB activation, while Rho signaling can induce integrin clustering that sustains Erk signaling, and consequently upregulates cyclin D1 [Giancotti and Tarone, 2003; Besson et al., 2004]. Rho is also able to inhibit Rac and Cdc42, allowing a switch between Rac and ERK-mediated cyclin D1 upregulation [Besson et al., 2004]. This upregulation of cyclin D1 and increased formation of cyclin D1–Cdk4/6 complexes contributes to cyclin E–Cdk2 activation via sequestration of p21 and p27. More directly, Rho activation can lead to the induction of Skp2, resulting in increased p27 turnover and cyclin E–Cdk2 activation [Mammoto et al., 2004].

Steroid and Peptide Hormone Regulation

In the normal breast the steroid hormones estrogen and progesterone have a crucial role in development and during pregnancy. The receptors for these hormones are ligand-activated nuclear transcription factors. Upon ligand binding they directly target transcription of genes with estrogen response elements or progesterone response elements but can also regulate separate sets of target genes by modulating the activities of other transcription factors, including AP-1, Sp1, and NF-κB [McDonnell and Norris, 2002]. In parallel to the direct regulation of gene transcription, estrogens and progestins also activate cytoplasmic signaling pathways including Src/Ras/Erk signaling [Edwards, 2005]. Both ER and PR interact with Src, but by different mechanisms [Edwards, 2005]. In breast cancer cells estrogen enhances proliferation, while progesterone has a biphasic effect where it initially accelerates cells into S phase and through the rest of the cell cycle, but ultimately causes cells to arrest in G_1 [Sutherland et al., 1998; Foster et al., 2001].

Cyclin D1 is a direct target of estrogen signaling, although no estrogen response elements have been identified in the cyclin D1 promoter [Foster et al., 2001]. Instead, activation appears to occur via ER interactions with AP-1 and Sp1 [Foster et al., 2001; Cicatiello et al., 2004]. Increased cyclin D1 expression enhances cyclin D1–Cdk4 complex formation and consequently pRb phosphorylation. Cyclin E–Cdk2 complexes are also activated within the first 4–6 h of estrogen treatment, through a mechanism that involves reduced association with p21 and complex formation with p130, a pRB-related pocket protein [Sutherland et al., 1998; Cicatiello et al., 2004]. The importance of cyclin D1 in estrogen stimulation of cell-cycle progression is illustrated by the ability of cyclin D1 induction to activate cyclin E–Cdk2 and induce cell-cycle progression, mimicking the effects of estrogen. Induction of c-Myc expression is also a rapid response to estrogen treatment, and leads to activation of cyclin E–Cdk2 via reduced association with p21 [Sutherland et al., 1998; Foster et al., 2001]. This has led to the hypothesis that estrogen induces cell-cycle progression through two initially independent pathways, one mediated by c-Myc and the other involving cyclin D1 [Sutherland et al., 1998].

The activation of cyclin E–Cdk2 by estrogen is not solely due to sequestration of p21 and p27 in cyclin D1–Cdk4 complexes, but also relies on decreased p21 protein synthesis, since maintenance of p21 levels prevents activation of cyclin E–Cdk2 [Foster et al., 2001]. The estrogen-mediated decrease in p21 requires c-Myc [Mukherjee and Conrad, 2005]. In addition, Cdc25A has been implicated in estrogen activation of cyclin E–Cdk2 [Foster et al., 2001]. Finally, p27 levels are downregulated by estrogen, by a mechanism that in part depends on estrogen induction of Skp2 and consequent increase in p27 proteolysis [Foster et al., 2003].

Progestins also rapidly increase expression of both c-Myc and cyclin D1 [Sutherland et al., 1998], with cyclin D1 induction occurring by a mechanism that at least in part overlaps with estrogen regulation of cyclin D1 [Cicatiello et al., 2004]. After the transient stimulation of S phase, cell proliferation is inhibited following a reduction in the activity of cyclin D1–Cdk4 and cyclin E–Cdk2 [Sutherland et al., 1998]. This is

mediated by decreased expression of cyclin D1 and cyclin E, since these proteins inhibit the antiproliferative effects of progestins when overexpressed [Musgrove et al., 2001], and by an increase in the proportion of cyclin E-Cdk2 complexes bound by p21 and p27. The latter response results from both decreased cyclin D1 expression and increased expression of p18 (INK4C) [Swarbrick et al., 2000]. A decrease in the levels of c-Myc reinforces this by increasing transcription of the p21 and p27 genes [Sutherland et al., 1998].

