

RESEARCH PAPER



The requirement for cyclin E in c-Myc overexpressing breast cancers

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ABSTRACT

Basal-like triple-negative breast cancers frequently express high levels of c-Myc. This oncoprotein signals to the core cell cycle machinery by impinging on cyclin E. High levels of E-type cyclins (E1 and E2) are often seen in human triple-negative breast tumors. In the current study, we examined the requirement for E-type cyclins in the c-Myc-driven mouse model of breast cancer (MMTV-c-Myc mice). To do so, we crossed cyclin E1- (E1^{-/-}) and E2- (E2^{-/-}) deficient mice with MMTV-c-Myc animals, and observed the resulting cyclin E1^{-/-}/MMTV-c-Myc and cyclin E2^{-/-}/MMTV-c-Myc females for breast cancer incidence. We found that mice lacking cyclins E1 or E2 developed breast cancers like their cyclin E wild-type counterparts. In contrast, further reduction of the dosage of E-cyclins in cyclin E1^{-/-}E2^{+/-}/MMTV-c-Myc and cyclin E1^{+/-}E2^{-/-}/MMTV-c-Myc animals significantly decreased the incidence of mammary carcinomas, revealing a role for E-cyclins in tumor initiation. We also observed that depletion of E-cyclins in human triple-negative breast cancer cell lines halted cell cycle progression, indicating that E-cyclins are essential for tumor cell proliferation. In contrast, we found that the catalytic partner of E-cyclins, the cyclin-dependent kinase 2 (CDK2), is dispensable for the proliferation of these cells. These results indicate that E-cyclins, but not CDK2, play essential and rate-limiting roles in driving the proliferation of c-Myc overexpressing breast cancer cells.

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Introduction


The locus encoding the transcription factor c-Myc represents one of the most frequently amplified genes across all human cancer types [1]. Overexpression of the c-Myc protein and activation of the c-Myc-dependent transcriptional program represents a characteristic feature of basal-like triple-negative breast cancers [2–7]. Among many different oncogenic functions of c-Myc, this protein was shown to drive cell division in part by impinging on E-type cyclins [8–12]. The two E-type cyclins, cyclins E1 and E2 collectively referred to as “cyclin E”, represent the key components of the core cell cycle machinery [13–16]. Upregulation of cyclins E1 and E2 is seen frequently in triple-negative, basal-like mammary carcinomas [17–21]. Targeted overexpression of c-Myc in the

mammary epithelium of transgenic mice results in mammary carcinomas [22–24], and these tumors express high levels of E-cyclins [25]. Also, mice engineered to overexpress cyclin E1 in mammary glands develop mammary hyperplasia and are prone to breast cancers [26], further underscoring the importance of the c-Myc → cyclin E axis in breast tumorigenesis.

E-type cyclins are essential during mouse embryonic development but are dispensable in an adult organism [27–29]. We previously demonstrated that cyclin E plays a rate-limiting role during the initiation and progression of liver cancers [27]. We also demonstrated that this function of cyclin E was independent of its ability to activate the cyclin-dependent kinase 2 (CDK2) [27]. In the current study, we extended these observations to the c-Myc-driven mouse breast cancer model as well as human triple-

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negative breast cancer cell lines expressing high levels of c-Myc.

Materials and methods

Mouse crosses

All mouse experiments were approved by the Dana Farber Cancer Institute Animal Care and Use Committee. Cyclin E1^{-/-} and E2^{-/-} mice were described previously [28]. MMTV-c-Myc mice were purchased from Charles River Laboratories. Mice were intercrossed to generate the following genotypes: cyclin E1^{-/-}/MMTV-c-Myc (n = 13), cyclin E2^{-/-}/MMTV-c-Myc (n = 20), cyclin E1^{-/-}E2^{+/-}/MMTV-c-Myc (n = 17) and cyclin E1^{+/-}E2^{-/-}/MMTV-c-Myc (n = 14). Cyclin E1^{+/+}E2^{+/+}/MMTV-c-Myc littermates (n = 20) served as wild-type controls. Mice were checked bi-weekly, by palpation, for the presence of breast tumors. Upon sacrifice of animals, tumors were excised, measured and analyzed by histology. Tumor volumes were calculated according to the formula $V = a \times b^2/2$ (where a is tumor length and b is tumor width)

Cell culture

Human breast cancer CAL-51 cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). HCC1806, HCC1143, HCC1395, HCC1937, MDA-MB-157, MDA-MB-231, MDA-MB-436, MDA-MB-468, HCC1954, HCC38, and HCC70 cells were obtained from the American Type Culture Collection (ATCC). All cells were cultured according to the recommendation of the supplier. To inhibit asCDK2, 10 μM 3MB-PP1 (529582, Millipore) was added to the culture medium.

