C/EBPδ targets cyclin D1 for proteasome-mediated degradation via induction of CDC27/APC3 expression

Snehalata A. Pawar[a,1,2], Tapasree Roy Sarkar[a,2], Kuppusamy Balamurugan[a], Shikha Sharan[a], Jun Wang[a], Youhong Zhang[a], Steven F. Dowdy[b], A-Mei Huang[a,3], and Esta Sterneck[a,4]

[a]Center for Cancer Research, National Cancer Institute, Frederick, MD 21702-1201; and [b]Department of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California, San Diego School of Medicine, La Jolla, CA 92093-0686

Edited* by George F. Vande Woude, Van Andel Research Institute, Grand Rapids, MI, and approved April 15, 2010 (received for review December 3, 2009)

The transcription factor CCAAT/enhancer binding protein δ (C/EBPδ, CEBPD, NFIL-6β) has tumor suppressor function; however, the molecular mechanism(s) by which C/EBPδ exerts its effect are largely unknown. Here, we report that C/EBPδ induces expression of the Cdc27 (APC3) subunit of the anaphase promoting complex/cyclosome (APC/C), which results in the polyubiquitination and degradation of the prooncogenic cell cycle regulator cyclin D1, and also down-regulates cyclin B1, Skp2, and Plk-1. In C/EBPδ knockout mouse embryo fibroblasts (MEF) Cdc27 levels were reduced, whereas cyclin D1 levels were increased even in the presence of activated GSK-3β. Silencing of C/EBPδ, Cdc27, or the APC/C coactivator Cdh1 (FZR1) in MCF-10A breast epithelial cells increased cyclin D1 protein expression. Like C/EBPδ, and in contrast to cyclin D1, Cdc27 was down-regulated in several breast cancer cell lines, suggesting that Cdc27 itself may be a tumor suppressor. Cyclin D1 is a known substrate of polyubiquitination complex SKP1/CUL1/F-box (SCF), and our studies show that Cdc27 directs cyclin D1 to alternative degradation by APC/C. These findings shed light on the role and regulation of APC/C, which is critical for most cellular processes.

breast cancer | tumor suppressor | cell cycle | anaphase promoting complex | Cdh1/FZR1

The transcription factor CCAAT/enhancer binding protein δ (C/EBPδ, CEBPD, NFIL-6β) is down-regulated in several types of tumors, including cervix, liver, and breast (1–5). In vitro, C/EBPδ inhibits the growth of tumor cell lines, is associated with G_0 growth arrest, or induces differentiation (6–8), and promotes genomic stability of mouse embryo fibroblasts (MEFs) (9). C/EBPδ expression correlates with low proliferation and histological grade in meningiomas (10) and was in a 70-gene signature predicting better survival of breast cancer patients (11). Despite this overwhelming evidence that C/EBPδ is a tumor suppressor and inhibits cell growth, the molecular mechanism for this actiity is largely unknown.

Mice with a C/EBPδ gene deletion undergo delayed postlactational mammary gland involution due to attenuated mammary epithelial cell (MEC) apoptosis. Gene expression analysis showed that C/EBPδ induction during involution correlated with repression of cyclin D1 expression (12). In vitro, C/EBPδ overexpression in a mouse MEC line accelerated the decline of cyclin D1 expression upon serum withdrawal (6). Cyclin D1, the regulatory subunit of cyclin-dependent kinases 4 and 6, promotes the G_1 to S transition of the cell cycle (13). In contrast to C/EBPδ, cyclin D1 is highly expressed in >50% of breast tumors (13), and mouse models have established cyclin D1 as a key driver of mammary oncogenesis (14, 15). Cyclin D1 promotes cancer even by additional, cell cycle independent functions (13). Thus, a cell's ability to control cyclin D1 is important to prevent tumor formation, and our understanding of how cyclin D1 expression is regulated can provide valuable insight into the mechanisms of oncogenesis and potential therapeutic avenues.

Results

C/EBPδ Down-Regulates Cyclin D1 Protein Expression. It was reported that C/EBPδ inhibits the growth of MCF-7 breast tumor cells (8) and that inhibition of cyclin D1 expression inhibits growth of breast tumor cell lines including MCF-7 (16). Addressing the possibility of a causal relationship, we found that exogenous C/EBPδ alone down-regulated endogenous cyclin D1 but not cyclin E2 in MCF-7 cells (Fig. 1*A*). An inverse correlation of C/EBPδ and cyclin D1 protein expression was also observed in MEFs from wild-type and *Cebpd* null mice (Fig. S1*A*). Next, we tested the role of target gene regulation by introducing an R198A mutation into the DNA binding domain (C/EBPδ-R198A), which does not affect nuclear localization (17) but prevents DNA binding of C/EBPδ (Fig. S2*A*). This mutation abolished the ability of C/EBPδ to down-regulate cyclin D1 in MCF-7 cells (Fig. 1*A*; see also Figs. 2*C* and 3*C*) and significantly diminished its ability to inhibit cell growth (Fig. 1*B*). Surprisingly, there was only a minor effect of C/EBPδ on cyclin D1 mRNA levels, which was not significantly altered by the R198A mutation (Fig. 1*C*). Similar data were obtained in the untransformed MCF-10A human breast epithelial cells (Fig. 1*D*) and MEFs (Fig. S1*B*), suggesting that C/EBPδ down-regulates cyclin D1 primarily at the protein level.

C/EBPδ Regulates Genome Integrity Through Modulation of Cyclin D1 Protein Levels. Cyclin D1 levels decline at S-phase entry, which is necessary for orderly DNA replication to occur. Excess cyclin D1 during S-phase can lead to DNA damage and genomic instability (18, 19). Because loss of C/EBPδ leads to genomic instability in primary MEFs (9), we asked whether this was due to the increased cyclin D1 protein level. Cells with damaged DNA exhibit histone 2AX phosphorylation (γH2AX) and nuclear γH2AX staining foci mark sites of DNA damage (20, 21). Accordingly, immortalized C/EBPδ KO MEF cultures harbor more cells with increased numbers of nuclear γH2AX foci than control cells and increased levels of γH2AX in cellular extracts (Fig. 2*A*). Knockdown of cyclin D1 to levels as in wild-type MEFs significantly decreased γH2AX levels (Fig. 2*B*). On the other hand, overexpression of cyclin D1 in WT MEFs phenocopied the elevated γH2AX levels of KO MEFs (Fig. S1*C*). Ectopic wild-type C/EBPδ but not the R198A mutant reduced cyclin D1 protein levels and H2AX phosphorylation in KO MEFs (Fig. 2*C*). These data suggest that C/EBPδ prevents DNA damage in part by controlling cyclin D1 protein levels.