The polypeptide hormone prolactin and its receptor are also important physiological regulators of mammary epithelial cell proliferation and differentiation that have been implicated in breast cancer but they have not been well-studied in the context of the cell-cycle machinery. However, prolactin treatment of breast cancer cells in culture leads to the induction of cyclin D1 and decreased expression of p21, but not p27 [Schroeder et al., 2002].

TGF-β Regulation

The cytokine TGF-β inhibits G_1/S progression in mammary epithelial cells through the induction of CDK inhibitors and modulation of CDK activity. TGF-β signals via a transmembrane serine/threonine kinase complex that phosphorylates SMAD proteins, thus stimulating the formation of SMAD heterocomplexes that regulate transcription of a large number of genes, including cell-cycle regulators. Specifically, SMAD complexes upregulate p21 and p15 (INK4B), and repress the expression of c-Myc, cyclin D1, and Cdc25A [Stull et al., 2004]. Upregulation of p15 leads to loss of cyclin D1–Cdk4 activity and p27 redistribution to cyclin E–Cdk2 complexes, and cyclin E–Cdk2 activity is also dampened through increased binding of p21 and loss of Cdc25A activation [Stull et al., 2004].

ROLE OF CELL-CYCLE REGULATORY MOLECULES IN BREAST CANCER DEVELOPMENT AND PROGRESSION

Cyclin D1

Cyclin D1 is overexpressed in up to 50% of primary breast cancers, in part due to amplification of the cyclin D1 gene, *CCND1*, and thus cyclin D1 is one of the most commonly overexpressed oncogenes in breast cancer [Sutherland and Musgrove, 2004; Arnold and Papanikolaou, 2005]. ER-positive breast cancers display cyclin D1 amplification and overexpression more commonly than ER-negative cancers, and it is in the ER-positive subgroup that the most consistent association between cyclin D1 amplification and poor outcome has been found. However, further data, ideally from prospective randomized clinical trials, will be necessary to resolve the issue of the relationship between cyclin D1 expression and response to therapy including disease outcome [Sutherland and Musgrove, 2004]. Cyclin D1 is overexpressed in ductal hyperplasias and ductal carcinoma in situ as well as invasive cancers, suggesting that it may play a role in the evolution of breast cancer. Studies in mice indicate that cyclin D1 overexpression can lead to the development of mammary carcinoma, but this occurs with long latency, suggesting that it likely cooperates with other oncogenes [Sutherland and Musgrove, 2004]. Although cyclin D1 overexpression might be expected to be associated with high proliferation rates, this is not the case in breast cancer where cyclin D1 overexpression is characteristic of slow-growing, more differentiated cancers. Furthermore, in a panel of breast cancer cell lines, Cdk4 activity was not strictly related to cyclin D1 expression, suggesting that functions of cyclin D1 other than its ability to activate Cdk4 and promote cell proliferation might contribute to its activity as a mammary oncogene [Sutherland and Musgrove, 2004]. Consistent with this hypothesis, transcript profiling of cells expressing a cyclin D1 mutant that cannot activate Cdk4 identified a gene expression signature that was also characteristic of cancers overexpressing cyclin D1. These experiments implicated the transcription factor C/EBPβ as a potential cyclin D1 effector molecule in this context, suggesting that the ability of cyclin D1 to modulate transcription may be important in mammary oncogenesis (reviewed in Sutherland and Musgrove, 2004; Arnold and Papanikolaou, 2005).