Immunoblotting and co-immunoprecipitation

For immunoblotting and co-immunoprecipitation (IP) experiments, cells were lysed in the lysis buffer (20 mM Tris-HCl pH 8.0, 0.1 M KCl, 5 mM MgCl₂, 10% glycerol, 0.1% Tween-20, 0.1% NP40) with protease cocktail inhibitor (4693159001, Millipore). For immunoblotting,

cell lysates were resolved on SDS-PAGE and transferred to PVDF membranes. For co-immunoprecipitation experiments, cell lysates were incubated with antibodies overnight. Immunoprecipitates were washed three times with the lysis buffer followed by SDS-PAGE. The following antibodies were used for immunoblotting: c-Myc (sc764, 1:1000, Santa Cruz), cyclin E1 (sc-247, 1:1000; Santa Cruz), cyclin E2 (EP454Y, 1:1,000; Millipore), CDK2 (sc-163, 1:1,000; Santa Cruz), tubulin (AC40, 1:3,000; Sigma), HSP90 (4874, 1:3,000; Cell Signaling), β-actin (A5441, 1:4,000, Sigma), GAPDH (D16H11, 1:1,000; Cell Signaling). For immunoprecipitation, the following antibodies were used: CDK1 (sc-54, Santa Cruz), CDK2 (sc-53,220, Santa Cruz), Flag (A2220, Sigma), IgG (5415S, Cell Signaling).

Viral transduction

The pLKO-puro containing shRNA against cyclin E1 (E1-sh: 5'-CGACATAGAGAACTGTGTCAA-3'), or cyclin E2 (E2-sh: 5'-CCAGACACATACAAACTATTT-3'), and pLentiCrispr-v2puro vector containing single-strand guide RNA against CDK2 (CDK2-sg: 5'-CAGAAACAAGTTGACGGGAG-3') were used as previously described [27]. 293 FT cells were used for the production of the virus. Briefly, lentiviral plasmids (E1-sh, E2-sh, Con-sh, Lenti-CDK2-sg, Lenti-con) were mixed with the packaging plasmid pCMV-delta8.9 and the envelope plasmid pCMV-VSV-G at the ratio of 10:9:1, and transfected into 293 FT cells using polyfect transfection reagent (301107, Qiagen). Two days later, virus-containing medium was collected, filtered, and used for infection in the presence of 10 μg/ml polybrene. After 24 h of infection, medium was changed and puromycin was added into the medium for 1 to 2 weeks.

Cell cycle analyses

Cells were cultured in a medium containing 75 μM 5-bromo-2'-deoxyuridine (BrdU) for 1 h, collected and fixed overnight in 90% ethanol. Cells were then treated with 2 N HCl containing 0.5%

Triton X-100, neutralized in borate buffer pH 8.5, incubated with an anti-BrdU antibody (556028, BD Biosciences), 5 $\mu\text{g}/\text{ml}$ propidium iodide and 200 $\mu\text{g}/\text{ml}$ RNase A for 30 min, and analyzed by FACS. Annexin V staining was performed using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen), following the manufacturer's protocol.

Growth curves

Cells were seeded in 12 well plates (2.5×10^4 cells per well, in triplicate) and cultured in appropriate medium. Cells were harvested at the indicated time-points, fixed in 10% buffered formalin, followed by crystal violet staining. 10% acetic acid was then used to dissolve the staining and the OD was measured at 590 nm. For assays shown in Figure 2(e) and 3(d), cells were plated in 12 well plates (2.5×10^4 per well), stained with crystal violet after culturing for 7 days and 9 days, respectively, and photographed.

Generation of cells expressing *asCDK2*

Mouse analog-sensitive (*as*) Cdk2 was constructed by cloning mouse Cdk2 cDNAs carrying the F80 G mutation [27] plus a C-terminal Flag-tag into pLenti-Neo vector (17392, Addgene). To generate HCC1806 cells expressing *asCdk2*, cells were transduced with lentiviruses expressing *asCdk2* and selected with neomycin (400 $\mu\text{g}/\text{ml}$) for 2 weeks. After that, the endogenous CDK2 was knocked out using CDK2-sg CRISPR virus followed by puromycin selection (2 $\mu\text{g}/\text{ml}$).

In vitro kinase assays

Analog-sensitive CDK2 was immunoprecipitated from CDK2-knockout HCC1806 cells engineered to ectopically express Flag-tagged *asCDK2*. For control, immunoprecipitation with IgG was used. Kinase assays were performed in the final volume of 30 μl of the kinase buffer (50 mM HEPES pH 7.5, 10 mM MgCl_2 , 1 mM DTT, 1 mM EGTA, 0.1 mM NaF), containing 10 μM ATP and 0.4 mCi [^{32}P] γATP (Perkin Elmer) and 1 μg of

recombinant histone H1 as a substrate, in the presence or absence of 10 μM 3MB-PP1. After 30 min incubation at 30°C, proteins were denatured, resolved on SDS-PAGE, transferred to nitrocellulose membranes and exposed to X-ray films.

Results

Analyses of the requirement for cyclin E in *c-Myc*-driven murine mammary carcinomas

To test the requirement for cyclin E in breast tumorigenesis *in vivo*, we took advantage of the MMTV-*c-Myc* transgenic mouse strain. Female mice of this strain develop mammary carcinomas due to overexpression of the *c-Myc* oncoprotein, which is targeted to mammary epithelium by the mouse mammary tumor virus (MMTV) promoter [22–24]. We first crossed MMTV-*c-Myc* mice with mice lacking cyclin E1 (cyclin E1^{-/-}) [28] or cyclin E2 (cyclin E2^{-/-}) [28] and observed the resulting cyclin E1^{-/-}/MMTV-*c-Myc* and cyclin E2^{-/-}/MMTV-*c-Myc* females for tumor incidence. We found that cyclin E1- and E2-deficient mice developed mammary carcinomas with very similar kinetics as control cyclin E1^{+/+}/E2^{+/+}/MMTV-*c-Myc* animals (Figure 1(a,c)). We concluded that neither cyclin E1 nor E2 is essential for the initiation of breast tumorigenesis in this experimental system.

