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Low Molecular Weight Cyclin E in Human Cancer: Cellular Consequences and Opportunities for Targeted Therapies

Joseph A. Caruso[1,*], **Mylinh T. Doung**[2], **Jason P. W. Carey**[3], **Kelly K. Hunt**[4], and **Khandan Keyomarsi**[3,*]

[1]Department of Pathology, University of California, San Francisco; San Francisco, CA USA

[2]Bellicum Pharmaceuticals, Houston, TX USA

[3]Department of Experimental Radiation Oncology; The University of Texas MD Anderson Cancer Center; Houston, TX USA

[4]Department of Breast Surgical Oncology; The University of Texas MD Anderson Cancer Center; Houston, TX USA

Abstract

Cyclin E, a regulatory subunit of cyclin-dependent kinase 2 (CDK2), is central to the initiation of DNA replication at the G_1/S-checkpoint. Tight temporal control of cyclin E is essential to the coordination of cell cycle processes and the maintenance of genome integrity. Overexpression of cyclin E in human tumors was first observed in the 1990s and led to the identification of oncogenic roles for deregulated cyclin E in experimental models. A decade later, low molecular weight cyclin E isoforms (LMW-E) were observed in aggressive tumor subtypes. Compared with full-length cyclin E, LMW-E hyperactivates CDK2 through increased complex stability and resistance to the endogenous inhibitors $p21^{CIP1}$ and $p27^{KIP1}$. LMW-E is predominantly generated by neutrophil elastase-mediated proteolytic cleavage, which eliminates the N-terminal cyclin E nuclear localization signal and promotes cyclin E's accumulation in the cytoplasm. Compared with full-length cyclin E, the aberrant localization and unique stereochemistry of LMW-E dramatically alters the substrate specificity and selectivity of CDK2, increasing tumorigenicity in experimental models. Cytoplasmic LMW-E, which can be assessed by immunohistochemistry, is prognostic of poor survival and predicts resistance to standard therapies in cancer patients. These patients may benefit from therapeutic modalities targeting the altered biochemistry of LMW-E or its associated vulnerabilities.

Keywords

cyclin E; low molecular weight cyclin E (LMW-E); cell cycle; neutrophil elastase

[*]Corresponding Authors: Khandan Keyomarsi, The University of Texas MD Anderson Cancer Center, 6565 MD Anderson Blvd, Unit 1052, Houston, TX 77030. Phone: 713-792-4845; Fax: 713-794-5369; kkeyomar@mdanderson.org; Joseph Caruso, The University of California, San Francisco, 513 Parnassus Ave, HSW 501 San Francisco, CA 94143; Phone: (415) 502-6117; joseph.caruso2@ucsf.edu.

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G_1-S Checkpoint Control

Fundamentally, cell division requires a cell to first replicate its genomic DNA and then segregate the replicated chromosomes into separate daughter cells. At the cellular level, the coordination of these processes is complex and requires precise temporal control to maintain genome integrity and cell viability. Tissue-level control of cell division is coordinated by a vast array of signals, including soluble factors, cell-cell interactions, and cell-matrix interactions. Individual cells integrate these social cues with readouts of cellular status (e.g. telomere length, DNA integrity, and metabolic state) to inform cell division processes. As Pardee first reported in 1974 (1), these varying cellular inputs converge on a core regulator governing the decision of a cell to replicate its genome or remain quiescent.

Although Pardee predicted a single R-factor underlying the G_1-S–phase restriction point, the point at which commitment to cell division is complete (2), subsequent research has revealed a more complicated regulatory network. At its core, cell cycle progression is orchestrated by the orderly expression of cyclins (regulatory subunits), which sequentially activate cyclin-dependent kinases (CDKs; catalytic subunits). Each phase of the cell cycle has a unique configuration of cyclins and CDKs that govern the cell division machinery. G_1 cyclin/CDK activity represents the major integration point of tissue-level controls on cell division (3). Sustained deviation in the activity of these CDKs, independent of tissue-level inputs, promotes tumor initiation and progression (4,5).

Cyclin E and Cell Cycle Control

In prevailing models of the mammalian cell cycle, cyclin D–CDK4/6 activity, stimulated by an abundance of mitogenic over inhibitory inputs, induces cells to enter G_1-phase from quiescence (G_0) by partially phosphorylating pRb (6). The E2F-family transcription factors are bound and sequestered by hypophosphorylated pRb in G_0 but are released by phosphorylated pRb during G1 progression and directly enhance cyclin E transcription (7). Cyclin D–CDK4/6 activity tapers off at the end of G1, signaling a shift in cell cycle regulation via cyclins from D-type to E-type. The cyclin E–CDK2 complex further phosphorylates pRb, enhancing the E2F-mediated upregulation of S-phase–specific genes (8). Cyclin E-CDK2 activity is subject to both intrinsic and extrinsic control throughout G_1-phase, in part through the binding of endogenous CDK inhibitors (CKi), including $p21^{CIP1}$ and $p27^{Kip1}$ (9). Cyclin E–CDK2 reaches peak activity in late G_1-phase through positive feedback mechanisms, including the enhanced E2F-mediated transcription of cyclin E and phosphorylation-induced inactivation of key inhibitors (e.g. p27, p21 and SMAD3) (10–12). Eventually, cyclin E–CDK2 becomes insensitive to intrinsic and extrinsic inputs mediating irreversible commitment to cell division (the restriction point) (2). As cells progress through S-phase, cyclin E undergoes rapid ubiquitin proteasome–mediated degradation (13,14). CDK2 activity is maintained by cyclin A throughout S-phase and into G_2-phase (14).

