HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2015 October 09.

Published in final edited form as:

Oncogene. 2015 April 9; 34(15): 1961–1967. doi:10.1038/onc.2014.137.

Cks Overexpression Enhances Chemotherapeutic Efficacy by Overriding DNA Damage Checkpoints

Sonia V. del Rincón[1,4], **Martin Widschwendter**[2,4], **Dahui Sun**[1], **Susanna Ekholm-Reed**[3], **John Tat**[3], **Leonardo K. Teixeira**[3], **Zdenka Ellederova**[1], **Elise Grolieres**[1], **Steven I. Reed**[3], and **Charles Spruck**[1]

[1]Tumor Initiation and Maintenance Program, Cancer Center, Sanford-Burnham Medical Research Institute, La Jolla, California, USA

[2]Department of Gynaecological Oncology, University College London, London, UK

[3]Department of Molecular Biology, The Scripps Research Institute, La Jolla, California, USA

Abstract

Cks1 and Cks2 are adaptor-like proteins that bind many cyclin-dependent kinases (Cdks). A wealth of clinical data has shown that Cks proteins are overexpressed in many types of human cancers and this often correlates with increased tumor aggressiveness. Previously, we showed that Cks overexpression abrogates the intra-S phase checkpoint, a major barrier to oncogene-mediated transformation. Interestingly, the intra-S phase checkpoint is crucial for the cellular response to replication stress, a major pathway of apoptosis induction by many chemotherapeutic agents. Here, we demonstrate cancer cells that overexpress Cks1 or Cks2 override the intra-S phase checkpoint in the presence of replication stress-inducing chemotherapies such as 5-Fluorourocil (5-FU) and methotrexate (MTX) leading to enhanced sensitivity *in vitro* and *in vivo*. Furthermore, enforced expression of Cks1 in a MTX-resistant breast cancer cell line was found to restore drug sensitivity. Our results suggest that Cks proteins are important determinants of apoptosis induction of replication stress-inducing chemotherapies such as 5-FU.

Keywords

Cks; Chemotherapy; 5-FU; Intra-S Phase Checkpoint

INTRODUCTION

Cdc kinase subunit (Cks) proteins are small (9 kD) highly conserved Cdk binding proteins that are ubiquitously expressed in eukaryotes. Although their precise functions in mammals

Corresponding Author: Charles Spruck, Sanford-Burnham Medical Research Institute, 10901 North Torrey Pines Road, La Jolla, CA, 92037. Phone: 858-646-3100; Fax: 858-713-6274; cspruck@sanfordburnham.org.

[4]These authors contributed equally to this work

CONFLICT of INTEREST

The authors declare no competing financial interests related to the work described in this study.

are not fully understood, studies in yeast and *Xenopus* suggest they likely act as adaptors in mediating Cdk functions, such as by targeting cyclin-Cdk complexes to their respective substrates or promoting their interaction with other cell division regulatory proteins (1–7). In *Saccharomyces cerevisae*, Cks proteins have also been shown to play a role in general transcription regulation by controlling nucleosome density in a process that is both Cdk1 and 19S proteosome-dependent (8, 9).

Humans express two closely related Cks paralogs (designated Cks1 and Cks2) that are highly similar at the structural level (81% amino acid identity). Knockout studies in mice have demonstrated that Cks proteins perform both essential redundant and specialized functions in cell division control and development. Cks2 is essential for the early stages of meiosis, with *CKS2*$^{-/-}$ knockout male and female mice arresting germ cell development in metaphase of the first meiotic division (10). Cks1 has been shown to perform a specialized Cdk-independent function as a cofactor of the SCF^{Skp2} ubiquitin ligase, which mediates the ubiquitin-dependent proteolysis of Cdk inhibitors p27, p21, and Rb family protein p130, among others (11–13). Targeted disruption of both *CKS1* and *CKS2* results in embryonic lethality with development arrested at or before the morula stage after only two to four cell divisions (14). This essential redundant function in mammalian development has been linked to impaired transcription of genes that encode mitotic regulators cyclin A, cyclin B1, and Cdk1, resulting in cell cycle arrest in G_2 phase.