Amplification of the cyclin D1 gene, *CCND1*, is present in only a minority of cyclin D1-overexpressing breast cancers. Given its role as a target of mitogenic signaling, one mechanism by which overexpression of cyclin D1 could occur in the absence of gene amplification is as a consequence of deregulation of RTKs and their downstream signaling pathways. Consistent with this idea, cyclin D1 has been implicated

in the oncogenic actions of Ras and Neu/erbB2. Murine mammary tumors induced by either oncogene display increased expression of cyclin D1, while cyclin D1 expression is necessary for Ras or erbB2-mediated mammary tumorigenesis [Fu et al., 2004; Sutherland and Musgrove, 2004]. This dependence likely reflects a requirement for the CDK-mediated functions of cyclin D1, since overexpression of p16 blocks erbB2-mediated tumor formation [Yang et al., 2004]. However, the relationship between cyclin D1 overexpression and erbB2 amplification in human breast cancer remains unclear [Arnold and Papanikolaou, 2005].

Cyclin E1

Cyclin E1 overexpression in the mouse mammary gland results in hyperplasias and the formation of carcinoma at low incidence and after long latency [Sutherland and Musgrove, 2004]. Whether other oncogenes require cyclin E has not been explored thoroughly, although fibroblasts lacking both E-type cyclins are resistant to transformation by oncogenic Ras in conjunction with other "immortalizing" oncogenes [Sherr and Roberts, 2004]. Cyclin E1 is frequently overexpressed in breast cancers, particularly ER-negative breast cancers, and this is associated with the presence of truncated isoforms, which appear to confer particularly poor outcome [Sutherland and Musgrove, 2004]. The low molecular weight isoforms display enhanced binding to Cdk2 and the resulting complexes are relatively resistant to inhibition by p21 and p27 [Wingate et al., 2005]. Furthermore, cyclin E1 overexpression is of greater prognostic significance when p27 expression is reduced and Cdk2 activation increased. Together with the association between cyclin E1 overexpression and markers of proliferation, these observations suggest that the CDK-dependent functions of cyclin E1 may be critical to its role in mammary oncogenesis.

p27 and p21

As negative regulators of the cell cycle both p21 and p27 are potential tumor suppressor genes. Experimental evidence supports this idea: mice lacking p27 develop pituitary adenomas, and are more susceptible to a number of carcinogens [Musgrove et al., 2004], while p21-null mice develop malignancies at long latency, although these did not include mammary cancers [Gartel and Radhakrishnan, 2005]. Mice lacking p21 do, however, display accelerated mammary tumor development after expression of Ras but not Myc [Bearss et al., 2002]. The role of p21 abundance as a modulator of outcome in primary breast cancer is not clear. Expression is generally undetectable in normal breast epithelial cells and variable in breast cancers, with conflicting data on the prognostic implications of p21 expression from a limited number of small patient series [Tsihlias et al., 1999]. The issue is confounded by functional interactions between p21 and known prognostic factors, for example the p53-mediated transcriptional regulation of p21, and the Akt-mediated phosphorylation and cytoplasmic localization of p21 in erbB2-overexpressing breast cancers. The latter raises the possibility that both localization and overall abundance may be of importance, and some recent clinical data suggest that cytoplasmic localization of p21, particularly in combination with erbB2 overexpression, confers poor outcome [Winters et al., 2003; Xia et al., 2004].

The utility of p27 as a prognostic marker has been more extensively examined. Normal human mammary duct epithelial cells exhibit nuclear immunoreactivity for p27, while breast cancers often exhibit reduced p27 expression and/or mislocalization of p27 to the cytosol [Alkarain et al., 2004]. Generally, reduced nuclear p27 expression is associated with high tumor grade and ER negativity, and in a small majority of studies, represents an independent prognostic indicator upon multivariate analysis [Alkarain et al., 2004]. Although loss of heterozygosity at band 13 of chromosome 12, which harbours the p27 gene, does occur in breast cancers, somatic mutations in this gene are rare [Alkarain et al., 2004], and the predominant mechanism for loss of protein expression is increased protein turnover. High expression of Skp2 in breast cancers correlates with low p27 levels, but a significant number of cancers with low p27 expression do not overexpress Skp2, indicating that other mechanisms must account for p27 degradation [Signoretti et al., 2002].