We previously observed that cyclins E1 and E2 play overlapping functions in normal mouse development [28]. Therefore, we hypothesized that the same may be true during *c-Myc*-driven mammary tumorigenesis. To test this, we interbred MMTV-*c-Myc*, cyclin E1^{-/-} and E2^{-/-} mice. Since mice entirely lacking E-cyclins (E1^{-/-}E2^{-/-}) die *in utero* [28,29], we generated animals expressing only one single allele of cyclin E, namely cyclin E1^{-/-}E2^{+/+}/MMTV-*c-Myc* and E1^{+/+}E2^{-/-}/MMTV-*c-Myc*. We previously demonstrated that female mice with a reduced dosage of cyclin E genes develop normally and are viable [28]. Analysis of tumor incidence revealed that the reduction of cyclin E copy number significantly diminished the incidence of breast cancers. Thus, by 13 months of age, 80% of control cyclin E1^{+/+}

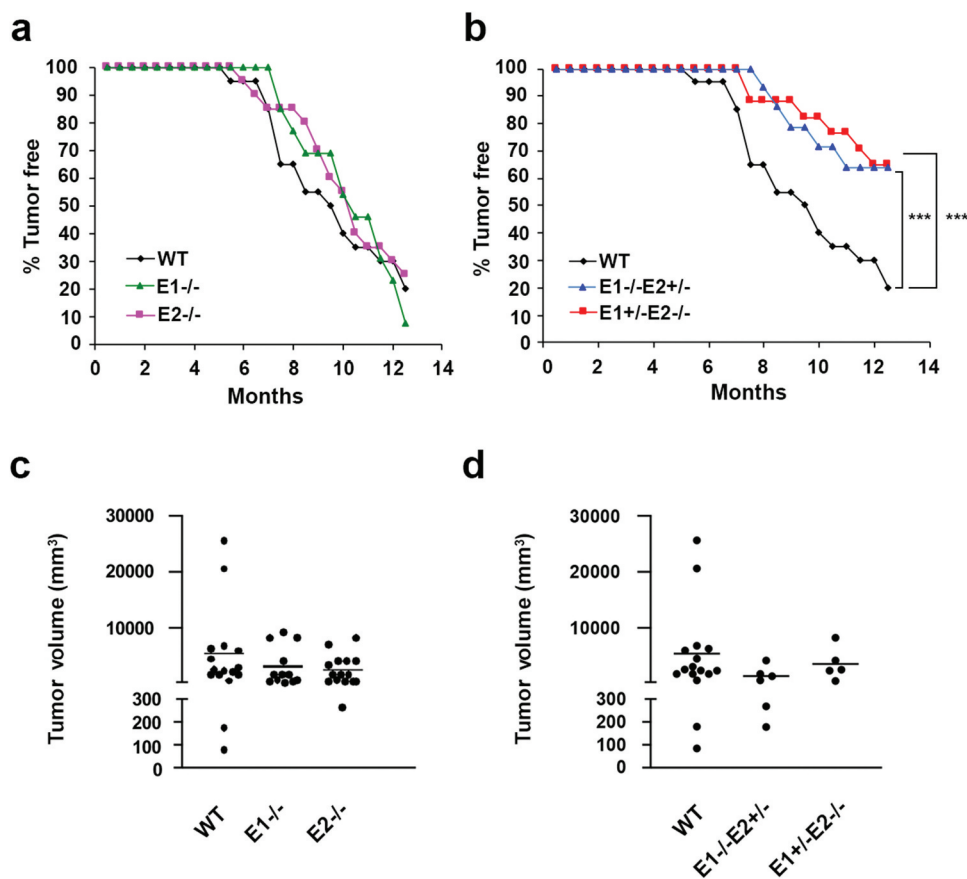


Figure 1. Analyses of breast cancer susceptibility in cyclin E-deficient mice. (a) Comparison of breast cancer incidence between control cyclin E1^{+/+}E2^{+/+}/MMTV-c-Myc (WT), cyclin E1^{-/-}/MMTV-c-Myc and cyclin E2^{-/-}/MMTV-c-Myc females. Mice were monitored by palpation for the presence of breast tumors. (b) A similar analysis using cyclin E1^{+/+}E2^{+/+}/MMTV-c-Myc (WT), cyclin E1^{-/-}E2^{+/-}/MMTV-c-Myc and cyclin E1^{+/-}E2^{-/-}/MMTV-c-Myc females (the same input for WT mice was used to generate panels a and b). ANOVA test for multiple comparisons (Friedman test) was used to calculate the *p*-values. ***, *p* < 0.001; in panel (a) differences were not significant. (c and d) The estimated total tumor burden (per mouse) in tumor-bearing animals of the indicated genotypes. Each dot corresponds to a different mouse and represents the combined tumor volume (the same input for WT mice was used to generate both panels). Tumor volumes were calculated according to the formula $V = a \times b^2 / 2$ (*a* is tumor length and *b* is tumor width). Horizontal lines depict mean values. There were no significant differences between the groups (Kruskal-Wallis test with Dunn's multiple comparison test).