A wealth of clinical studies has shown that Cks proteins likely play important causative roles in human tumorigenesis. Overexpression of Cks1 has been reported in cancers of the breast, colon, lung, stomach, bladder, kidney, mouth, esophagus, and ovary, and this phenotype is often associated with down-regulation of SCF^{Skp2} target protein p27 and increased tumor aggressiveness (15–25). Cks1 has also been shown to be transcriptionally activated by oncoproteins c-Myc, B-Raf, and cyclin D1 (26–27). Overexpression of Cks2 has been observed in cancers of the breast, colon, bladder, esophagus, stomach, brain, and bile duct, and is often associated with an increased risk of metastasis and tumor recurrence (15, 28–37).

Previously, we showed that overexpression of Cks proteins abrogates the intra-S phase checkpoint induced by replication stress, potentially alleviating a critical barrier of oncoprotein-mediated transformation (38). Interestingly, several widely used chemotherapy drugs promote apoptosis of cancer cells by creating nucleotide pool imbalances or forming crosslinks in DNA which induce DNA damage and replication stress. We therefore sought to determine whether Cks overexpression could possibly influence the efficacy of this class of anti-cancer drugs. Here, we show Cks overexpressing cancer cells override DNA damage checkpoints when treated with replication stress-inducing chemotherapies, leading to enhanced apoptosis *in vitro* and *in vivo*.

RESULTS

Cks overexpression sensitizes cancer cells to replication stress-inducing chemotherapies

Our previous study showed Cks overexpressing cells override the intra-S-phase checkpoint in the presence of replication stress (hydroxyurea (HU) treatment or oncogene

overexpression) suggesting these cells could be particularly vulnerable to chemotherapies that function, at least in part, through this mode of action. To test this, we generated MCF-7 breast cancer cells that stably overexpressed Cks1 at levels comparable to high-expressing breast cancer specimens and evaluated their sensitivity to chemotherapeutic drugs known to induce replication stress, such as 5-FU and MTX. 5-FU is converted to several active metabolites in cells, including fluorodeoxyuridine monophosphate (FdUMP), fluorodeoxyuridine triphosphate (FdUTP), and fluorouridine triphosphate (FUTP), the former of which functions as an irreversible inhibitor of thymidylate synthase (TS). TS inhibition promotes an imbalance in nucleotide pools favoring the synthesis of deoxyuridine triphosphate (dUTP) over deoxythymidine triphosphate (dTTP) and replication stress. MTX is an inhibitor of dihydrofolate reductase (DHFR) which is involved in thymidylate (dTMP) production. 5-FU or MTX treatment of stable Cks1 overexpressing MCF-7 cells was found to increase the levels of apoptotic markers such as Parp, Puma, and Caspase 7, compared to control cells (Fig. 1A). Furthermore, we observed a dose-dependent effect of Cks1 expression on MCF-7 growth inhibition in the presence of 5-FU, but not in cells treated with the mitotic inhibitor paclitaxel (Supplementary Fig. 1). To ensure these results were not due to cell line differences and could also be extended to the paralog Cks2, we generated Cks1 or Cks2 overexpressing MCF-7 cell populations by lentiviral transduction and evaluated their sensitivity to 5-FU. Treatment of the transduced cells with 5-FU showed overexpression of Cks1 or Cks2 induced a significant increase in apoptosis, as evidenced by increased cleaved Parp and caspase 9, compared to control (empty vector) cells (Fig. 1B). In addition, Cks1 and Cks2 overexpressing MCF-7 cells displayed a significantly reduced growth rate in the presence of 5-FU ($p<0.01$) (Fig. 1C). Microscopic analysis of the 5-FU treated cells showed increased pyknosis, an irreversible condensation of chromatin associated with apoptosis, in the Cks1 and Cks2 overexpressing cells compared to control cells (Fig. 1D). Moreover, clonogenic assays revealed a significantly reduced ability of Cks1 or Cks2 overexpressing cells to form colonies in the presence of 5-FU ($p<0.0001$ and $p<0.01$, respectively) (Fig. 1E), consistent with increased cell death induction. Extensive attempts to confirm these results using a reverse experimental strategy in which Cks1/2 expression was knocked down in Cks1/2 overexpressing cancer cells were unsuccessful, since knockdown of both genes was found to induce significant apoptosis that dwarfed drug treatment in all cell lines tested, possibly indicating the cells are addicted to Cks1/2 overexpression for their survival (data not shown). However, knockdown of Cks1 in low Cks1/2 expressing MCF-7 cells was found to abrogate Puma expression following 5-FU treatment (data not shown). Collectively these results showed that overexpression of Cks1 or Cks2 conferred sensitivity to replication stress-inducing chemotherapy drugs such as 5-FU and MTX.