At the G_1-S–phase transition, cyclin E participates in the initiation of DNA replication through several mechanisms beyond the E2F-mediated activation of S-phase gene transcription. [1] Cyclin E–CDK2 phosphorylates p220NPAT, thereby stimulating *histone biosynthesis* (15,16); [2] Cyclin E triggers *centrosome duplication* through a CDK2-

dependant mechanism mediated by the phosphorylation of centrosomal proteins (e.g. nucleophosmin B23 and CP110) (17,18) and through a CDK2-independent mechanism dependent on a centrosome localization signal within cyclin E (19); [3] Cyclin E–CDK2 regulates *DNA replication licensing and the origin of replication firing* through interactions with the origin replication complex subunits (e.g. CDC6, CDT1, and the minichromosome maintenance protein complex), in a manner that is predominantly kinase independent (20–22).

Evidence from studies with knockout mice demonstrates considerable redundancy among different cyclin/CDK complexes. Cyclin E/CDK2 is largely dispensable for mammalian cell cycles. Cyclin E1/E2 double-knockout mice are viable, although the failure of endoreduplication requires placental rescue during gestation (23–26). Cells derived from these animals demonstrate specific defects in their ability to enter G_1-phase from G_0-phase, which are due in part to defective DNA replication licensing. CDK2 knockout mice are also viable, but they are infertile (27). Critically, cyclin E–null mouse embryonic fibroblasts are resistant to oncogenic transformation, indicating that cyclin E may play a critical role in tumor development (26). In established cancer cell lines, dominant-negative CDK2 dramatically inhibits cell cycle progression (28). Collectively, experimental evidence demonstrating that cyclin E is largely dispensable for adult cell cycle progression but critical to cancer cell cycle progression suggests that specifically targeting cyclin E–CDK2 could have a favorable therapeutic index.

Cyclin E Overexpression in Human Cancers

Cyclin E expression is commonly deregulated in many cancer types, including breast, lung, colorectal, gastric, lymphoma, leukemia, and osteosarcoma (29–38). Cyclin E overexpression has several underlying mechanisms including gene amplification, transcriptional upregulation, and disruption of cyclin E degradation. [1] Cyclin E *gene amplification* is observed in many common tumor types, particularly ovarian (22%) (39), esophageal/gastric (18%) (40,41), endometrial (14%) (42), bladder (7%) (43), pancreatic (6%) (44), and non-small cell lung cancer (5%) (45) (**Supplementary Figure 1A**). [2] *Transcriptional upregulation* of cyclin E occurs in tumors with inactivated pRb, due to an increase in E2F-mediated transcription. Inactivation of pRb is a frequent event during tumorigenesis, as a consequence of excessive CDK4/6 activity (due to the activation of certain oncogenes, such as cyclin D1 and RAS), the genetic inactivation of the pRb gene, or the introduction of viral oncoproteins (e.g. HPV16-E7) mediating degradation or sequestration of pRb (46). The transcriptional upregulation of cyclin E can also result from c-MYC overexpression or amplification, which directly activates the cyclin E promoter (47,48). [3] *Disruption of cyclin E degradation* is frequently observed following genetic inactivation of FBW7, a critical subunit of the ubiquitin protein ligase complex responsible for directing cyclin E degradation (49–51). Inactivation of FBW7 in several tumor types is commonly observed in Cancer Genome Atlas (TCGA) databases (52), including endometrial (20%), colorectal (17%), cervical, (12%), bladder (9%), and head and neck cancer (8%) (Supplementary Figure 1B).

Cyclin E overexpression leads to enhanced CDK2 activity and cell cycle progression, thereby reducing the ability of cells to regulate the G_1-S transition. Owing to functional redundancy, cyclin E–CDK2 overexpression has the ability to phosphorylate pRb, even if cyclin D–CDK4/6 complexes have been rendered inactive by the overexpression of p16 (53). In experimental models, cyclin E overexpression is sufficient to induce tumorigenesis *in vivo*. Approximately 10% of transgenic mice with overexpression of human cyclin E in the mammary gland develop mammary carcinomas (54). Cyclin E overexpression also dramatically enhances the transformation by oncogenes such as HRAS (55). A likely key mechanism of cyclin E–mediated oncogenesis is the induction of genomic instability (56–58), which was not observed following the overexpression of cyclin D1 or cyclin A (57). However, recent studies suggest that cyclin D1 overexpression can also induce genomic instability in a CDK-independent fashion by promoting the transcriptional induction of a specific set of genomic instability–inducing genes (59–62). This suggests that deregulation of cyclins and CDKs may act synergistically to cause genomic instability. Mechanistically, cyclin E overexpression increases DNA replication beyond the capacity of cellular nucleotide pools, causing replication stress and replication-induced DNA damage (63). In p53-deficient cells, cyclin E overexpression causes centrosome amplification, leading to polyploidy (64). Cyclin E mutants incapable of activating CDK2 or initiating the G_1-S-transition are nonetheless able to transform rat embryonic fibroblasts in cooperation with HRAS as long as the centrosome localization signal is intact (65).