⁺E2^{+/+}/MMTV-c-Myc mice developed mammary carcinomas, compared to only 35% in the cyclin E1^{-/-}E2^{+/-}/MMTV-c-Myc and cyclin E1^{+/-}E2^{-/-}/MMTV-c-Myc groups (Figure 1(b,d)). These observations indicate that E-type cyclins play a rate-limiting role in the initiation of c-Myc-driven breast tumorigenesis *in vivo* in the MMTV-c-Myc mouse model.

Analyses of the requirement for cyclin E in human TNBC cell lines

To extend these analyses to human breast cancers, we chose several human triple-negative breast cancer (TNBC) cell lines expressing relatively high levels of c-Myc (Figure 2(a)). We verified that

these cell lines also express high levels of E-cyclins (Figure 2(a)). We next depleted both E-cyclins using previously validated anti-cyclin E1 and -E2 shRNAs [27] (Figure 2(b) and Supplemental Figure S1), and examined the effect on cell cycle progression. Specifically, we pulsed cells with BrdU, stained with an anti-BrdU antibody and propidium iodide, and analyzed by flow cytometry. We observed that depletion of E-cyclins resulted in an accumulation of cells in the G1 phase of the cell cycle and a strong reduction of the fraction of cells undergoing DNA synthesis (Figure 2(c) and Supplemental Figure S2). Consistent with these findings, we determined that the depletion of E-cyclins virtually extinguished tumor cell proliferation (Figure 2(d,e)).