Author contributions: S.A.P., T.R.S., K.B., S.S., J.W., Y.Z., and E.S. designed research; S.A.P., T.R.S., K.B., S.S., J.W., and Y.Z. performed research; S.F.D. and A.-M.H. contributed new reagents/analytic tools; S.A.P., T.R.S., K.B., S.S., J.W., Y.Z., and E.S. analyzed data; and S.A.P. and E.S. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]Present address: Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR 72205.

[2]S.A.P. and T.R.S. contributed equally to this work.

[3]Present address: Institute of Biochemistry, College of Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan.

[4]To whom correspondence should be addressed. E-mail: sternecg@mail.nih.gov.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0913813107/-/DCSupplemental.

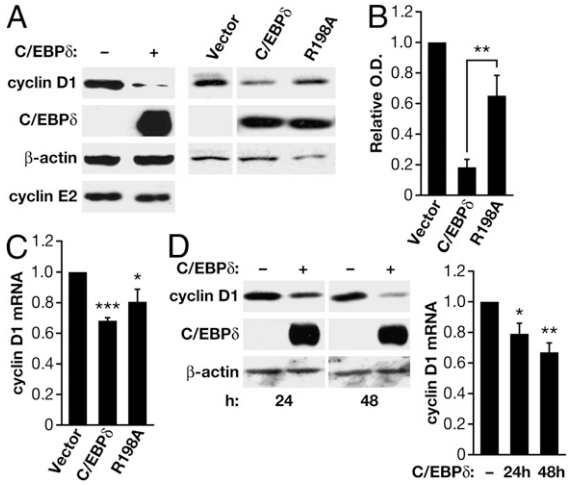

Fig. 1. C/EBPδ inhibits cyclin D1 expression and cell growth. (*A*) Western blot analyses of MCF-7 cells 24 h after transfection with vector or a wild-type or R198A mutant C/EBPδ expression construct as indicated. *A Right* is derived from one membrane with intermediate lanes deleted. Note the lower level of protein loading (β-actin) in the R198A lane. (*B*) MCF-7 cell survival after transfection with the indicated C/EBPδ expression constructs or vector control and 2 weeks of drug selection. Living cells were quantified by spectrophotometry of GIEMSA stain by OD at 630 nm. Data from seven independent experiments are expressed relative to vector control (mean ± SEM). **, $P < 0.005$. (*C*) Q-PCR analysis of cyclin D1 mRNA levels in MCF-7 cells treated as in *A*. Data (mean ± SEM) are from five independent experiments analyzed in triplicates, normalized to actin, and relative to vector control. ***, $P < 0.0001$; *, $P < 0.05$. The difference between the two C/EBPδ constructs was not statistically significant. (*D*) MCF-10A cells were transiently transfected with a C/EBPδ expression construct or empty vector, and RNA and whole-cell protein extracts were prepared 24 and 48 h later. Q-PCR analysis of cyclin D1 mRNA expression levels was normalized to actin and relative to vector-transfected cells (means ± SEM from four independent experiments done in triplicates). *, $P < 0.05$, **, $P < 0.005$. Western blot data are from one membrane with intermediate lanes deleted.

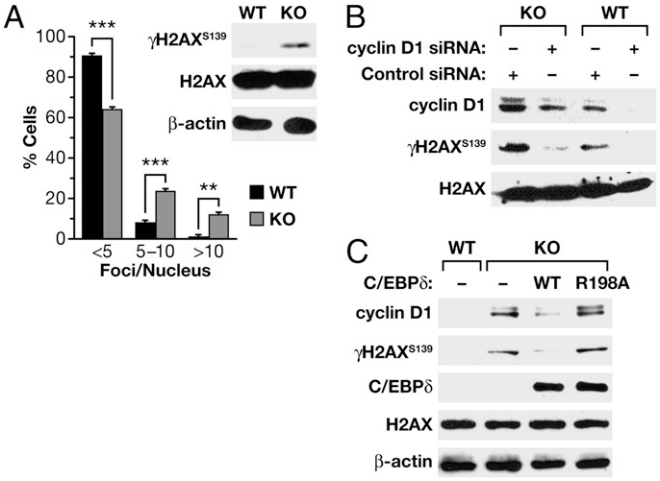

Fig. 2. Elevated levels of γH2AX in C/EBPδ-deficient MEFs are due to cyclin D1. (*A*) Representative Western blot analysis of total H2AX and Ser139 phosphorylated H2AX (γH2AX) in whole cell extracts of exponentially growing wild-type (WT) and C/EBPδ null (KO) MEFs and distribution of cells according to number of γH2AX foci in their nuclei. Data are from three independent cell preparations per genotype with at least 100 cells counted each (mean ± SEM). **, $P < 0.01$; ***, $P < 0.001$. (*B*) Western blot analysis of MEFs 48 h after nucleofection with siRNA against cyclin D1 or nontargeting control siRNA as indicated. (*C*) Western blot analysis of whole cell lysates from WT or KO MEFs 48 h after transient transfection with vector (-) or expression constructs for human wild-type C/EBPδ (WT) or the R198A mutant (R198A). Transfection efficiency was estimated at 60% of the cultures based on a cotransfected GFP-expression construct.