Since erbB2 overexpression associates with low p27 levels in breast cancer patients overall [Newman et al., 2001] or in the lymph node negative subset [Spataro et al., 2003], activation of signaling pathways downstream of this receptor may promote p27 degradation. However, RTK signaling can also lead to restriction of p27 to the cytosol. Indeed, in studies of primary

breast cancer specimens, the presence of cytosolic p27 was associated with its phosphorylation on T157 and the presence of active Akt [Alkarain et al., 2004]. Furthermore, in cancers with either low or high p27 expression, the presence of cytosolic p27 was associated with worse disease-free and overall survival [Alkarain et al., 2004].

Elegant studies by the Arteaga laboratory reveal how p27 regulates erbB2-induced mammary tumorigenesis (reviewed in [Musgrove et al., 2004]). Haploinsufficiency of p27 enhanced cell proliferation and anchorage-independent growth of the erbB2-overexpressing cells in vitro relative to wild-type controls. Furthermore, MMTV-Neu/p27-heterozygous mice exhibited a shorter latency for mammary tumor development than the corresponding p27 wild-type animals. Interestingly, in these studies complete loss of p27 expression in p27-null animals was associated with decreased cyclin D1 expression and Cdk4 activity, decreased cell proliferation and increased tumor latency in response to mammary-specific expression of erbB2. However, other studies have not found decreased cyclin D1 expression in mammary epithelial cells from p27-null mice, but rather documented activation of cyclin E–Cdk2 activity and increased proliferation [Musgrove et al., 2004]. Consequently it may be informative to investigate erbB2-mediated tumorigenesis on a genetic background where the absence of p27 enhances rather than impairs mammary epithelial cell proliferation. These studies have some parallels with the decreased incidence of MMTV–Myc-induced tumors on a p21-null background, which is accompanied by decreased proliferation and reduced CDK activity, in contrast with the increased proliferation and CDK activity in MMTV-Ras-induced tumors on a p21-null background [Bearss et al., 2002]. For reasons that are not yet fully understood, it appears that p21 and p27 can behave as context-dependent inhibitors or promoters of cell-cycle progression in mammary epithelium.

p16

Recent studies have highlighted the importance of p16 in resistance to oncogenic transformation in mammary epithelium (reviewed in Tlsty et al., 2004). When normal human mammary epithelial cells are cultured, they initially proliferate but this ceases after approximately 20 population doublings. When proliferation ultimately resumes the proliferating cells are characterized by loss of p16 expression due to methylation of the *INK4A* gene promoter. Furthermore, they can be immortalized by expression of telomerase, in contrast with the early passage cells that retain p16 expression. Importantly, hypermethylation of the *INK4A* promoter occurs in 20–30% of breast cancers, with additional breast cancers displaying LOH or allelic imbalance, suggesting that this culture model mimics at least some processes involved in the development of human breast cancer. The issue of the relationship between p16 expression and prognosis is complicated by the substantial increase in p16 expression that occurs when pRB is deleted or mutated, such that reduced p16 expression, "normal" expression and overexpression may each be associated with different phenotypes. In general high expression of p16 is associated with poor prognosis (for example, Hui et al., 2000), although it is difficult to draw firm conclusions since the few published series examining this question typically include <100 patients.

CELL-CYCLE REGULATORY GENES AND RESPONSE TO THERAPY

As endpoints of diverse signaling pathways targeted by existing and emerging therapeutics, cell-cycle regulatory genes are potential mediators of resistance when aberrantly expressed. In breast cancer, this possibility has been examined in the context of endocrine therapies and those targeted at erbB receptors (i.e., monoclonal antibodies and tyrosine kinase inhibitors).