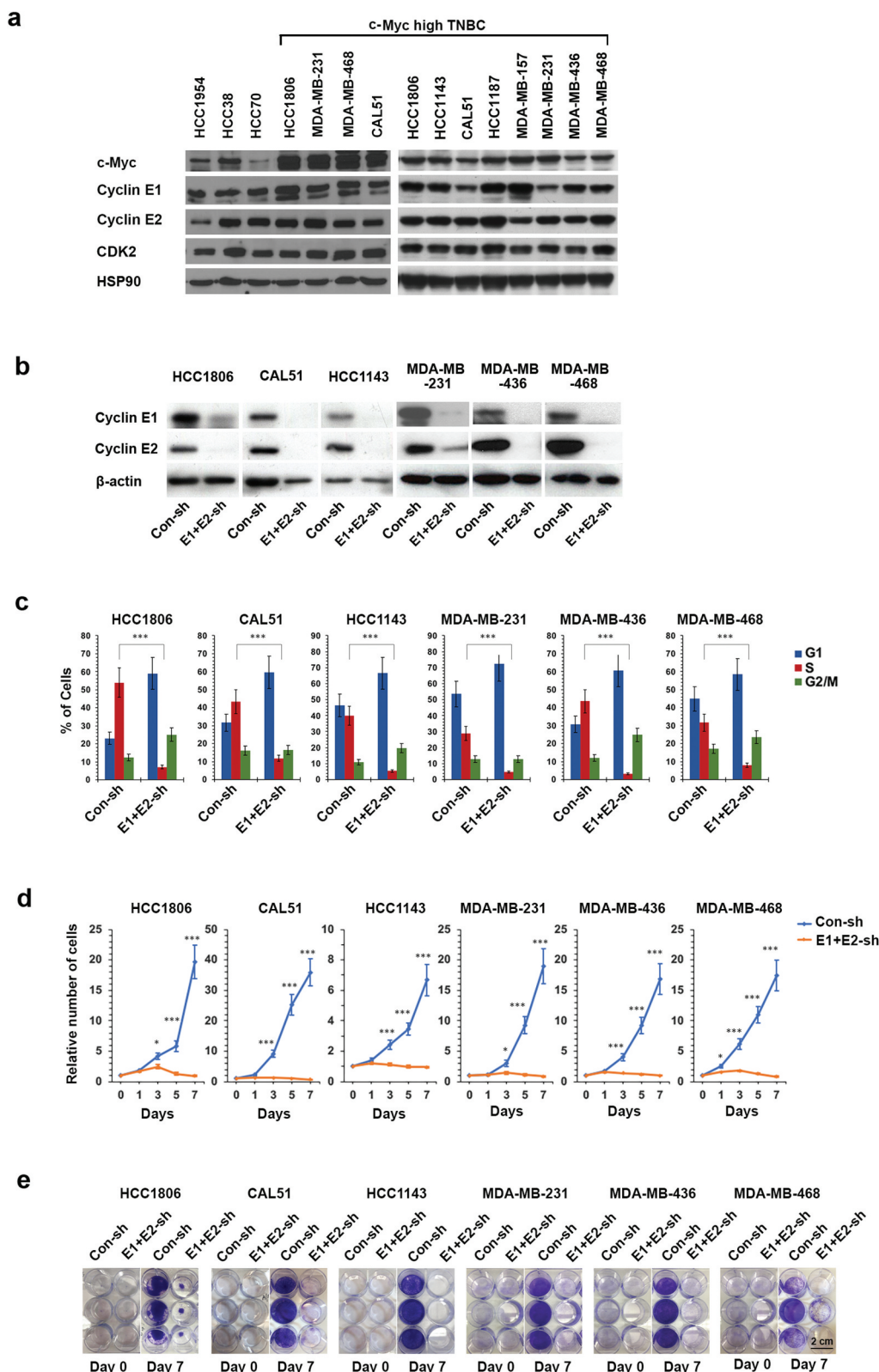


Figure 2. Cyclins E1 and E2 are required for proliferation of triple-negative breast cancer (TNBC) cell lines with high c-Myc expression. (a) Immunoblot analysis of c-Myc, cyclin E1, E2 and CDK2 levels in the indicated human breast cancer cell lines. HSP90 was used as a loading control. (b) Immunoblot analysis of cyclin E1 and E2 levels in the indicated six TNBC cell lines following transduction with viruses encoding anti-cyclin E1 and E2 shRNAs (E1+ E2-sh), or control shRNA (Con-sh). β -actin was used as a loading control. (c) The percentage of cells in the indicated phases of the cell cycle following depletion of E-cyclins as in panel b. Cells were pulsed with BrdU, stained with an anti-BrdU antibody and propidium iodide and analyzed by FACS. Shown are mean values, error bars indicate SD, $n = 3$. Unpaired two-tailed Student's *t* test was used to evaluate the differences between S-phase

fractions in Con-sh versus E1+ E2-sh groups. $***, p < 0.001$. (d) Growth curves of the indicated human TNBC cell lines following depletion of E-cyclins as in panel b. Shown are relative numbers of cells on the indicated days; the number of plated cells (day 0) was set as 1. Error bars indicate SD, $n = 3$. Unpaired two-tailed Student's t test was used for the statistical analysis of the differences between Con-sh versus E1+ E2-sh groups at the indicated time-points. $*, p < 0.05$; $***, p < 0.001$. (e) The indicated TNBC cell lines were plated at 2.5×10^4 cells per well in 12 well plates following depletion of cyclins E1 and E2, as in panel b. Cells were stained and photographed after 7 days. Scale bar, 2 cm.

These results indicate that E-cyclins play an essential role in driving cell cycle progression of all six TNBC cell lines analyzed.

Analyses of the requirement for CDK2 in human TNBC cell lines

The best-documented function of E-cyclins is the ability to activate their catalytic partner, CDK2 [13]. Therefore, we verified the interaction of cyclin E1 with CDK2 in two TNBC cell lines (CAL51 and HCC1806). Specifically, we immunodepleted CDK2 using an anti-CDK2 antibody, and probed the supernatants for the presence of cyclin E1. We observed that immunodepletion of CDK2 removed essentially all cyclin E1 pool, indicating that in these cells cyclin E1 stoichiometrically binds to CDK2 (Figure 3(a)). In contrast, very little cyclin E1 interacted in these cells with a related cell cycle kinase, CDK1 (Figure 3(a)).

To test the requirement for CDK2 in the proliferation of TNBC cells, we used CRISPR/Cas9 to knock out CDK2 in six TNBC cell lines (Figure 3(b)). In contrast to the depletion of E-cyclins, which arrested cell growth (Figure 2(d,e)), knockout of CDK2 had only a very modest effect on cell proliferation (Figure 3(c,d)). Collectively, these observations indicate that E-cyclins, but not CDK2, are essential for TNBC cell proliferation.

To further probe the requirement for CDK2 in driving the proliferation of TNBC cells, we knocked out CDK2 in a TNBC cell line HCC1806 and ectopically expressed the analog-sensitive (*as*) version of CDK2, which we have previously engineered and characterized [27] (Figure 4(a,b)). Analog-sensitive kinases contain a single amino acid substitution within the kinase ATP binding pocket, which renders this kinase inhibitable by *as*-specific inhibitors, such as 3MB-PP1 [30,31] (Figure 4(a)). Indeed, we verified that the addition of 3MB-PP1 strongly inhibited the kinase activity of *as*CDK2 (Figure 4(c)). We next cultured cells expressing *as*CDK2 in the presence of 3MB-PP1, and evaluated the effect on cell growth. As it was the case upon CDK2 ablation, inhibition of

CDK2 kinase had only a modest effect on cell proliferation (Figure 4(d)). These observations further support our conclusion that CDK2 is not required for the growth of TNBC cells.

Discussion

Our earlier work revealed that E-cyclins seem to be dispensable for the physiology of adult mice, as an acute, global shutdown of cyclin E did not have any obvious adverse consequences [27]. In the current study, we have shown that cyclin E is essential for the initiation of mammary carcinomas in mouse MMTV-*c-myc* model. We observed that mice expressing only one remaining cyclin E allele ($E1^{-/-}E2^{+/-}$, and $E1^{+/-}E2^{-/-}$) displayed reduced breast cancer incidence.