C/EBPδ Regulates Degradation of the Cyclin D1 Protein. Cyclin D1 protein expression is regulated by multiple mechanisms including degradation by the proteasome (22). Treatment with the proteasome inhibitor MG132 stabilized the endogenous cyclin D1 protein ≈2-fold in MCF-7 cells (Fig. 3*A*). However, in the presence of C/EBPδ, MG132 increased cyclin D1 protein levels ≈5-fold and almost abolished down-regulation by C/EBPδ, suggesting that C/EBPδ promotes proteasomal degradation of cyclin D1. Furthermore, C/EBPδ efficiently down-regulated exogenous wild-type cyclin D1 in MCF-7 and HEK293 cells (Fig. 3*B*). These data confirm that the cyclin D1 promoter is not required for regulation of cyclin D1 by C/EBPδ. We used this system to also assess the effect of other C/EBP isoforms. C/EBPβ had been shown to act downstream of cyclin D1 and to physically interact with cyclin D1 (23). C/EBPβ also down-regulated ectopic cyclin D1 protein in HEK293 cells (Fig. S1*D*). However, its effect was less pronounced compared with C/EBPδ, whereas C/EBPα had no effect on cyclin D1 protein levels (Fig. S1*D*). Furthermore, silencing of endogenous C/EBPδ increased endogenous cyclin D1 protein levels in MCF-10A cells, whereas silencing of C/EBPα or C/EBPβ had no effect on cyclin D1 expression (Fig. S1*E*). These results led us to focus our efforts on the mechanism by which C/EBPδ down-regulates cyclin D1 protein expression.

To assess the effect of C/EBPδ on cyclin D1 stability more directly, we expressed both proteins in HEK293 cells and inhibited protein synthesis with cycloheximide. We used HEK293 cells because they contain very low levels of endogenous cyclin D1 and C/EBPδ, and exogenous C/EBPδ does not inhibit growth of HEK293 cells (Fig. S3). Therefore, indirect effects of growth inhibition on cyclin D1 protein expression can be ruled out. Indeed, this experiment showed that the stability of cyclin D1 protein was reduced by wild-type C/EBPδ but not by the R198A mutant form of C/EBPδ (Fig. 3*C*). These data confirm that C/EBPδ promotes degradation of cyclin D1.

C/EBPδ Induces Cdc27 Expression. Cyclin D1 is targeted for degradation by the SKP1/CUL1/F-box (SCF) ubiquitin ligase during the cell cycle and also in response to DNA-damage (18, 22). However,

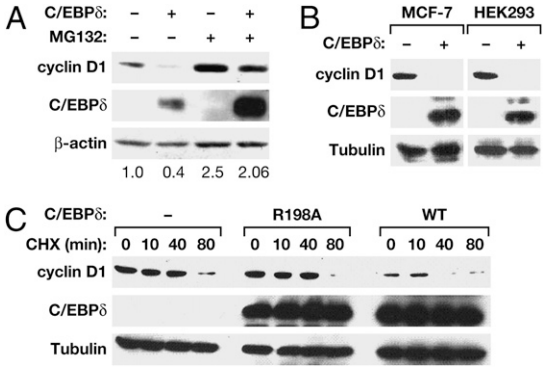

Fig. 3. C/EBPδ regulates cyclin D1 protein stability. (*A*) Western blot analysis of MCF-7 cells transfected with C/EBPδ expression constructs or vector control and treated with MG132 (100 μM) for 1 h before preparation of whole cell lysate. DMSO was used as solvent control. Densitometric quantification of cyclin D1 is shown below. (*B*) Western blot analysis of total protein extracts from MCF-7 and HEK293 cells 24 h after transfection with expression constructs for cyclin D1, and C/EBPδ as indicated. (*C*) Western blot analysis of whole cell lysates from HEK293 cells transfected with expression constructs for cyclin D1 and WT-C/EBPδ (WT), R198A-C/EBPδ (R198A), or vector control (-) and treated with cycloheximide (CHX, 100 μM) for the indicated times.

another multisubunit ubiquitin ligase, the anaphase promoting complex/cyclosome (APC/C) containing the Cdc27/APC3 subunit, also can mediate degradation of cyclin D1 in response to ionizing radiation (24). We found that C/EBPδ induced expression of Cdc27 and, therefore, focused our investigations on this pathway. First, Cdc27 and C/EBPδ were coexpressed and, hence, inversely related to cyclin D1 expression when comparing wild-type and C/EBPδ null MEFs, or the untransformed breast epithelial cell line MCF-10A and the breast adenocarcinoma cell line MCF-7 (Fig. 4*A*). Silencing of endogenous C/EBPδ in MCF-10A cells reduced Cdc27 protein levels and concomitantly increased cyclin D1 protein levels (Fig. 4*B*). Exogenous C/EBPδ induced expression of endogenous Cdc27 in MCF-7 or HEK293 cells, unless C/EBPδ was mutated and unable to bind DNA (R198A) (Fig. 4*C*). This result is in agreement with the observation that C/EBPδ−R198A was unable to trigger degradation of ectopic cyclin D1 in HEK293 cells (Fig. S2*B*). Because Cdc27 is known for targeting cyclin B1 for degradation (25), we asked whether C/EBPδ would also elicit cyclin B1 degradation. MCF-7 and MCF-10A cells do not express appreciable levels of cyclin B1. However, in MDA-MB-231 cells, where transient expression of C/EBPδ also induced Cdc27, cyclin B1 and cyclin D1 were down-regulated, whereas cyclin E2 was not affected (Fig. 4*C*). To obtain a more comprehensive picture of APC/C target regulation by C/EBPδ, we also analyzed expression of other APC/C substrates (26). C/EBPδ expression in MCF-7 cells down-regulated Skp2 and Plk-1 along with cyclin D1. However, Aurora-A and Cdc25 protein levels were in fact induced by C/EBPδ (Fig. S4). These data indicate that C/EBPδ does not affect all APC/C targets in the same way. However, in summary, the data show that C/EBPδ leads to down-regulation of at least four APC/C substrates, cyclin D1, cyclin B1, Plk-1, and Skp2, which are all consistent with inhibition of the cell cycle by C/EBPδ.

To address the mechanism of Cdc27 induction by C/EBPδ, we analyzed its RNA expression. We found that C/EBPδ did not affect Cdc27 mRNA levels in HEK293 cells, and Cdc27 mRNA levels were similar between WT and KO MEFs (Fig. S5), suggesting that C/EBPδ does not regulate Cdc27 expression at the mRNA level. However, because a C/EBPδ mutant, that cannot bind DNA, does not induce Cdc27 expression, the mechanism is likely by transcriptional regulation of another target gene, which then modulates Cdc27 protein expression directly.