Enhanced DNA damage response in Cks-overexpressing cells treated with 5-FU is dependent on Cks binding to Cdks

5-FU treatment promotes an increase in the dUMP/dTMP ratio leading to polymerase-catalyzed incorporation of uracil and 5-FU derivative 5-F-dUMP into genomic DNA. Uracil and 5-F-dUMP are removed from DNA by uracil DNA glycosylases leading to DNA fragmentation, thus triggering the DNA damage response. To attempt to explain the enhanced apoptosis observed in Cks 1/2 overexpressing cells treated with 5-FU, we first

evaluated whether Cks1 overexpression might affect the rate of incorporation of 5-FU derivatives into genomic DNA. Stable Cks1 overexpressing and control MCF-7 cells were treated with 2-^{14}C-labeled 5-FU for 48 hrs and incorporation of 2-^{14}C-F-dUMP into genomic DNA quantified by scintillation counting. A 40% increase in 2-^{14}C-F-dUMP incorporation per μg of genomic DNA was observed in Cks1 overexpressing cells compared to control cells ($p < 0.001$) (Fig. 2A). We next analyzed the DNA damage response in these cells following treatment with 5-FU. Cks1 overexpressing cells were found to contain increased phospho-Ser15-p53 and phospho-Ser1981-ATM, markers of the DNA damage response, compared to control cells (Fig. 2B), suggesting more robust DNA damage. Moreover, knockdown of ATM expression by siRNA in the Cks1 overexpressing cells reduced the expression of apoptotic markers cleaved Parp and caspase 7, indicating the apoptosis induced was a direct result of enhanced genotoxic stress (Supplementary Fig. 2). A similar enhancement of the DNA damage response was observed in HCT116 colorectal cancer cells engineered to stably overexpress Cks1, demonstrating the effect was not breast cancer cell specific (Supplementary Fig. 3). These results suggested that Cks1 overexpression enhances the apoptotic effects of 5-FU through genotoxic stress, possibly by increasing the incorporation of 5-FU derivative 5-F-dUMP into genomic DNA.

Previously, we showed that the ability of Cks proteins to override of the intra-S phase checkpoint was dependent on the interaction with Cdks (38). Since Cdk-independent functions have been reported for Cks proteins (described above) we next sought to determine whether the 5-FU sensitivity of Cks1 overexpressing cells was Cdk binding dependent. MCF-7 cells were transiently transduced with adenoviruses that expressed control (β-gal), Cks1, or Cks1^{E63Q}, a mutant that cannot bind Cdks (39). Treatment of these transduced cell populations with 5-FU revealed enhanced DNA damage response and increased apoptosis only in the cells that expressed wild-type Cks1 (Fig. 2C). The reduced apoptosis observed in this experiment compared to previous experiments (*i.e*. Figs. 1A, 1B, and 2B) was likely due to lower exogenous Cks1 expression compared to the endogenous protein. These results demonstrated the 5-FU sensitivity phenotype is mediated through a direct interaction of Cks proteins with Cdks.