Our analyses of tumor-bearing mice indicated that the total tumor burden per mouse was not different between cyclin E-deficient versus the control group (Figure 1(c,d)). This might indicate that once a tumor has developed, cyclin E is not required for breast cancer progression. However, a more likely scenario is that there is a differential threshold of cyclin E activity required for tumor initiation versus progression. Stated differently, we hypothesize that reduced levels of cyclin E in cyclin $E1^{-/-}E2^{+/-}$ and $E1^{+/-}E2^{-/-}$ mice become rate-limiting for tumor initiation, but they are sufficient to maintain tumor cell proliferation. This hypothesis can be tested using conditional cyclin E knockout mice, which will allow a complete shutdown of cyclin E in already developed tumors. We predict that the ablation of cyclin E in tumors *in vivo* will arrest tumor cell proliferation. Our analyses of human breast cancer cell lines are consistent with this notion. Specifically, we observed that cyclin E is essential for driving tumor cell proliferation, as depletion of cyclin E arrested the cell cycle.

Amplification of the cyclin E gene and overexpression of cyclin E protein have been documented in a large number of tumor types [13,32].

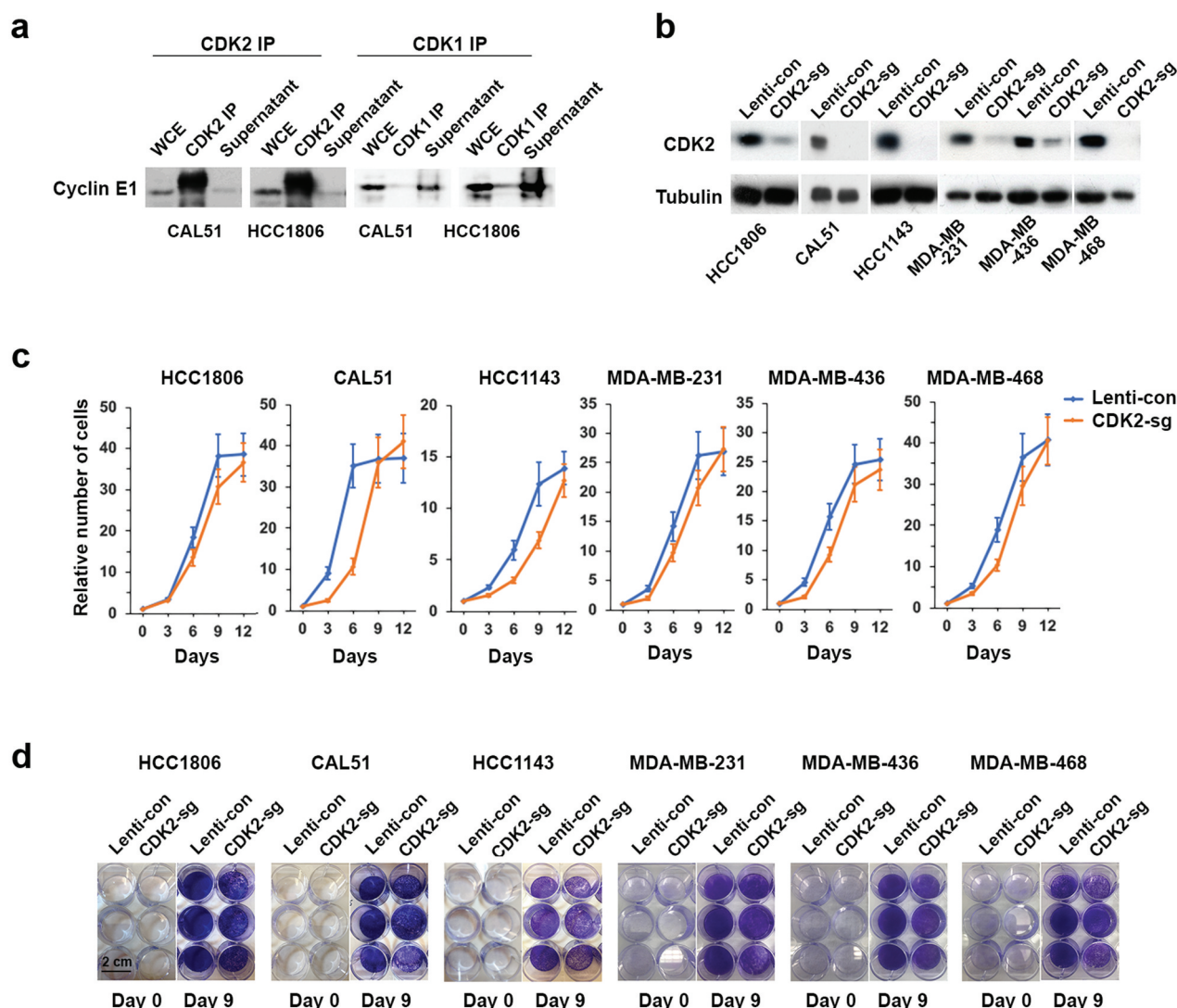


Figure 3. CDK2 is dispensable for proliferation of TNBC cell lines. (a) Immunoprecipitation (IP) using anti-CDK2 (left) or anti-CDK1 (right) antibodies, followed by immunoblotting with an anti-cyclin E1 antibody in CAL51 and HCC1806 cells. Note that immunoprecipitated CDK2 brought down large amounts of cyclin E1, and that essentially no cyclin E1 protein was left in the supernatants after CDK2 IP. In contrast, very little cyclin E1 was detected after CDK1 IP, and cyclin E1 remained in the supernatant. Whole cell extracts (WCE) were also immunoblotted. (b) Immunoblot analysis of CDK2 levels in the indicated human TNBC cell lines after CRISPR-mediated knockout of CDK2 (CDK2-sg), compared with cells transduced with control viruses (Lenti-con). Tubulin was used as a loading control. (c) Growth curves of the indicated human TNBC cell lines following knockout of CDK2 (CDK2-sg) compared with cells transduced with control viruses (Lenti-con). Shown are relative numbers of cells on the indicated days; the number of plated cells (day 0) was set as 1. Error bars indicate SD, $n = 3$. (d) The indicated TNBC cell lines were plated at 2.5×10^4 cells per well in 12 well plates following knockout of CDK2, as in panel b. Cells were stained and photographed after 9 days. Scale bar, 2 cm.