Cdc27 Mediates C/EBPδ-Induced Cyclin D1 Degradation. To address the role of Cdc27 in cyclin D1 regulation more directly, we depleted Cdc27 by RNA knockdown in HEK293 cells. This approach abolished the ability of C/EBPδ to down-regulate cotransfected cyclin D1 expression (Fig. 4*D*). Importantly, silencing of endogenous Cdc27 expression in MCF-10A cells increased endogenous cyclin D1 levels (Fig. 4*E Upper*), confirming a role for Cdc27 in regulation of cyclin D1 protein levels. However, Cdc27 does not bind substrates directly but rather through coactivator molecules such as Cdh1/FZR1 (27). Indeed, silencing of Cdh1 in MCF-10A cells also increased endogenous cyclin D1 protein levels (Fig. 4*E Lower*). These data demonstrate that the Cdh1 and Cdc27 components of the APC/C are critical for the low levels of cyclin D1 expression in these cells. Consistent with these results, we detected association

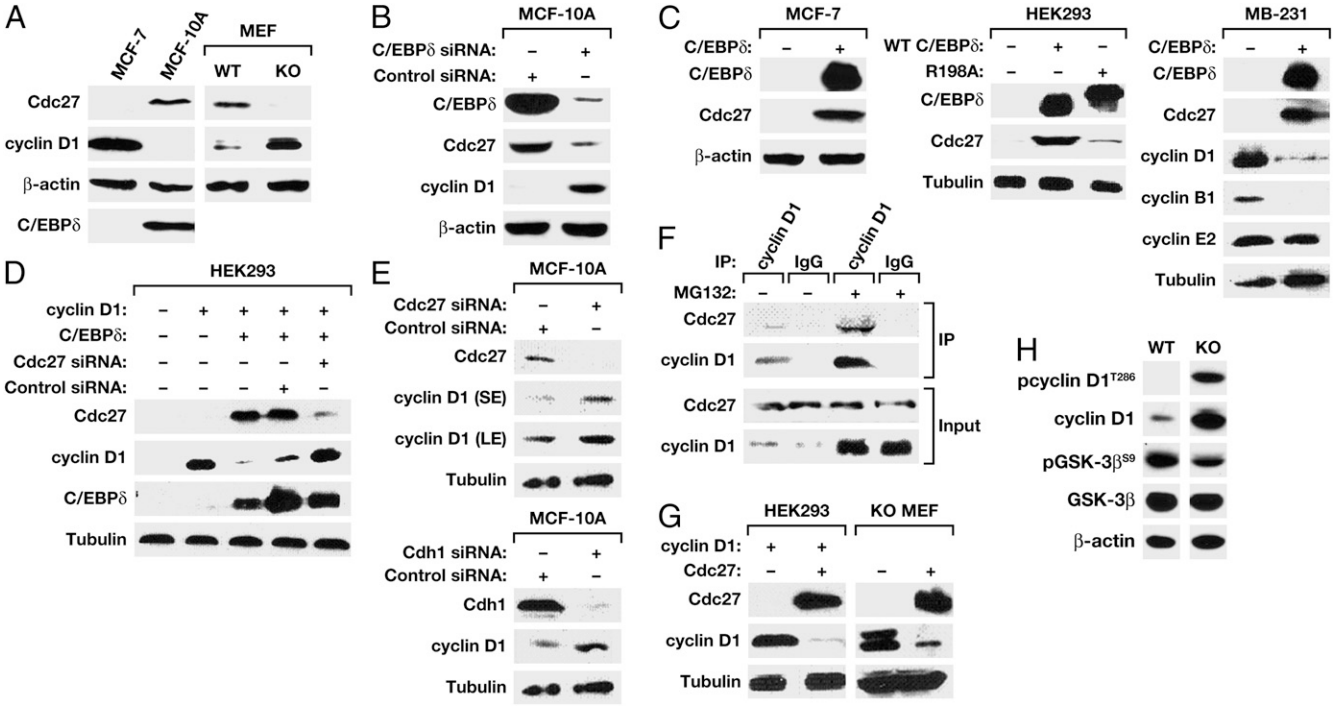

Fig. 4. C/EBPδ mediated cyclin D1 degradation requires induction of Cdc27 expression. (*A*) Western blot analysis comparing endogenous Cdc27, cyclin D1, and C/EBPδ expression in whole cell extracts from MCF-7 and MCF-10A cells or immortalized WT and KO MEFs. (*B*) Western blot analysis of whole cell lysates from MCF-10A cells 48 h after nucleofection with siRNA against C/EBPδ or nontarget control. (*C*) Western blot analysis of whole cell extracts from MCF-7, HEK293, and MDA-MB-231 cells, 24 h after transfection with the indicated flag-tagged C/EBPδ expression constructs or vector control. (*D*) Western blot analysis of whole cell extracts from HEK293 cells 48 h after nucleofection with siRNA against Cdc27 or nontargeting control siRNA along with expression constructs of cyclin D1 and C/EBPδ when indicated. (*E*) Western blot analysis of whole cell extracts from MCF-10A cells 48 h after nucleofection with siRNA against Cdc27 (*Upper*) or Cdh1 (*Lower*) and nontargeting control siRNA. SE, short exposure; LE, long exposure. (*F*) Western blot analysis of cyclin D1 and Cdc27 from MCF-10A cells after immunoprecipitation with anti-cyclin D1 antibodies or IgG as control. Cells were treated with MG132 (50 μM) for 3 h before harvest when indicated. A fraction of the input samples is shown at the bottom. (*G*) Western blot analysis of whole cells extracts from HEK293 cells and KO MEFs 24 h after transfection with the indicated expression constructs. (*H*) Western blot analysis of whole cell lysates from WT and KO MEFs with the indicated antibodies. Data are from one membrane with intermediate lanes deleted.

of endogenous cyclin D1 and Cdc27 by coimmunoprecipitation from MCF-10A cells, which was increased by proteasome inhibition with MG132 (Fig. 4*F*). These data show that Cdc27 levels modulate cyclin D1 expression and that Cdc27 is necessary for cyclin D1 degradation in response to C/EBPδ.