Override of the intra-S-phase checkpoint in Cks1 overexpressing cells is required for 5-FU sensitivity

The increased incorporation of 5-FU derivatives into genomic DNA of Cks1 overexpressing cells (Fig. 2A) suggested that the enhanced apoptosis could be due to an override of the intra-S-phase checkpoint, as we previously reported for replication stress induced by oncogene expression (38). To test this, we first analyzed molecular markers of the cell cycle in Cks1 and Cks2 overexpressing MCF-7 cell populations treated with 5-FU. 5-FU treatment is known to invoke a G_1/S phase or intra-S-phase checkpoint arrest of cell division. However, we observed increased levels of cyclin B1, a marker of late S, G_2, and M phases, in MCF-7 cells that overexpressed either Cks1 or Cks2 (Fig. 3A). We then evaluated the effects on cell cycle kinetics by flow cytometry. In contrast to control cells which showed arrest mainly in G_1 and S phases, a significant proportion of Cks1 overexpressing MCF-7 cells had progressed into S phase 24 hrs after 5-FU treatment, as indicated by a broadening of the G_1 peak shoulder as well as an increase in the S phase population (total S phase cells:

39.4%-control vs. 56.9% for Cks1-overexpressing cells) (Fig. 3B). A similar effect on cell cycle kinetics was observed previously for Cks overexpressing cells treated with HU (38). These results confirmed that Cks1 overexpressing cells failed to arrest cell cycle progression and continued to replicate their DNA in the presence of 5-FU. To evaluate the significance of the failure to arrest cell cycle on the enhanced apoptosis of 5-FU treated Cks overexpressing cells, we arrested cell cycle progression in late G_1 phase by incubation with mimosine prior to 5-FU treatment. Mimosine has been shown to arrest cell cycle progression prior to the establishment of DNA replication forks (40). Mimosine treatment of Cks1 overexpressing cells was found to abrogate the enhanced apoptosis induced by 5-FU treatment (Fig. 3C). These data suggested that Cks overexpression promotes sensitivity to 5-FU by overriding DNA damage checkpoints.

Cks-overexpression re-sensitizes chemotherapy resistant cells and promotes improved chemotherapy response in vivo

To evaluate the potential clinical significance of Cks proteins in the chemotherapy response, we explored whether Cks overexpression could overcome resistance developed to replication stress-inducing chemotherapies. MTX-resistant MDA-MB-231 breast cancer cells were engineered to overexpress Cks1 at a level similar to overexpressing breast cancers, treated with MTX or vehicle, and apoptosis induction analyzed by Annexin V staining and Western blotting. Cks1 overexpressing cells displayed a >2-fold increase in Annexin V positive cells and increased cleaved Parp compared to control cells, suggesting re-sensitization to MTX treatment (Figs. 4A & B). We next employed an orthotopic breast cancer model to determine whether the chemotherapy sensitivity phenotype could be extended to cancers *in vivo*. Stable Cks1 overexpressing and control MCF-7 cells were implanted into the mammary fat pads of nude mice and 5-FU administered after the tumor volumes reached 100 mm^3. A significant reduction in tumor growth was observed for the Cks1 overexpressing tumors indicating they were indeed sensitized to 5-FU treatment ($p<0.05$) (Fig. 4C). Molecular analysis of the resultant tumors showed increased cleaved Parp and Puma expression in the Cks1 overexpressing tumors compared to control tumors (Fig. 4D). Quantification of the Western blot signals demonstrated a significant increase in cleaved Parp in Cks1 overexpressing breast cancers treated with 5-FU ($p<0.01$). These results confirmed *in vivo* that Cks overexpression is a clinically important determinant of the response of breast cancers to replication stress-inducing chemotherapies.

DISCUSSION

Our results demonstrate that Cks1/2 overexpression sensitizes cancer cells to replication stress-inducing chemotherapies such as 5-FU and MTX by overriding DNA damage checkpoints, including the replication stress checkpoint (also known as the intra-S phase checkpoint). 5-FU has been shown to induce replication stress by promoting misincorporations of its derivatives (dUMP and FdUMP) into genomic DNA resulting in the accumulation of DNA repair intermediates and fragmentation, and inhibiting TS leading to imbalances in nucleotide pools. Both of these mechanisms activate the intra-S phase checkpoint mediated by ATR-Chk1 signaling, which in turn functions to down-regulate Cdk activity through targeted degradation of the Cdk activating phosphatase Cdc25A (41). We

previously showed that Cks overexpression overrides the intra-S phase checkpoint induced by HU treatment or oncogene expression (38). Therefore, 5-FU sensitivity of Cks overexpressing cancer cells is likely caused, at least in part, by the failure of cells to invoke G_1 and intra-S phase checkpoints in response to replication stress, leading to enhanced induction of apoptosis through DNA damage overload. This hypothesis is supported by our data which showed treatment of Cks overexpressing cells with 5-FU results in a higher proportion of cells entering S phase, increased incorporation of 5-FU intermediates, and enhanced activation of ATM-checkpoint signaling.