Tumor-Specific Low-Molecular-Weight Cyclin E isoforms

A 1993 study characterizing deregulated cyclin expression in breast cancer cell lines revealed that the cells accumulate several truncated forms of the cyclin E protein of unknown biological consequence (29). These truncated isoforms, collectively termed low-molecular-weight cyclin E (LMW-E), are restricted to tumor cell lines and do not occur in human mammary epithelial cells (HMECs) obtained from disease-free donors (29). In both normal and tumor cells and tissues full length cyclin E (which, hereafter is referred to as FL-cyclin E) is expressed at approximately 50 kDa, as determined by western blot and mass spectrometry (66). Five tumor-specific LMW-E isoforms with molecular weights ranging from 45 to 33 kDa have been identified (66,67). Digestion of cyclin E with neutrophil elastase (NE) recapitulates the characteristic LMW-E pattern seen in cancer cells (66,68) (Figure 1a). In addition, at least two other proteases, calcium-dependent calpain (69) and calpain-2 (70), have been linked to the generation of LMW-E in tumor cells.

Clinically, LMW-E isoforms (71,72) have been observed in multiple tumor types including breast cancer (29), ovarian cancer (73,74), melanoma (75), colorectal cancer (76–79), lung cancer (80), bladder cancer, and renal cell carcinomas (81). However these isoforms have not been detected in matched adjacent histologically normal tissues. (82). One analysis of total cyclin E (FL-cyclin E + LMW-E) by western blot in 395 breast cancer patients revealed a statistically significant association with distant metastases and reduced overall survival (83). At a median follow-up of 6.4 years, 91.7% of patients with total cyclin E overexpression developed either local and/or distant metastases compared with 7% of patients without total cyclin E overexpression ($p < 0.001$). In multivariable analysis, total cyclin E expression was an independent prognostic variable and a better predictor of disease-

specific and overall survival compared with nodal status, estrogen receptor (ER) status, or even IIIB-IV stage. Before this study, the prognostic value of cyclin E expression had been evaluated only on the basis of nuclear staining in immunohistochemistry studies. These studies revealed no consistent association between cyclin E overexpression and survival (84). These results led us to study the features distinguishing LMW-E from FL-cyclin E, in order to better understand their roles in the development of aggressive cancers.

Generation of LMW-E by intracellular Neutrophil Elastase

The observation that tumor-specific LMW-E could be generated from FL-cyclin E by the serine protease NE was an unexpected finding (Figure 1a). In breast cancer patients, high levels of NE have been shown to be prognostic for poor overall, metastasis-free, and disease-specific survival, and also predictive of resistance to standard therapies (85–88). High levels of NE have also been found in bladder cancer, lung cancer, and pancreatic cancer (86,88–93). One study using the *loxP*-Stop-*loxP* K-ras^{G12D} mouse model of lung cancer showed that NE knockout severely limits tumor growth and proliferation. This study also identified another intracellular target of NE in mouse and human cells, the insulin receptor substrate-1 (IRS-1), which increases phosphatidylinositol 3-kinase (PI3K) activity through enhanced association with the platelet-derived growth factor receptor, ultimately promoting Akt activation (94). Our group has evaluated the role of NE in the C3(1)/Tag mouse model of triple-negative breast cancer (TNBC), reproducing the finding in the lung cancer model that NE-knockout reduces tumor growth and proliferation with no obvious effect on metastasis (95). In these models, the relative importance of NE-mediated pro-tumorigenic mechanisms (activation of PI3K, generation of LMW-E, enhanced inflammation etc.) is an active area of investigation.

In vitro models demonstrate that NE can undergo endocytosis following its association with the cancer cell surface receptor neurophilin-1 (96) in a clathrin- and dynamin-dependent manner (97). Uptake of NE from the extracellular environment has been shown to result in generation of LMW-E (68), as well as, degradation of IRS-1 (98). Serine protease inhibitors are essential to the regulation of NE activity. Elafin, an endogenous WAP-domain-containing NE inhibitor, is downregulated in invasive breast cancer compared with breast tissue from disease-free donors (99,100). Downregulation of elafin sensitizes HMECs to exogenous NE-induced proliferation, suggesting that elafin is a counterbalance against the mitogenic effects of NE, including the intracellular generation of LMW-E (95).

Oncogenic Functions of LMW-E

Overexpression of LMW-E and FL-cyclin E are not mutually exclusive, and both have oncogenic properties. However, we posit that LMW-E has several properties that make it a particularly potent mediator of cell cycle deregulation and tumorigenesis when compared with FL-cyclin E. These properties of LMW-E include hyperactivation of CDK2, altered substrate interactions, and novel interactions within the cytoplasmic compartment, as described in more detail below.