To address whether Cdc27 is sufficient to target cyclin D1 for degradation, we coexpressed Cdc27 with exogenous cyclin D1 in HEK293 cells. Indeed, Cdc27 alone down-regulated the cyclin D1 protein (Fig. 4*G*). Furthermore, "reconstitution" of C/EBPδ KO MEFs with exogenous cdc27 "corrected" the excess cyclin D1 protein levels (Fig. 4*G*). Collectively, these data show that C/EBPδ promotes degradation of cyclin D1 through induction of Cdc27 expression and that Cdc27 expression levels determine cyclin D1 protein levels.

The profound effect of exogenous Cdc27 on cyclin D1 protein levels in C/EBPδ null MEFs was particularly interesting in light of our finding that these cells harbor hyperactive GSK-3β kinase, as evidenced by reduced levels of its inhibitory phosphorylation at Ser9 (28) (Fig. 4*H*). GSK-3β phosphorylates cyclin D1 at residue T286, which marks it for polyubiqutination by the SCF complex (22). However, despite hyperactive GSK-3β in C/EBPδ null MEFs, total cyclin D1 protein levels were increased and, indeed, also the amount of T286 phosphorylation (Fig. 4*H*). These data raise the notion that Cdc27 may also augment degradation of cyclin D1 by the GSK-3β/SCF pathway.

Cdc27-Mediated Cyclin D1 Degradation Requires the D-Box and an Intact "Repressor Box". The RXXL motif, or D-box, at residues 29–32 of cyclin D1 is required for its degradation in response to gamma irradiation (24) and is a conserved motif of APC/C targets that is recognized by the Cdh1 coactivator (27, 29). Mutation of this sequence to QXXA abolished degradation of cyclin D1 by C/EBPδ, and Cdc27 in cotransfection experiments in MCF-7 (Fig. 5*A*) and HEK293 cells (Fig. S6). In addition, we investigated the potential role of a central "repressor box" domain, which interacts with transcriptional corepressors and transcription factors (30–32). We found that mutation of the conserved amino acids RK to EE at positions 179/180 within this domain of cyclin D1 abolished its degradation by both C/EBPδ and Cdc27 (Fig. 5*A* and Fig. S6).

Cdc27 anchors adaptor and coactivator subunits such as Cdh1 with the ubiquitination substrate to the APC/C (27). We determined that interaction of cyclin D1 with Cdc27 in fact depended on an intact RXXL motif. Wild-type cyclin D1 but not the QXXA mutant cyclin D1 coimmunoprecipitated with Cdc27 or Cdh1 in both MCF-7 (Fig. 5*B*) and HEK293 cells (Fig. S7*A*). This result is consistent with the RXXL motif being the docking site for Cdh1 (29). Indeed, Cdh1 also did not associate with the QXXA mutant cyclin D1 (Fig. S7*B*). However, the RK-EE mutant that was resistant to degradation still interacted with Cdc27 and Cdh1 (Fig. 5*B* and Fig. S7). Therefore, we asked whether this mutation affected polyubiquitination by the APC/C. In fact, wild-type cyclin D1 transfected into HEK293 cells was associated with polyubiquitination, which was significantly enhanced by cotransfected Cdc27 (Fig. 5*C*). On the other hand, the RK-EE mutant cyclin D1 was completely refractory to association with polyubiquitin in response to Cdc27. Interaction of Cdc27 and cyclin D1 (WT and RK-EE) was also confirmed in this experiment (Fig. 5*C*). These data show that Cdc27 targets cyclin D1 for degradation by a mechanism that requires the D-box at residues 29–32 of cyclin D1 for complex formation with the APC/C and the RK residues at position 179/180 in the repressor box of cyclin D1 for successful polyubiquitination.

Cdc27 Expression Is Down-Regulated in Cancer Cells. Comparison of MEFs, three human untransformed breast epithelial cell lines, and eight human breast tumor cell lines revealed that the untransformed breast epithelial cell lines MCF-10A, MCF-12A, and HBL100 along with wild-type MEFs expressed the highest amount of C/EBPδ and the lowest amount of cyclin D1 (Fig. 6). All eight breast cancer cell lines analyzed expressed comparatively high levels of cyclin D1. Expression levels of Cdc27 were also highest in WT MEFs and untransformed breast epithelial cell lines (3/3) and lowest in breast cancer cell lines (7/8). Only the breast tumor cell line T47D expressed Cdc27 levels comparable with those of untransformed cells, yet displayed high levels of cyclin D1. These cells may have developed mechanisms that allow cyclin D1 to escape degradation by Cdc27. In summary, these data confirm the inverse relationship of C/EBPδ and cyclin D1 expression and show that like C/EBPδ, Cdc27 expression is down-regulated in most tumor cell lines.

Discussion

The multisubunit ubiquitin ligases SCF and APC/C play pivotal roles in all major cellular processes and also regulate each other. Yet, comparatively little is known about the function of individual subunits and the identity of most substrates (33). Our study shows that C/EBPδ promotes Cdc27/APC3 expression, which then trig-