Overexpression of either Cks1 or Cks2 was found to sensitize cancer cells to 5-FU treatment, suggesting this phenotype is mediated through a redundant function of Cks proteins. Previously we showed that Cks overexpression can partially restore Cdk2-associated kinase activity even in the presence of inhibitory (Tyr15) phosphorylations (38). Although the precise molecular mechanism(s) for this is unclear Cks proteins have been proposed to function in Cdk substrate targeting likely mediated through their anion binding site, which can bind phosphate (7, 39). Therefore, high level expression of Cks proteins could override the intra-S phase checkpoint invoked by 5-FU treatment by enhancing the targeting of Cdk2 substrates that promote continued cell cycle progression in the presence of replication stress. Further research is needed to identify these Cdk substrates and determine their essentiality in overriding the intra-S phase checkpoint.

The molecular cause(s) of Cks1 and Cks2 overexpression in human cancers are not well defined. Cks1 has been shown to be a transcriptional target of oncoproteins c-Myc, B-RAF, and cyclin D1 (26–27), which are frequently overexpressed in many types of human cancers. In addition, the *CKS1* locus is localized to chromosome region 1q21 which is frequently amplified in cancers, including those of the breast (42). However, information regarding the mechanism of Cks2 overexpression is lacking. It will be of interest to determine the predominant causes of Cks overexpressions in human cancers and whether specific oncoproteins might play prominent roles in promoting Cks-dependent chemotherapy sensitivity.

Cks proteins could have therapeutic implications as both clinical biomarkers and molecular targets for enhancing chemotherapeutic efficacy. 5-FU is routinely used to treat breast as well as head and neck cancers, and is a main chemotherapy for the treatment of colorectal cancers. Given the results of our study, assessment of Cks1/2 levels in clinical specimens is warranted, with the potential to provide a new biomarker for response to 5-FU and possibly other drugs that function through replication stress. Moreover, Cks1 has been shown to be targeted for degradation by the ubiquitin proteasome system (UPS) (43). Therefore, drugs that effectively block this process in cancers might be useful to enhance the efficacy of replication stress-inducing chemotherapies, or possibly restore sensitivity to cancers that have developed resistance to these drugs. Additional research is also needed to determine whether the therapeutic benefits of Cks overexpression might extend to other chemotherapies that induce DNA damage and replication stress through alternative mechanisms, such as formation of DNA crosslinks (*e.g.* Cisplatin), topoisomerase inhibition (*e.g.* Irinotecan), or prevention of DNA replication elongation (*e.g.* gemcitabine). Furthermore, it was recently shown that 5-FU misincorporated bases in DNA are excised by

thymine DNA glycosylase (TDG), and TDG overexpression sensitizes cancer cells to 5-FU treatment (44). Therefore, it will be of interest to determine if Cks overexpression can synergize with TDG to enhance the efficacy of 5-FU and other replication stress-inducing drugs for cancer treatment.

MATERIALS and METHODS

Reagents

Antibodies used in this study included: anti-PUMA (Cell Signaling Technology); anti-cleaved poly ADP-ribose polymerase (PARP; Cell Signaling Technology); anti–β-actin (Sigma); anti-ATM (Abcam); anti-p-Ser1981-ATM (Abcam); anti-cleaved Caspase 7 (Stressgen Bioreagents Corp.); anti-p-Ser15-p53 (Cell Signaling Technology); anti-p53 (Oncogene Research); anti-Cdk2 (Santa Cruz Biotechnology); anti-Ku86 (Santa Cruz Biotechnology); anti-Tubulin (Santa Cruz Biotechnology); and anti-Flag (Sigma). 5-FU and MTX were purchased from Sigma. 2-^{14}C-5-FU was purchased from Moravek Biochemicals. Cks expressing adenoviruses were described previously (13).