[1] Hyperactivation of CDK2 (Figure 1b):

Compared with FL-cyclin E, LMW-E has a higher binding affinity for CDK2 (101). Furthermore, LMW-E/CDK2 complexes cannot be inhibited by $p21^{CIP1}$ or $p27^{KIP1}$ (102). These factors contribute to significantly higher levels of CDK2-associated kinase activity when CDK2 is in complex with LMW-E compared to FL-cyclin E (66,103). Furthermore, LMW-E is less susceptible than FL-cyclin E to nuclear FBW7-mediated ubiquitin-directed proteosomal degradation, and thus is more stable than FL-cyclin E (104,105). Although LMW-E accumulates in the cytoplasm, it can shuttle to the nucleus when bound to CDK2 and phosphorylate conventional targets required for S-phase progression (e.g. pRB). In a three-dimensional mammary epithelial culture system, the overexpression of hyperactive LMW-E disrupted acinar morphogenesis, enhanced proliferation, and increased tumorigenicity as compared with FL-cyclin E. In this system, LMW-E phenotypes were dependent on their ability to hyperactivate CDK2 (106). In HMECs, the sustained phosphorylation of histone acetyltransferase HBO1 by hyperactive LMW-E-CDK2, but not FL-cyclin E-CDK2, promotes the enrichment of cells with epithelial-to-mesenchymal transition (EMT) and cancer-stem-like phenotypes in an HBO-1-dependent manner (Figure 1c) (107).

[2] Altered Substrate Interactions:

Novel and atypical properties that arise from the altered structure of LMW-E may be similarly consequential in enhancing tumorigenesis. Although CDKs are the catalytic subunit, interactions with substrates are governed in part by the individual cyclins. Given that LMW-E isoforms are missing a significant part of the N-terminus, one would predict that LMW-E and FL-cyclin E would have different substrate interactions when in complex with CDK2. This is illustrated by the interaction between CDK2 and CDC25c (Figure 1c). The inducible overexpression of either LMW-E or FL-cyclin E induces CDC25c phosphorylation, increasing its activity. CDC25c promotes the activation of cyclin-B-CDK1 and PLK1, causing premature mitotic entry in cyclin E overexpressing cells (108,109). LMW-E-overexpressing cells, however, fail to sustain CDC25c phosphorylation/activity and thus exit mitosis much faster than control cells (not overexpressing any form of cyclin E). In stark contrast, cells overexpressing FL-cyclin E maintain high levels of CDC25c phosphorylation and activity, which sustains hyperactivation of CDK1 and PLK1 and thereby substantially delays mitotic exit as compared with control cells (108). The significant shortening of mitosis in LMW-E overexpressing cells prevents proper chromosome segregation, promotes cytokinesis failure, and leads to the generation of multinucleated cells with supernumerary centrosomes (Figure 1d). These defects are not seen at high levels with overexpression of FL-cyclin E (109,110). One study of 331 breast cancer patients revealed a significant correlation between high LMW-E expression, centrosome amplification, and polyploidy ($p = 0.0003$). Patients with these features had significantly lower rates of disease-specific survival ($p = 0.02$) (109).

[3] Novel Interactions of Cytoplasmic LMW-E (Figure 1e):

FL-cyclin E is normally translated in the cytoplasm, where it forms a complex with CDK2, and is then transported to the nucleus via the importin-dependent pathway (71,72,111). The

nuclear localization signal sequence is found at the cyclin E gene's N-terminus, which is cleaved off in the LMW-E isoforms (112). Fractionation and protein complementation assays demonstrate that the LMW-E-CDK2 complexes preferentially accumulate in the cytoplasm (104). Accumulation of LMW-E-CDK2 in the cytoplasm provides this complex the opportunity to interact with novel partners that FL-cyclin E-CDK2 would not encounter in the nucleus. One recent study identified ATP-citrate lyase (ACLY), an enzyme required for the conversion of citrate to oxaloacetic acid and acetyl-CoA, as a novel LMW-E–binding protein in the cytoplasmic compartment (113). Acetyl-CoA is required for a diverse set of cellular processes, including the synthesis of lipids for membrane biogenesis and histone acetylation. ACLY activity is higher in tumors than in disease-free tissues and it plays an important role in tumorigenesis (114–116). Interaction with LMW-E increases ACLY activity, thereby increasing lipid levels in the cells in a CDK2-independent fashion. That LMW-E has CDK2-independent functions is not surprising, as FL-cyclin E has several such roles as well (e.g., in the initiation of DNA replication and centrosomal duplication) (26). Experimentally, purified FL-cyclin E can also interact with ACLY. In cells, however, FL-cyclin E is largely confined to the nucleus and ACLY resides in the cytoplasm, therefore LMW-E has greater opportunity to alter ACLY activity than FL-cyclin E. Significantly, ACLY activity has important roles in tumorigenesis, and its knockdown attenuates several oncogenic features of LMW-E-expressing cells (113).

Cytoplasmic mislocalization may be a common feature of cyclin deregulation in cancer. Cyclin D1, which lacks a canonical nuclear localization signal, is known to shuttle in complex with CDKs between the nucleus and cytoplasm at certain stages of the cell cycle. Cyclin D1 overexpression dramatically increases the cytoplasmic pool of cyclin D1 (117). Evidence suggests that excessive cytoplasmic cyclin D1 has important oncogenic effects, including increased cell migration and invasion (118,119), through direct interactions with several components of the cytoskeleton and cell migration machinery, including paxillin phosphorylation, which increases Rac1 activity (120,121).