Cyclin E is frequently overexpressed in breast cancers, in particular in triple-negative subtype (TNBC), where it was shown to confer poor prognosis [33,34]. Several different mechanisms were shown to result in cyclin E overexpression in human tumors, including gene amplification as well as the loss of FBXW7, an F-box protein that is responsible for cyclin E degradation [13]. Moreover, some breast cancer cell lines and

tumors were shown to express truncated, proteolytically cleaved cyclin E species. These truncated cyclin E molecules bind CDK2 with higher affinity and are resistant to inhibition by KIP/CIP cell cycle inhibitors [35,36].

It is not entirely clear how overexpressed cyclin E contributes to tumorigenesis. Since in normal cells cyclin E regulates cell division by phosphorylating several cell cycle proteins, overexpressed cyclin

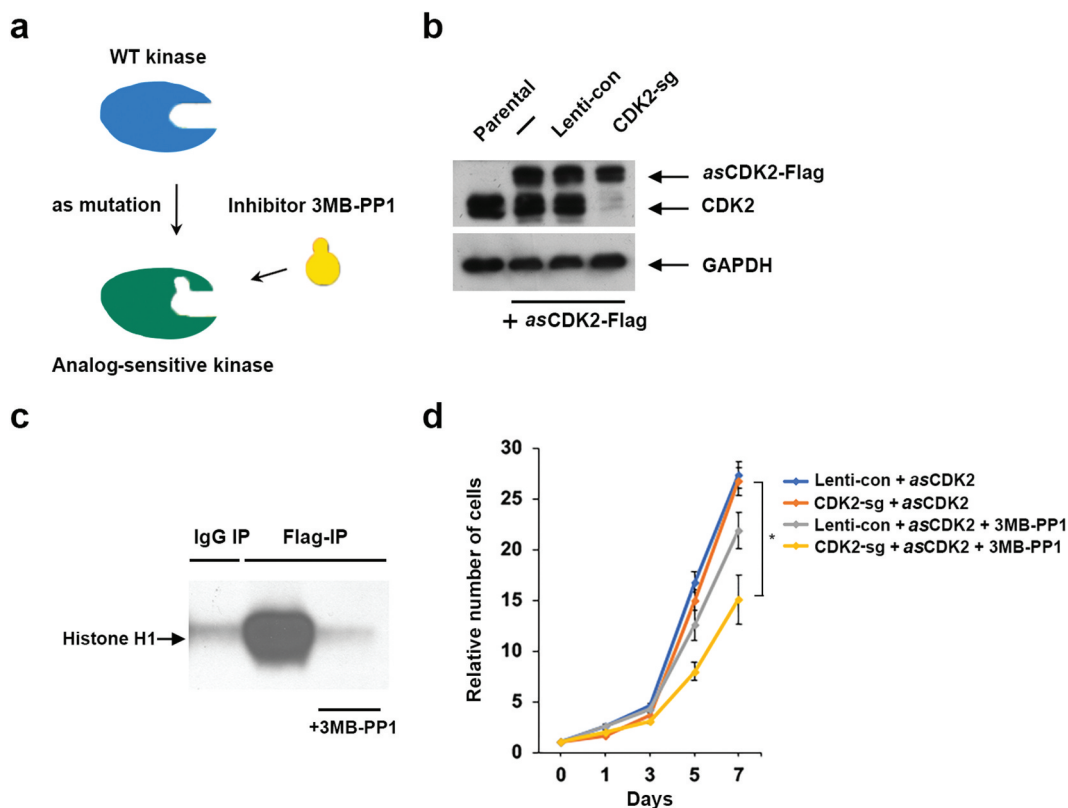


Figure 4. Analyses of TNBC cells expressing analog-sensitive CDK2. (a) A diagram illustrating the principle of analog-sensitive kinases. (b) Immunoblot analysis of the levels of ectopically expressed analog-sensitive CDK2 (asCDK2-Flag) and the endogenous CDK2 (CDK2) in parental HCC1806 cells (first lane) or cells expressing asCDK2 (lanes 2–4). In lane 3, cells were transduced with a control lentivirus (Lenti-con), in lane 4 cells underwent CRISPR-mediated knockout of CDK2 (CDK2-sg). GAPDH was used as loading control. (c) asCDK2 was immunoprecipitated from CDK2-knockout HCC1806 cells expressing asCDK2 using an anti-Flag antibody, and subjected to *in vitro* kinase reactions using histone H1 as a substrate in the presence or absence of 3MB-PP1. As a negative control, *in vitro* kinase reaction was performed using immunoprecipitates obtained with IgG (IgG IP). (d) Growth curves of HCC1806 cells transfected with a control lentivirus and expressing asCDK2 in addition to the endogenous CDK2 (Lenti-con + asCDK2), or CDK2 knockout cells expressing asCDK2 (CDK2-sg + asCDK2). Cells were cultured in the presence or absence of 10 μ M 3MB-PP1 (+3MB-PP1). Shown are relative numbers of cells on the indicated days; the number of plated cells (day 0) was set as 1. Error bars indicate SD, $n = 3$. Unpaired two-tailed Student's *t* test was used for the statistical analysis, comparing CDK2-sg + asCDK2 versus CDK2-sg + asCDK2 + 3MB-PP1; *, $p < 0.05$.