Endocrine Resistance

Since antiestrogens, an effective and widely used therapy for hormone-responsive breast cancer, rapidly downregulate cyclin D1, deregulated expression of this cyclin might be expected to impact on sensitivity to these agents. Some limited clinical data support the idea that response to therapy with the antiestrogen tamoxifen is of shorter duration in patients with high cyclin D1, and overexpression of cyclin D1 in breast cancer cells in culture leads to acute antiestrogen resistance [Butt et al., 2005]. Further data from experimental models and primary breast cancers will be necessary to resolve the question of whether the level of cyclin D1 expression affects response to antiestrogens. Although cyclin E overexpression in

breast cancer cells exerts at best a weak effect on antiestrogen sensitivity in vitro, one study found that high cyclin E expression was associated with poor relapse-free survival in patients treated with endocrine therapies (reviewed in [Sutherland and Musgrove, 2004]). Again, further studies are required.

p27 plays a key role in mediating the cell-cycle arrest of breast cancer cells by clinically relevant pharmacological agents that block estrogen action (reviewed in [Sutherland and Musgrove, 2004]). For example, treatment of MCF-7 breast cancer cells with the steroidal antiestrogen ICI 182780 (Faslodex) results in increased expression of p27, enhanced association of p27 with cyclin E–Cdk2, and cell-cycle arrest. Furthermore, the strong induction of p27 by this antiestrogen contributes to the induction of a quiescent, growth factor-insensitive state. Downregulation of p21 or p27, by treatment with antisense oligonucleotides or overexpression of Skp2, confers antiestrogen resistance in breast cancer cells in culture. Interestingly, MEK inhibition of antiestrogen-resistant breast cancer cells restores p27 inhibition of cyclin E–Cdk2 complexes and drug sensitivity, suggesting that antiestrogen sensitivity might be restored in resistant cells by treatment with particular signal transduction inhibitors. Tumor p27 and p21 status may be predictive of antiestrogen sensitivity in a clinical setting. Thus, in a study of premenopausal women with early stage breast cancer receiving combination endocrine therapy of tamoxifen and goserelin, high p27 expression was associated with improved relapse-free and overall survival [Pohl et al., 2003], and p21 levels have been associated with response to antiestrogens in some, but not all, clinical studies [Butt et al., 2005].

Resistance to erbB-Targeted Therapies

In erbB2-overexpressing breast cancer cells, administration of the selective EGF receptor kinase inhibitor AG1478 (that, in this scenario, inhibits the erbB2 kinase indirectly by inducing the formation of inactive erbB1/erbB2 heterodimers) or the anti-erbB2 monoclonal antibody 4D5/herceptin led to a decrease in cyclin D1 expression and redistribution of p27 into cyclin E/Cdk2 complexes, resulting in their inhibition and G_1 arrest [Lane et al., 2000; Lenferink et al., 2001]. These findings predict that increased expression of cyclin D1, or reduced expression of p27, might lead to resistance to anti-erbB therapies. In support of this hypothesis, our laboratory has recently demonstrated that deregulated cyclin D1 expression is associated with resistance to the EGF receptor inhibitor gefitinib in head and neck squamous cell carcinoma cell lines [Kalish et al., 2004]. Since cyclin D1 overexpression is relatively infrequent in erbB2-overexpressing breast cancers [Arnold and Papanikolaou, 2005], and since overexpression of cyclin D1 in breast cells did not confer resistance to the effects of EGF receptor inhibition [Chou et al., 1999], this may not be a major mechanism of resistance in breast cancer. However, in a cell culture model, herceptin-resistant, erbB2-overexpressing breast cancer cells exhibited lower p27 expression than the herceptin-sensitive parental line, and sensitivity to this antibody-based therapy could be restored by ectopic p27 expression [Nahta et al., 2004]. These findings warrant further studies in a clinical setting.