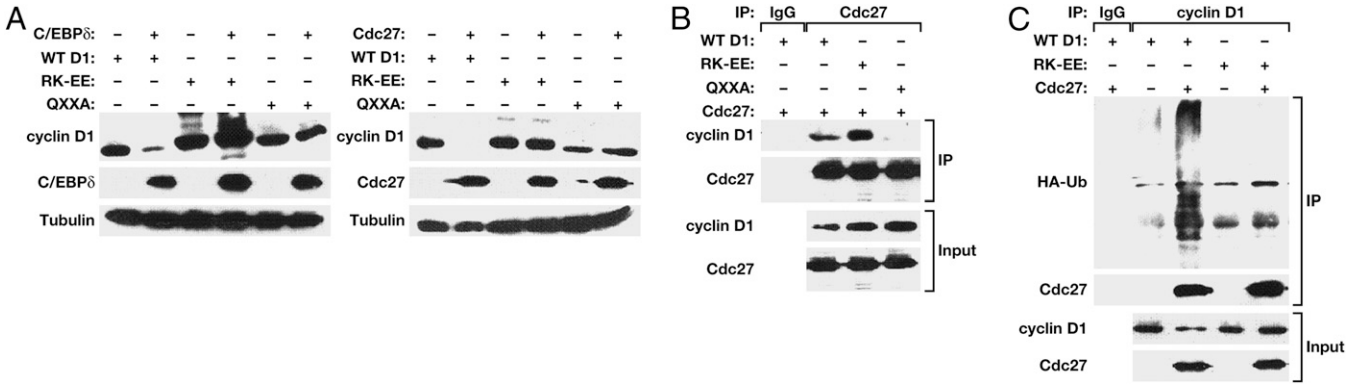

Fig. 5. The RK residues at positions 179/180 of cyclin D1 are required for polyubiquitination and degradation by C/EBPδ or Cdc27. (*A*) Western blot analysis of whole cell extracts from MCF-7 cells 24 h after transfection with expression constructs of wild-type cyclin D1 (WT D1) or cyclin D1 mutants (RK-EE, QXXA) together with either C/EBPδ (*Left*) or Cdc27 (*Right*) expression constructs as indicated. (*B*) Western blot analysis of cyclin D1 and Cdc27 from MCF-7 cells transfected with the indicated constructs and after immunoprecipitation with anti-Cdc27 antibodies. IgG antibodies were used as a negative control with an equivalent aliquot of the same extract used for Cdc27. A fraction of the input samples is shown at the bottom. (*C*) Western blot analysis of HEK293 cells transfected with HA-ubiquitin expression constructs along with constructs as indicated. Immunoprecipitate with anti-cyclin D1 antibodies was analyzed for HA-ubiquitin and presence of Cdc27. A fraction of the input samples is shown at the bottom. IgG antibodies were used as a negative control with an equivalent aliquot of the same extract used for cyclin D1.

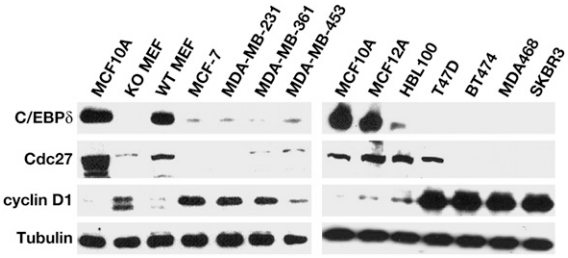

Fig. 6. Cdc27 is down-regulated in breast cancer cell lines. Western blot analysis of endogenous C/EBPδ, Cdc27, and cyclin D1 expression in whole cell extracts from WT and KO MEFs, the untransformed human breast epithelial cell lines MCF-10A, MCF-12A, and HBL100, and eight human breast cancer cell lines (MCF-7; MDA-MB-231, -361, -453, -468; SKBR3, BT474, and T47D). MCF-10A cell lysate was analyzed on both sets of samples for cross-reference.

gers degradation of cyclin D1 as well as cyclin B1, Plk-1, and Skp2, identifying a molecular pathway by which C/EBPδ can act as a growth inhibitor and tumor suppressor. Furthermore, the data reveal a unique role for Cdc27 in determining cyclin D1 stability and strongly suggest that Cdc27 itself may be a potent tumor suppressor protein.

Although cyclin D1 association with Cdc27 had been described before (24), cyclin A and cyclin B1, which are involved in the G_2/M transition of the cell cycle, were the only known cyclins targeted by the APC/C during the cell cycle (27). Targeting of cyclin D1, which regulates the G_1/S transition of the cell cycle, by Cdc27 and Cdh1 suggests further complexity of APC/C-mediated cell cycle regulation. In particular, the T286 phosphorylation of cyclin D1 was shown to trigger association of cyclin D1 with FBX4 and αB crystalline, or FBXW8 to initiate SCF-mediated polyubiquitination (22). Furthermore, as we reported here for Cdc27, FBX4 and αB crystalline expression also is down-regulated in breast tumor cells (34). It remains to be determined whether and how Cdc27 intersects with SCF-mediated cyclin D1 degradation. However, our gene deletion, protein depletion, and overexpression experiments in different cell lines have clearly shown that Cdc27 modulates cyclin D1 protein levels and that Cdc27 interacts with cyclin D1 and promotes its polyubiquitination. Degradation by this pathway required intact RK residues at position 179/180 of cyclin D1. In addition, we confirmed that Cdh1 interacts with cyclin D1 and that Cdh1 silencing results in increased cyclin D1 protein expression. This result identifies a further molecular mechanism for the role of Cdh1 as a tumor suppressor and in maintenance of genomic stability (29).

Cdc27 is one of five tetratrico-peptide repeat (TPR) subunits considered core components of the APC/C, which secure the interaction with substrate/coactivator complexes (27). However, protein depletion studies in *Drosophila* showed that several of these TPR subunits are functionally not equivalent and may determine unique functions of the APC/C (35). Interestingly, not all known APC/C targets were down-regulated as a result of C/EBPδ expression. Thus, specific up-regulation of Cdc27 by C/EBPδ may signal a shift in the preference of substrates targeted by the APC/C. This is a plausible mechanism for the observed up-regulation of Aurora A by C/EBPδ, because to our knowledge there is currently no evidence that Cdc27 is required for APC/C-mediated degradation of Aurora A. On the other hand, Aurora A can be targeted by the SCF as well (36), which may also be modulated by CEBPδ. Hence, additional, yet-to-be-discovered functions of C/EBPδ may contribute to further fine-tuning of APC/C substrate expression.