Tumorigenic and pro-metastatic activity of LMW-E—Both primary mammary tumor formation and metastasis are markedly enhanced in LMW-E transgenic mice as compared with FL-cyclin E-overexpressing mice. LMW-E overexpression in the mammary gland is sufficient to induce mammary adenocarcinomas in 27% of mice, whereas FL-cyclin E expression in the mammary gland induces mammary adenocarcinomas in only 10.4% of the transgenic mice (54,122). Metastasis was observed in 25% of LMW-E tumor-bearing animals compared with only 8.3% of animals with tumors arising in the FL-cyclin E background. In this model, LMW-E overexpression results in the spontaneous loss of heterozygosity at the $p14^{ARF}$ locus, suggesting that LMW-E cooperates with p53 inactivation to generate an aggressive tumor phenotype. CDK2 wild-type or heterozygous mice succumbed to mammary tumors with mean latencies of 16 and 19.5 months, respectively, whereas CDK2 nullizygous littermates did not display tumors through 24 months of observation (123). Furthermore, continuous administration of two different CDK inhibitors significantly delayed LMW-E-induced mammary tumor formation. Examination of breast cancer specimens using immunohistochemistry reveal that about half the patients whose tumors express LMW-E (i.e., cytoplasmic cyclin E) but no detectable levels of FL-

cyclin E (nuclear) (described in more detail in the section to follow) (124–126). Therefore, a critical direction for future research should be the development of novel mouse models of LMW-E expression in the absence of endogenous mouse cyclin E, which may reveal new biology and increase our understanding of the clinical problem (71).

Future Directions: The Translational Potential of LMW-E

The prognostic significance of LMW-E:

Western blot analysis, the primary assay for evaluating LMW-E in experimental systems, is impractical for routine clinical use. Given the cytoplasmic mislocalization of LMW-E, we hypothesized that an immunohistochemical assay designed to score the nuclear and cytoplasmic compartments for expression of cyclin E and phosphorylated CDK2 (p-CDK2) would provide a surrogate measure of LMW-E expression in human tumor specimens with the potential for clinical application. Once developed, this immunohistochemical analysis was applied to tumor samples from 1676 breast carcinoma patients (124). In this study, cytoplasmic cyclin E strongly correlated with cytoplasmic p-CDK2, high tumor grade, estrogen receptor (ER)-negative status, progesterone receptor (PR)-negative status, and human epidermal growth factor receptor 2 (HER2)-positive status (all $p < 0.0001$) (124). In multivariable analysis, LMW-E and p-CDK2 predicted recurrence-free and overall survival, suggesting that this assay could be used to identify patients with aggressive breast cancers (124). Recently (126), we expanded this analysis to 2,494 breast cancer patients from four different patient cohorts. In multivariable analysis, cytoplasmic cyclin E staining was associated with the greatest risk of recurrence across all breast cancer subtypes. Collectively, these studies suggest that cytoplasmic cyclin E is likely to identify patients with the highest likelihood of recurrence consistently across different patient cohorts and breast cancer subtypes. Standardization and Clinical Laboratory Improvement Amendments (CLIA) certification of this assay will facilitate the stratification of patients for LMW-E or FL-cyclin E-directed therapies.

Cytoplasmic cyclin E as a predictive biomarker:

In experimental models, LMW-E mediates resistance to endocrine therapies such as aromatase inhibitors and fulvestrant (110,127). In one study, breast cancer patients with LMW-E overexpressing tumors (cytoplasmic cyclin E) who received aromatase inhibitors in the neo-adjuvant setting had significantly worse recurrence-free survival than did patients whose tumors did not express cytoplasmic cyclin (128). Recently, three CDK4/6 inhibitors (palbociclib, ribociclib, and abemaciclib) in combination with either letrozole or fulvestrant have been evaluated in clinical trials for the treatment of advanced hormone receptor positive (HR+) breast cancer (129,130). These trials showed that patients who received a CDK4/6 inhibitor in addition to letrozole or fulvestrant had progression-free survival durations that were at least twice those of patients who received letrozole or fulvestrant alone (131–133). The US Food and Drug Administration (FDA) has now approved all three CDK4/6 inhibitors for the treatment of post-menopausal women with HR positive, HER2-negative metastatic breast cancer (134–137). Despite these promising clinical advances, a major limitation in the use of CDK4/6 inhibitors is the lack of reliable biomarkers to identify patients with intrinsic and/or acquired resistance to these agents. Although previous *in vitro*

studies have shown that pRb, cyclin D, and p16 could predict response to palbociclib (138–140), results from phase II/III trials have not shown a significant correlation between response and the expression of p16, pRb (131), and Ki67 expression; *CCND1* amplification (141); or (3) *PIK3CA* and ESR1 (142) mutational status (143). A recent study from our group demonstrated that together pRb and LMW-E status predicts response in HR-positive breast cancer patients treated with palbociclib (144). Specifically, a multivariable Cox proportional hazards model showed that pRb and LMW-E were the only factors significantly associated with the progression-free interval in both patients treated with palbociclib plus letrozole and those treated with palbociclib plus fulvestrant, with hazard ratios of 0.2 and 0.09 (pRb-negative), 3.2 and 5.2 (LMW-E–positive), and 9.2 and 23.8 (both pRb-negative and LMW-E–positive) respectively (144). These studies suggest that an immunohistochemical assay for pRb and LMW-E can be used clinically to identify patients who are likely to have a sustained response to CDK4/6 inhibitors in combination with endocrine therapy.