E likely drives uncontrolled cell proliferation. An additional mechanism was provided by the observations that high levels of cyclin E in tumor cells lead to chromosomal instability [37,38]. Moreover, the hyperactive, truncated cyclin E species were detected in tumor cell cytoplasm (while full-length cyclin E is nuclear) [39], suggesting that cyclin E might regulate additional cytoplasmic targets. Lastly, depletion of cyclin E in cyclin E1-amplified ovarian cancer cell lines was shown to trigger apoptosis, suggesting that cyclin E may play a pro-survival role in these cells [40].

It should be noted that while the role of cyclin E in tumorigenesis is very well established, the requirement for CDK2 in this process remains unclear. In a mouse breast cancer model driven by truncated cyclin E, Cdk2 was shown to play

a rate-limiting role, as genetic ablation of Cdk2 blocked tumorigenesis [41]. Analyses of mouse liver cancers revealed that Cdk2 is required for tumor initiation, but it is dispensable for tumor progression and maintenance [42]. Also, analyses of several human cancer cell lines revealed that CDK2 is dispensable for their proliferation [43]. On the other hand, CDK2 was shown to play an essential role in the context of cyclin E1 amplified ovarian cancer cell lines as well as in melanoma cells [44,45].

Our analyses of TNBC cell lines revealed that while depletion of cyclin E blocked cell cycle progression, knockout of cyclin E catalytic partner, CDK2, has very little effect on cell proliferation. This observation might be hard to reconcile with

the current cell cycle model indicating that cyclin E forms a stoichiometric complex with, and serves as a regulatory subunit of CDK2.

One possible explanation might be provided by the observation that in addition to CDK2, cyclin E can bind and activate a related kinase CDK1 [46]. Consistent with this notion, Aleem et al. demonstrated that in cells of *Cdk2*^{-/-} mice, cyclin E interacts with Cdk1 [47]. Hence, the normal proliferation of CDK2-knockout TNBC cells could be explained by the compensatory formation of catalytically active cyclin E-CDK1 complexes in CDK2-knockout cells. However, our study argues against this possibility. First, we found that in TNBC cells under normal conditions (when CDK2 is present), cyclin E predominantly binds CDK2, not CDK1. Second, we engineered TNBC cells to express analog-sensitive CDK2 in place of wild-type CDK2. This scenario should not leave any room for compensation from CDK1, as cyclin E is bound to analog-sensitive CDK2 forming active complexes. Yet, we observed that inhibition of analog-sensitive CDK2 had only a very minor effect on cell proliferation. One possible caveat is that there might be a small pool of cyclin E bound to CDK1 (or to another kinase) in TNBC cells, and this pool might be sufficient to drive tumor cell proliferation even when the predominant partner of cyclin E, CDK2, has been inhibited.

An alternative explanation is that cyclin E might play a CDK2-independent, kinase-independent role in driving the proliferation of tumor cells. There are several observations to support this possibility. Thus, it was shown that cyclin E mutants that are unable to activate CDK2 can transform rat embryonic fibroblasts in collaboration with Ha-Ras [48]. Another study reported that a fraction of cyclin E localizes to centrosomes and promotes S phase entry in a CDK2-independent fashion. Ectopic expression of cyclin E mutant deficient in activating CDK2 was able to accelerate S phase entry, as long as the centrosomal localization sequence was present in the mutant [49]. Moreover, cyclin E was shown to promote MCM loading in cells exiting quiescence in a kinase-independent fashion [50]. Our recent study of human liver cancer cell lines revealed that cyclin E-associated kinase is not required for tumor

cell proliferation [27]. Lastly, it was reported that in human cancer cell lines, the vast majority of cyclin E protein is present in catalytically inactive complexes [51]. These observations raise a possibility that cyclin E might play a kinase-independent role in tumorigenesis. However, the molecular basis of such possible function remains unknown.

Regardless of these unresolved insights, our study indicates that targeting cyclin E expression might represent an attractive therapeutic approach in TNBC. We predict that the so-called degrader compounds [52], which would selectively deplete cyclins E1 and E2, might be particularly efficacious, as they would extinguish both kinase-dependent and -independent functions of cyclin E, thereby assuring a therapeutic effect. The apparent lack of requirement for cyclin E in postnatal physiology [27] raises a possibility that such degrader compounds might selectively target cyclin E-dependent cancer cells while sparing normal, non-transformed tissues.

Disclosure statement

P.S. has been a consultant at Novartis, Genovis, Guidepoint, The Planning Shop, ORIC Pharmaceuticals, Syros and Exo Therapeutics; his laboratory receives research funding from Novartis.

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