C/EBPδ arrests growth of many cell lines in vitro, and several studies suggest that it acts as a tumor suppressor (see Introduction). Interestingly, the effect of C/EBPδ on cell growth is cell-type specific. In contrast to MEC, C/EBPδ expression is not associated with growth arrest of fibroblasts (37). In osteoblasts, C/EBPδ has been linked to proliferation (38), and we found that C/EBPδ does not affect proliferation of HEK293 cells. We hypothesize that the ability of C/EBPδ to arrest cell growth and act as a tumor suppressor may be modulated by cell-type specific cofactors for Cdc27 expression or function and by the cell's dependence on cyclin D1 for proliferation. Recently, it was shown that C/EBPδ is required for limbal stem cell renewal. Loss of C/EBPδ resulted in an accelerated cell cycle of stem cells (39). Because cyclin D1 promotes S-phase entry and accelerates the cell cycle, we speculate that inhibition of cyclin D1 expression might be a relevant mechanism for C/EBPδ in this cell system as well and raises the possibility of a role for Cdc27 in stem cell renewal.

Cyclin D1 is overexpressed in many cancers, and down-regulation of cyclin D1 is a promising antitumor mechanism (40). Indeed, specific ablation of cyclin D1 is a validated strategy against breast cancer (22, 41). Many structurally unrelated chemotherapeutic compounds down-regulate cyclin D1, and cyclin D1 expression can be a surrogate marker of therapeutic efficacy (22). However, the exact mechanism(s) by which these compounds act on cyclin D1 proteins is largely unknown (22, 41). Specific knowledge about the various modalities of cyclin D1 regulation will be critical to understand why some cell lines or cancers are responsive while others are not and how to manage cancers with mutations and, therefore, resistance against certain therapeutic agents. GSK-3β inhibitors usually lead to accumulation of cyclin D1, whereas activation of GSK-3β triggers cyclin D1 degradation (22). Pharmacological down-regulation of cyclin D1 including targets within the GSK-3β pathway is an active area of research for cancer therapeutics (42). Our data suggest that expression of genes such as Cdc27 may determine whether cyclin D1 is sensitive to GSK-3β activation in a particular cell type. Knowledge about Cdc27 expression and its regulation may benefit the selection of patients for targeted therapies, and exploration of mechanisms that lead to Cdc27 expression could aid future therapeutic approaches against cancer. Notably, although much attention has been given to the cross-regulation of APC/C and SCF activators and inhibitors, this study reveals an additional level of control via regulated expression of an APC/C core component by a tumor suppressor. Our data suggest that silencing of C/EBPδ or Cdc27 will allow cancer cells to escape cyclin D1 down-regulation, and their expression could be relevant for the success of targeted therapeutic approaches.

Materials and Methods

Cell Survival and Growth Assays. MCF-7 cells were transfected with 5 μg of pcDNA3.1-based expression vectors containing the neomycin resistance gene along with 0.5 μg of pMaxGFP (Amaxa) by using FuGENE 6 (Roche Biochemicals). After 24 h, two thirds of cells were used to verify expression from the transfected constructs by Western blot analysis. One-third of the cells were reseeded and selected with G418 (400 μg/mL). After 2 weeks, the cells were fixed with methanol and stained with Giemsa. The retained dye was extracted with 1% SDS in 0.2 M NaOH and quantified by spectrophotometry at 630 nm absorbance. See *SI Materials and Methods* for further details on cell culture conditions and expression constructs used for transient transfection.

Analysis of γH2AX Foci. WT and KO MEFs were seeded on coverslips. Sixteen hours later, the cells were fixed with 2% formaldehyde, permeabilized with 0.3% Triton X-100, and quenched with 1% glycine, all in PBS. γH2AX was stained with polyclonal anti-γH2AX (1:600; Millipore) and Alexa Fluor 488 goat anti-rabbit IgG (1:1,000; Invitrogen) with 3% BSA. Cell nuclei were detected with 0.1% 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen). Cells were visualized with an LSM 510 META confocal fluorescent microscope (Zeiss), and data were collected blinded to the sample identity.

Quantitative RT-PCR, RNAi, protein isolations, immunoprecipitations, and Western analyses were performed according to standard procedures. See *SI Materials and Methods* for details.

ACKNOWLEDGMENTS. We thank Glenn Summers, Jennifer Beachley, and Linda Miller for outstanding assistance with animal work and Jiro Wada for preparation of the figures. For generously sharing valuable reagents, we

thank Drs. Andrew Arnold (University of Connecticut School of Medicine); Ching Shih Chen and James W. DeWille (The Ohio State University); J. Alan Diehl (Abramson Family Cancer Research Institute); Philip Hieter (The University of British Columbia); Peter F. Johnson, Brad St. Croix, Allan M. Weissman, and Kyung S. Lee (CCR, National Cancer Institute); Muthusamy Thangaraju (Medical College of Georgia); and Cynthia Zahnow (The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University). For discussion and constructive comments, we thank Drs. Ira Daar, J. Alan Diehl, Philipp Kaldis, Deborah Morrison, and Allan Weissman. This research was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute. BD69319 and 69320 mouse monoclonal antibodies against C/EBPδ were provided by BD Biosciences Pharmingen through an Antibody Co-development Collaboration between the National Cancer Institute and BD Bioscience.