Cell lines

MCF-7 and MDA-MB-231 human breast cancer cells were maintained in RPMI and DMEM media, respectively. HCT116 human colorectal carcinoma cells were maintained in McCoy's media. All medias were supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and streptomycin. All cell lines were purchased from ATCC. For generation of stable Cks overexpressing cell lines, the cells were co-transfected with pcDNA3.1-Flag-Cks1 or empty vector (control) and pPur (Clontech) using Lipofectamine 2000 (Invitrogen). Cell clones were isolated and Cks protein expression screened in comparison with Cks overexpressing breast cancer specimens (Supplementary Fig. 1; data not shown). For generation of Cks1/2 overexpressing cell populations, the cells were transduced with lentiviruses that expressed Cks1 or Cks2 with C-terminal Flag-tags. Lentiviral expression vectors were generated based on pLenti CMV Puro DEST (Addgene). Viral productions and cell transductions were performed as recommended by the supplier.

Apoptosis assays

Pyknosis was quantified by treating cells seeded on glass cover slips with 5-FU for 48 hrs followed by incubation with fresh medium for 72 hrs. Cells were fixed with 3.7% formaldehyde and stained with DAPI. Cells were analyzed using a fluorescence microscope and the percentage of cells with apoptotic bodies, pyknotic and fragmented nuclei, calculated. At least 150 cells were counted for each experiment. Clonogenic assays were performed by treating cells with vehicle (DMSO) or 2.5–5 μM 5-FU for 48 hrs post-plating onto 10cm dishes. After 21 days in fresh medium, the colonies were fixed with cold methanol and stained with crystal violet stain (Sigma). Annexin V analyses were performed using the FITC Apoptosis Detection Kit I (BD Biosciences). Cells were co-stained with propidium iodide and analyzed using a FACScan Flow Cytometer. Cell cycle kinetics was analyzed using CellQuest software.

5-FU incorporation assays

Cells were grown in media containing 10 μM 2-^{14}C-5-FU (57.2 mCi/mmol) for 48 hrs and genomic DNA isolated using the QIAamp DNA Blood Midi Kit (Qiagen). Incorporated 2-^{14}C-5-FU was measured using a scintillation counter and expressed as counts/μg genomic DNA.

Mouse xenograft experiments

Female nude (*nu/nu*) mice (Charles River Laboratories) were implanted with an 17β-estradiol pellet (0.72 mg; Innovative Research of America) prior to inoculation with 3×10^6 MCF-7 cells (stable Cks1 overexpressing or control) re-suspended in Matrigel (BD Biosciences) into the mammary fat pads. For drug treatments, mice were randomly divided into 2 groups and administered 5-FU (40mg/kg/week) or PBS (control) once tumor volume reached 100 mm^3. Tumor volume was calculated as length ($width^2$)/2=mm^3. A portion of each tumor was frozen in liquid N_2 for Western blot analysis.

Statistical analyses

Statistical significance was evaluated in most cases using the student's t-test. Differences in tumor growth were assessed using non-linear regression analysis. All calculations were performed using GraphPad Prism v5.0 software (GraphPad Software, Inc.)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We wish to thank C. Torres and Y. Altman (Sanford-Burnham Medical Research Institute) for technical assistance with flow cytometry analyses. This work was supported by grants from the American Cancer Society (RSG-06-020-01) and National Institutes of Health (7R01HD049539-05) to CS, Department of Defense Breast Cancer Research Program (W81XWH-06-1-0344) to SVdR, and National Cancer Institute (CA074224) to SIR. Additional support came from a UCLH/UCL Comprehensive Biomedical Research Centre grant and was undertaken at UCLH/UCL, which received a portion of its funding from the Department of Health NIHR Biomedical Research Centres funding scheme.