Targeting LMW-E overexpressing tumors (Figure 2).

Studies in pre-clinical models suggest that tumors that are resistant to CDK4/6 inhibition (e.g. pRb-negative and LMW-E positive tumors) (144) or that become resistant to endocrine therapy (145) are likely to respond to CDK2 inhibitors such as roscovitine or dinaciclib. CDK2 may also be a therapeutic target in other aggressive tumor types. In a model of treatment-naïve aggressive HER2-positive breast cancer cells expressing LMW-E, the combination of trastuzumab (targeting HER2) and roscovitine led to synergistic cell killing (146). The administration of roscovitine or dinaciclib prior to treatment with doxorubicin is also synthetically lethal in both TNBC xenografts (147) and inflammatory breast cancer cell lines (125) expressing high levels of LMW-E. In addition, the specific knockdown of CDK2 significantly inhibits LMW-E driven tumor proliferation and increases apoptosis (123). The development of inhibitors that specifically target CDK2 without inhibiting other CDKs is critical to a comprehensive therapeutic approach targeting the deregulated cell cycle. Such inhibitors could be translated into biomarker (cytoplasmic cyclin E)-driven clinical trials.

A significant problem with the current generation of CDK2 inhibitors is their lack of specificity and their associated toxicity. A combination therapy approached could be used to reduce the toxicity of current CDK inhibitors by maximizing the therapeutic effect and thereby shortening the treatment duration. For example, the combination of the PLK4 inhibitor CFI-400945 and roscovitine can, by generating supernumerary centrosomes and inhibiting centrosome clustering respectively, synergistically cause multipolar anaphase catastrophe and death in lung cancer cells (148). Another possibility is to target the specific vulnerabilities of tumors with high LMW-E and FL-cyclin E expression. Such tumors, for example, have high levels of replication stress and DNA damage. The inhibition of Wee1 kinase, which normally blocks mitosis by specifically phosphorylating CDK1, would force LMW-E and FL-cyclin E-expressing tumor cells with high levels of unrepaired DNA damage into the cell cycle, causing many to undergo mitotic catastrophe and cell death. NE-directed therapies (149), developed for the treatment of inflammatory disease (e.g. chronic obstructive pulmonary disease), could also be repurposed to specifically inhibit the generation of LMW-E and the activation of other pro-tumorigenic pathways in tumor cells.

Overall, LMW-E-expressing tumors, which can be identified on the basis of cytoplasmic cyclin E expression, are among the most aggressive tumors, particularly in breast cancer patients. Identifying treatment strategies that are effective for patients with these tumors is a top priority.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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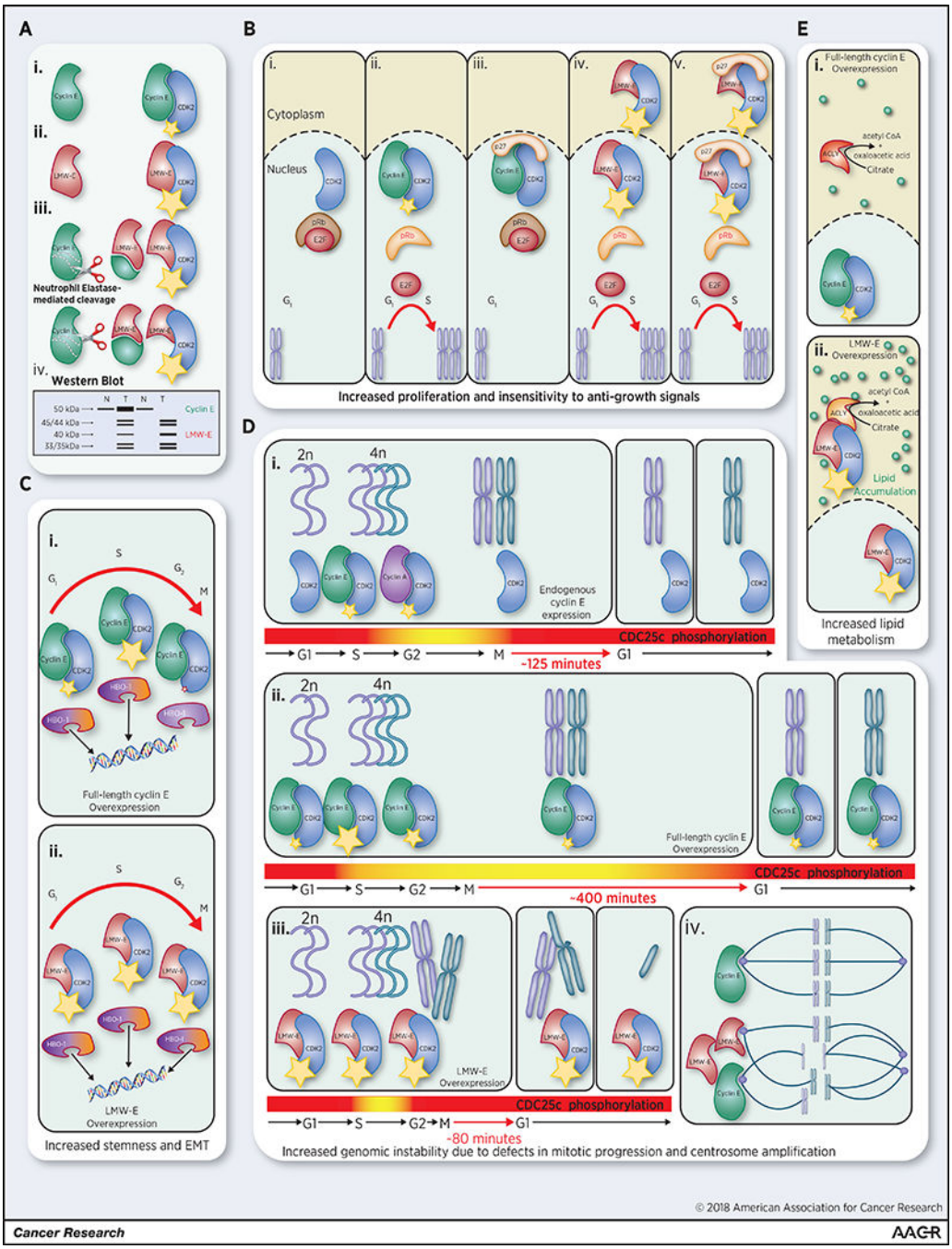