1. Agrawal S, et al. (2007) The C/EBPdelta tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. *Blood* 109:3895–3905.
2. Ko CY, Hsu HC, Shen MR, Chang WC, Wang JM (2008) Epigenetic silencing of CCAAT/enhancer-binding protein delta activity by YY1/polycomb group/DNA methyltransferase complex. *J Biol Chem* 283:30919–30932.
3. Porter D, et al. (2003) Molecular markers in ductal carcinoma in situ of the breast. *Mol Cancer Res* 1:362–375.
4. Tang D, Sivko GS, DeWille JW (2006) Promoter methylation reduces C/EBPdelta (CEBPD) gene expression in the SUM-52PE human breast cancer cell line and in primary breast tumors. *Breast Cancer Res Treat* 95:161–170.
5. Neve RM, et al. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. *Cancer Cell* 10:515–527.
6. O'Rourke JP, Newbound GC, Hutt JA, DeWille J (1999) CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G0 growth arrest and apoptosis. *J Biol Chem* 274:16582–16589.
7. Gery S, Tanosaki S, Hofmann WK, Koppel A, Koeffler HP (2005) C/EBPdelta expression in a BCR-ABL-positive cell line induces growth arrest and myeloid differentiation. *Oncogene* 24:1589–1597.
8. Ikezoe T, et al. (2005) CCAAT/enhancer-binding protein delta: A molecular target of 1,25-dihydroxyvitamin D3 in androgen-responsive prostate cancer LNCaP cells. *Cancer Res* 65:4762–4768.
9. Huang AM, et al. (2004) Loss of CCAAT/enhancer binding protein δ promotes chromosomal instability. *Oncogene* 23:1549–1557.
10. Barresi V, Vitarelli E, Cerasoli S, Barresi G (2010) The cell growth inhibitory transcription factor C/EBPdelta is expressed in human meningiomas in association with low histological grade and proliferation index. *J Neurooncol* 97:233–240.
11. Naderi A, et al. (2007) A gene-expression signature to predict survival in breast cancer across independent data sets. *Oncogene* 26:1507–1516.
12. Thangaraju M, et al. (2005) C/EBPdelta is a crucial regulator of pro-apoptotic gene expression during mammary gland involution. *Development* 132:4675–4685.
13. Roy PG, Thompson AM (2006) Cyclin D1 and breast cancer. *Breast* 15:718–727.
14. Wang TC, et al. (1994) Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 369:669–671.
15. Yu Q, Geng Y, Sicinski P (2001) Specific protection against breast cancers by cyclin D1 ablation. *Nature* 411:1017–1021.
16. Grillo M, et al. (2006) Validation of cyclin D1/CDK4 as an anticancer drug target in MCF-7 breast cancer cells: Effect of regulated overexpression of cyclin D1 and siRNA-mediated inhibition of endogenous cyclin D1 and CDK4 expression. *Breast Cancer Res Treat* 95:185–194.
17. Miller M, Shuman JD, Sebastian T, Dauter Z, Johnson PF (2003) Structural basis for DNA recognition by the basic region leucine zipper transcription factor CCAAT/enhancer-binding protein alpha. *J Biol Chem* 278:15178–15184.
18. Pontano LL, et al. (2008) Genotoxic stress-induced cyclin D1 phosphorylation and proteolysis are required for genomic stability. *Mol Cell Biol* 28:7245–7258.
19. Shields BJ, Hauser C, Bukczynska PE, Court NW, Tiganis T (2008) DNA replication stalling attenuates tyrosine kinase signaling to suppress S phase progression. *Cancer Cell* 14:166–179.
20. Bonner WM, et al. (2008) GammaH2AX and cancer. *Nat Rev Cancer* 8:957–967.
21. Mah LJ, El-Osta A, Karagiannis TC (2010) gammaH2AX: A sensitive molecular marker of DNA damage and repair. *Leukemia* 24:679–686.
22. Alao JP (2007) The regulation of cyclin D1 degradation: Roles in cancer development and the potential for therapeutic invention. *Mol Cancer* 6:24.
23. Lamb J, et al. (2003) A mechanism of cyclin D1 action encoded in the patterns of gene expression in human cancer. *Cell* 114:323–334.
24. Agami R, Bernards R (2000) Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* 102:55–66.
25. King RW, et al. (1995) A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* 81:279–288.
26. Sumara I, Maerki S, Peter M (2008) E3 ubiquitin ligases and mitosis: Embracing the complexity. *Trends Cell Biol* 18:84–94.
27. Peters JM (2006) The anaphase promoting complex/cyclosome: A machine designed to destroy. *Nat Rev Mol Cell Biol* 7:644–656.
28. Luo J (2009) Glycogen synthase kinase 3beta (GSK3beta) in tumorigenesis and cancer chemotherapy. *Cancer Lett* 273:194–200.
29. Wäsch R, Robbins JA, Cross FR (2010) The emerging role of APC/CCdh1 in controlling differentiation, genomic stability and tumor suppression. *Oncogene* 29:1–10.
30. Wang C, et al. (2003) Cyclin D1 repression of peroxisome proliferator-activated receptor gamma expression and transactivation. *Mol Cell Biol* 23:6159–6173.
31. Inoue K, Sherr CJ (1998) Gene expression and cell cycle arrest mediated by transcription factor DMP1 is antagonized by D-type cyclins through a cyclin-dependent-kinase-independent mechanism. *Mol Cell Biol* 18:1590–1600.
32. Petre-Draviam CE, et al. (2005) A central domain of cyclin D1 mediates nuclear receptor corepressor activity. *Oncogene* 24:431–444.
33. Skaar JR, Pagano M (2009) Control of cell growth by the SCF and APC/C ubiquitin ligases. *Curr Opin Cell Biol* 21:816–824.
34. Lin DI, et al. (2006) Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF (FBX4-alphaB crystallin) complex. *Mol Cell* 24:355–366.
35. Pál M, Nagy O, Ménesi D, Udvardy A, Deák P (2007) Structurally related TPR subunits contribute differently to the function of the anaphase-promoting complex in Drosophila melanogaster. *J Cell Sci* 120:3238–3248.
36. Fujii Y, et al. (2006) Fbxw7 contributes to tumor suppression by targeting multiple proteins for ubiquitin-dependent degradation. *Cancer Sci* 97:729–736.
37. O'Rourke J, Yuan R, DeWille J (1997) CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. *J Biol Chem* 272: 6291–6296.
38. Umayahara Y, Ji C, Centrella M, Rotwein P, McCarthy TL (1997) CCAAT/enhancer-binding protein delta activates insulin-like growth factor-I gene transcription in osteoblasts. Identification of a novel cyclic AMP signaling pathway in bone. *J Biol Chem* 272:31793–31800.
39. Barbaro V, et al. (2007) C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells. *J Cell Biol* 177:1037–1049.
40. Kim JK, Diehl JA (2009) Nuclear cyclin D1: An oncogenic driver in human cancer. *J Cell Physiol* 220:292–296.
41. Freemantle SJ, et al. (2007) Cyclin degradation for cancer therapy and chemo-prevention. *J Cell Biochem* 102:869–877.
42. Takahashi-Yanaga F, Sasaguri T (2008) GSK-3beta regulates cyclin D1 expression: A new target for chemotherapy. *Cell Signal* 20:581–589.