References

1. Reynard GJ, Reynolds W, Verma R, Deshaies RJ. Cks1 is required for G(1) cyclin-cyclin-dependent kinase activity in budding yeast. Mol Cell Biol. 2000; 20:5858–64. [PubMed: 10913169]
2. Patra D, Dunphy WG. Xe-p9, a Xenopus Suc1/Cks homolog, has multiple essential roles in cell cycle control. Genes Dev. 1996; 10:1503–15. [PubMed: 8666234]
3. Patra D, Dunphy WG. Xe-p9, a Xenopus Suc1/Cks protein, is essential for the Cdc2-dependent phosphorylation of the anaphase-promoting complex at mitosis. Genes Dev. 1998; 12:2549–59. [PubMed: 9716407]
4. Murray AW. Recycling the cell cycle: cyclins revisited. Cell. 2004; 116:221–34. [PubMed: 14744433]
5. Patra D, Wang SX, Kumagai A, Dunphy WG. The xenopus Suc1/Cks protein promotes the phosphorylation of G(2)/M regulators. J Biol Chem. 1999; 274:36839–42. [PubMed: 10601234]
6. Kaiser P, Moncollin V, Clarke DJ, Watson MH, Bertolaet BL, Reed SI, et al. Cyclin-dependent kinase and Cks/Suc1 interact with the proteasome in yeast to control proteolysis of M-phase targets. Genes Dev. 1999; 13:1190–202. [PubMed: 10323869]

7. Harper JW. Protein destruction: adapting roles for Cks proteins. Curr Biol. 2001; 11:R431–5. [PubMed: 11516665]
8. Chaves S, Baskerville C, Yu V, Reed SI. Cks1, Cdk1, and the 19S proteasome collaborate to regulate gene induction-dependent nucleosome eviction in yeast. Mol Cell Biol. 2010; 30:5284–94. [PubMed: 20855529]
9. Yu VP, Baskerville C, Grunenfelder B, Reed SI. A kinase-independent function of Cks1 and Cdk1 in regulation of transcription. Mol Cell. 2005; 17:145–51. [PubMed: 15629725]
10. Spruck CH, de Miguel MP, Smith AP, Ryan A, Stein P, Schultz RM, et al. Requirement of Cks2 for the first metaphase/anaphase transition of mammalian meiosis. Science. 2003; 300:647–50. [PubMed: 12714746]
11. Spruck C, Strohmaier H, Watson M, Smith AP, Ryan A, Krek TW, et al. A CDK-independent function of mammalian Cks1: targeting of SCF(Skp2) to the CDK inhibitor p27Kip1. Mol Cell. 2001; 7:639–50. [PubMed: 11463388]
12. Bornstein G, Bloom J, Sitry-Shevah D, Nakayama K, Pagano M, Hershko A. Role of the SCFSkp2 ubiquitin ligase in the degradation of p21Cip1 in S phase. J Biol Chem. 2001; 278:25752–7. [PubMed: 12730199]
13. Tedesco D, Lukas J, Reed SI. The pRb-related protein p130 is regulated by phosphorylation-dependent proteolysis via the protein-ubiquitin ligase SCF(Skp2). Genes Dev. 2002; 16:2946–57. [PubMed: 12435635]
14. Martinsson-Ahlzen HS, Liberal V, Grunenfelder B, Chaves SR, Spruck CH, Reed SI. Cyclin-dependent kinase-associated proteins Cks1 and Cks2 are essential during early embryogenesis and for cell cycle progression in somatic cells. Mol Cell Biol. 2008; 28:5698–709. [PubMed: 18625720]
15. Wang JJ, Fang ZX, Ye HM, You P, Cai MJ, Duan HB, et al. Clinical significance of overexpressed cyclin-dependent kinase subunits 1 and 2 in esophageal carcinoma. Dis Esophagus. 2013; 26:729–36. [PubMed: 23301842]
16. Lee SW, Kang SB, Dee DS, Lee JU. Akt and Cks1 are related with lymph node metastasis in gastric adenocarcinoma. Hepatogastroenterology. 2013; 60(127) [Epub ahead of print].
17. Martin-Ezquerra G, Salgado R, Toll A, Baro T, Mojal S, Yebenes M, et al. CSC28 protein kinase regulatory subunit 1B (CKS1B) expression and genetic status analysis in oral squamous cell carcinoma. Histol Histopathol. 2011; 26:71–7. [PubMed: 21117028]
18. Wang XC, Tian LL, Tian J, Wu HL, Meng AM. Overexpression of Cks1 is associated with poor survival by inhibiting apoptosis in breast cancer. J Cancer Res Clin Oncol. 2009; 135:1393–401. [PubMed: 19352700]
19. Liu Z, Fu Q, Lv J, Wang F, Ding K. Prognostic implication of p27Kip1, Skp2 and Cks1 expression in renal cell carcinoma: a tissue microarray study. J Exp Clin Cancer Res. 2008; 27:51. [PubMed: 18922157]
20. Kawakami K, Enokida H, Tachiwada T, Nishiyama K, Seki N, Nakagawa M. Increased SKP2 and CKS1 gene expression contributes to the progression of human urothelial carcinoma. J Urol. 2007; 178:301–7. [PubMed: 17499794]
21. Slotky M, Shapira M, Ben-Izhak O, Linn S, Futerman B, Tsalic M, et al. The expression of the ubiquitin ligase subunit Cks1 in human breast cancer. Breast Cancer Res. 2005; 7:R737–44. [PubMed: 16168119]
22. Shapira M, Ben-Izhak O, Linn S, Futerman B, Minkov I, Hershko DD. The prognostic impact of the ubiquitin ligase subunits Skp2 and Cks1 in colorectal carcinoma. Cancer. 2005; 103:1336–46. [PubMed: 15717322]
23. Masuda TA, Inoue H, Nishida K, Sonoda H, Yoshikawa Y, Kakeji Y, et al. Cyclin-dependent kinase 1 gene expression is associated with poor prognosis in gastric carcinoma. Clin Cancer Res. 2003; 9:5693–8. [PubMed: 14654553]
24. Inui N, Kitagawa K, Miwa S, Hattori T, Chida K, Nakamura H, et al. High expression of Cks1 in human non-small cell lung carcinomas. Biochem Biophys Res Commun. 2003; 303:978–84. [PubMed: 12670508]