CONCLUSIONS AND FUTURE DIRECTIONS

The increased understanding of cell-cycle control mechanisms has led to increasingly detailed answers to questions about the pathways through which breast epithelial cells proliferation is regulated, and how they are deregulated in breast cancer. However, a new series of questions has been raised, some of which are highlighted here. For example, much attention is currently directed at the issue of whether the activity of different CDKs is essential for proliferation, and consequently whether the therapeutics directed at CDK inhibition that are currently being developed [Swanton, 2004] are likely to be effective. The prevailing view, supported by a substantial body of evidence (reviewed in [Sherr and Roberts, 2004]), has been that both cyclin D- and cyclin E-dependent kinases are essential for progression through G_1 and into S phase. However, a recent series of publications documenting mice in which all three D-type cyclins, both Cdk4 and Cdk6, both E-type cyclins, or Cdk2 have been deleted, calls this view into question. These mice develop to at least embryonic day 14.5, and fibroblasts derived from them proliferate in culture, although they are all impaired to some degree in the ability to resume proliferation from quiescence and are resistant to oncogenic transformation in vitro

(reviewed in Sherr and Roberts, 2004). These data indicate that neither cyclin D–Cdk4/6 nor cyclin E–Cdk2 is strictly essential for cell-cycle progression, corroborating the earlier finding that the proliferation of several cancer cell lines is unaffected by siRNA-mediated knockdown of Cdk2 [Tetsu and McCormick, 2003]. It is important to note several caveats in interpreting these studies. First, acute inhibition of CDK activity may have consequences different to those observed in knockout animals, as demonstrated by investigations of the effects of Rb inactivation [Sage et al., 2003]. Divergent mechanisms of cell-cycle control in stem cells and more differentiated adult cells are one reason for such differences in response. Second, not all strategies for inhibition of CDK activity are equivalent; for example, a "kinase dead" CDK will bind cyclins, CDK inhibitors, and substrates that would be available to complex with other proteins if it was deleted. This is illustrated by the ability of expression of p27 or a dominant-negative form of Cdk2 to inhibit the proliferation of cell lines unaffected by knockdown of Cdk2 [Tetsu and McCormick, 2003]. Finally, tissue specificity in dependence on particular components of the cell cycle is suggested by the diversity in the frequency of aberrant expression of individual genes in different cancers and by the phenotypes of mice lacking individual cell-cycle genes [Pagano and Jackson, 2004], arguing for caution in extrapolating data obtained using fibroblasts to other cell and tissue types. Thus, it will be important to determine the degree to which these intriguing recent data apply to other model systems including breast epithelial cells and breast cancers.

Continuously cycling cells do not strictly require cyclin D–Cdk4/6 or cyclin E–Cdk2, but cell-cycle re-entry from serum starvation does depend on these CDKs, indicating that the cell cycle can be governed by context-dependent control mechanisms. This concept is supported by recent evidence for differential activation of the DNA damage response in normal cells compared with precancerous cells [Venkitaraman, 2005]. An implication of these observations is that it may be possible to specifically target the control mechanisms that operate in abnormal cell cycles. In addition, since antiproliferative signals must be over-ridden for both transformation and exit from quiescence, CDK inhibition may be more effective as a preventive strategy than as a therapy for established cancers. Although questions have been raised over the likely efficacy of therapies targeting cyclin E–Cdk2, most cancer cell lines that continued to proliferate in the absence of Cdk2 remained sensitive to inhibition of cyclin D1–Cdk4 [Tetsu and McCormick, 2003], and the strong evidence for a central role of cyclin D1 in breast cancer argues for investigation of cyclin D1 as a therapeutic target in this disease [Arnold and Papanikolaou, 2005]. Alternatively, it may be useful to investigate therapies that target both cyclin D–Cdk4/6 and cyclin E–Cdk2. This is demonstrably successful in the cases of antiestrogens and herceptin, which regulate cyclin D1 and p21/p27 and hence have effects on both major G_1 CDK complexes in breast cancer cells. The limitation that resistance places on the clinical application of both these agents has fueled investigations of potential mechanisms of resistance, but deregulation of cell-cycle molecules remains relatively understudied in this context.

One additional area that merits more detailed investigation is the relative contributions of various actions of cyclins and CDK inhibitors to cell-cycle control and oncogenesis. In the case of cyclin D1, for example, activation of Cdk4/6, sequestration of p21/p27, and CDK-independent interactions with transcription factors including the ER may all contribute to the development or progression of breast cancer. Similarly, p21 and p27 have effects on cytoskeletal organization and cell migration as well as on CDK assembly and activity.

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