Figure 1: The unique biochemical activities of LMW-E versus FL cyclin E and their consequences.

(A) (i) Cyclin E (FL 50 kDa) is an activating subunit of CDK2 that promotes kinase activity (small yellow star) during the G_1-S-phase transition. Tumor-specific LMW-E isoforms are generated by (ii) alternative translation from methionine 46 (40 kDa) and (iii) NE-mediated cleavage of full-length cyclin E at two N-terminal sites (45/44 kDa and 35/33 kDa; doublets due to phosphorylation events) (66,105). LMW-E isoforms demonstrate higher binding affinity for CDK2, promoting hyperactivation of the kinase (large yellow star). (iv) In western blot analysis of normal (N) and tumor (T) tissue (using a C-terminally-directed

antibody), LMW-E isoforms characteristically resolve as five distinct bands beneath FL cyclin E. In half of LMW-E-expressing breast tumors, cyclin E is also overexpressed; in the other half, however, LMW-E is expressed in the absence of full-length cyclin E (B). (i) When the pRb pathway is unaltered by oncogenic events, hypophosphorylated pRb binds to and sequesters E2F family transcription factors in G_0-phase. (ii) FL cyclin E activates CDK2, leading to the hyperphosphorylation and inactivation of pRb, thereby releasing E2Fs to activate S-phase gene expression and progression. (iii) However, CDK inhibitors (e.g. p27) can inhibit the FL cyclin E-CDK2 complex (even if FL cyclin E is overexpressed) and prevent S-phase progression. (iv) Hyperactive LMW-E-CDK2 complexes can localize to the nucleus and hyperphosphorylate pRB; (v) even in the presence of CDK inhibitors, thereby promoting insensitivity to negative growth signals. (C) Hyperactive LMW-E-CDK2 complexes have other consequences. (i) FL Cyclin E-CDK2 can phosphorylate the substrate HBO-1; however, (ii) only constitutive hyperphosphorylation by LMW-E-CDK2 can promote HBO-1-dependant EMT and stemness properties, suggesting that cell cycle context-independent phosphorylation of HBO-1 alters its histone acetyltransferase activity in a pro-tumorigenic manner. (D) The proper timing of DNA replication and mitosis is essential to genome integrity. (i) One mechanism to ensure the fidelity of this process is feedback control through CDC25c, which promotes the proper timing of mitotic entry and exit through the activation of cyclin B-CDK1 and PLK1. (ii) Overexpression of FL cyclin E results in the improper phosphorylation of CDC25c and premature mitotic entry, but maintains the phosphorylation of CDC25c, delaying mitotic progression to cytokinesis and thereby largely preventing genomic instability. (iii) LMW-E overexpression also initiates premature mitotic entry; however unlike FL cyclin E, LMW-E cannot sustain CDC25c phosphorylation, resulting in faster mitotic exit and genomic instability. (iv) Genomic instability is further promoted by centrosome amplification induced by both FL cyclin E and LMW-E overexpression. (E) (i) FL cyclin E is largely restricted to the nucleus and therefore has limited opportunities to interact with cytoplasmic proteins. (ii) In contrast, LMW-E lacks an N-terminal nuclear localization signal promoting its accumulation in the cytoplasm where it can interact with novel binding partners including ACLY. LMW-E-CDK2 enhances ACLY activity (independent of phosphorylation), thereby promoting intracellular lipid accumulation and pro-tumorigenic phenotypes, including migration and invasion.

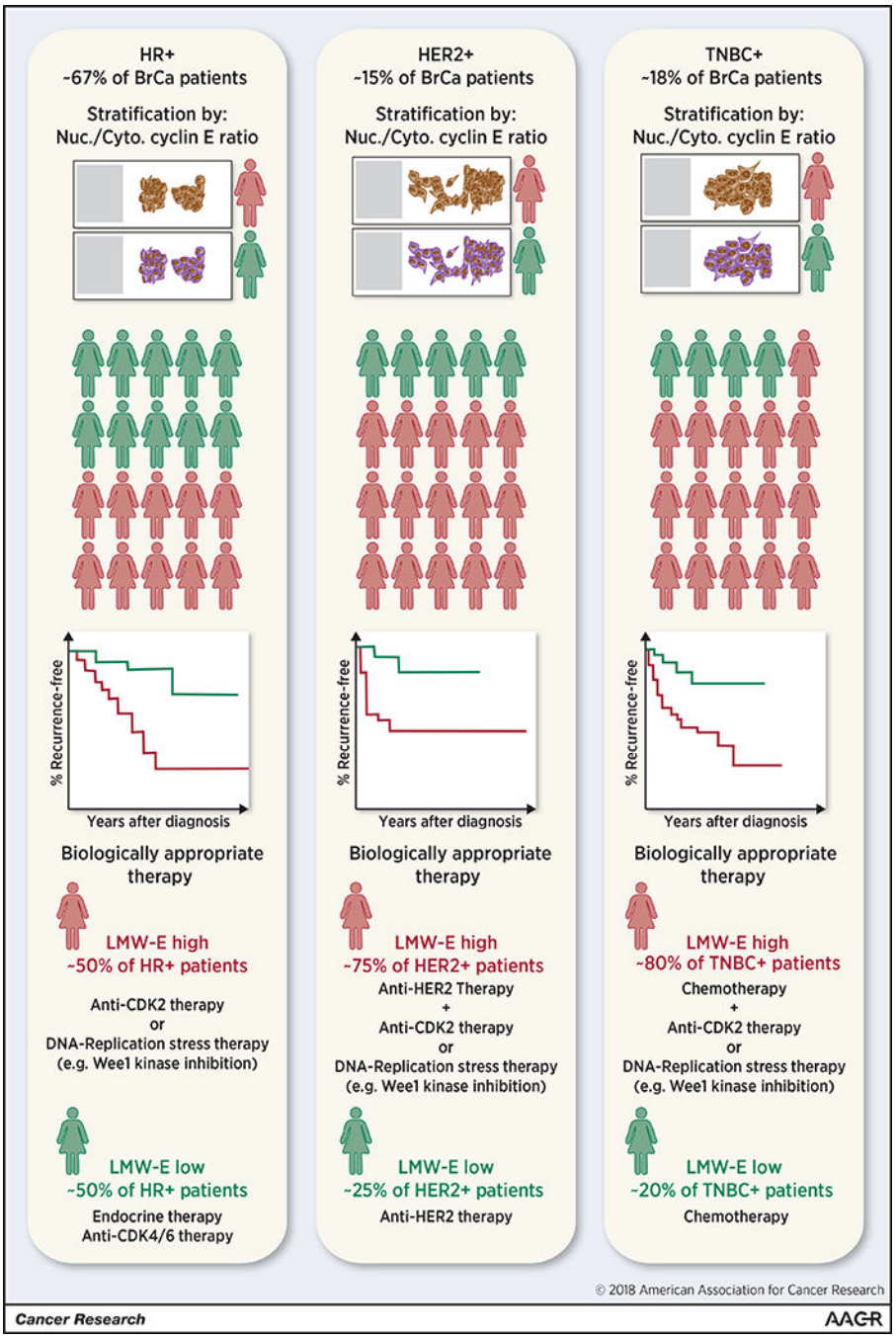

Figure 2: The prognostic significance of LMW-E.

Clinically, breast cancers are stratified into three groups, HR positive breast cancer, HER2 positive breast cancer, and TNBC based on the pathohistological assessment the of ER, PR, and HER2 expression. These breast tumor types are characterized by dramatic differences in clinical course and are treated using tailored therapeutic approaches. Immunohistochemical analysis of cyclin E or phosphorylated CDK2 and scoring according to the nuclear (FL)-to-cytoplasmic (LMW) cyclin E ratio identifies a patient population in each breast cancer subtype expressing high levels of LMW-E relative to FL cyclin E (approximately 50% of

HR positive cancers, 75% of HER2 positive cancers, and 80% of TNBC). Within each of these subtypes, patients whose tumors express high LMW-E relative to FL-cyclin E have significantly worse survival outcomes than patients whose tumors predominantly express FL cyclin E. Data reviewed here suggest that LMW-E-expressing tumors are resistant to commonly used targeted therapeutics and may benefit from a combination of current therapeutic approaches with either anti-CDK2 based therapy or therapeutic strategies targeting specific vulnerabilities of LMW-E overexpressing tumors.