25. Yamamoto S, Tsuda H, Miyai K, Takano M, Tamai S, Matsubara O. Cumulative alterations of p27-related cell-cycle regulators in the development of endometriosis-associated ovarian clear cell adenocarcinoma. Histopathology. 2010; 56:740–9. [PubMed: 20546339]
26. Keller UB, Old JB, Dorsey FC, Nilsson JA, MacLean KH, Chung L, et al. Myc targets Cks1 to provoke the suppression of p27Kip1, proliferation, and lymphomagenesis. EMBO J. 2007; 26:2562–74. [PubMed: 17464290]
27. Bhatt KV, Hu R, Spofford LS, Aplin AE. Mutant B-RAF signaling and cyclin D1 regulate Cks1/S-phase kinase-associated protein 2-mediated degradation of p27Kip1 in human melanoma cells. Oncogene. 2007; 26:1056–66. [PubMed: 16924241]
28. Shen DY, Zhan YH, Wang QM, Rui G, Zhang ZM. Oncogenic potential of cyclin kinase subunit-2 in cholangiocarcinoma. Liver Int. 2013; 33:137–48. [PubMed: 23121546]
29. Perez-Magan E, Campos-Martin Y, Mur P, Fiano C, Ribalta T, Garcia JF, et al. Genetic alterations associated with progression and recurrence in meningiomas. J Neuropathol Exp Neurol. 2012; 71:882–93. [PubMed: 22964784]
30. Menghi F, Orzan FN, Eoli M, Farinotti M, Maderna E, Pisati F, et al. DNA microarray analysis identifies CKS2 and LEPR as potential markers of meningioma recurrence. Oncologist. 2011; 16:1440–50. [PubMed: 21948653]
31. Chen R, Feng C, Xu Y. Cyclin-dependent kinase-associated protein Cks2 is associated with bladder cancer progression. J Int Med Res. 2011; 39:533–40. [PubMed: 21672358]
32. Tanaka F, Matsuzaki S, Mimori K, Kita Y, Inoue H, Mori M. Clinicopathological and biological significance of CDC28 protein kinase regulatory subunit 2 overexpression in human gastric cancer. Int J Oncol. 2011; 39:361–72. [PubMed: 21617860]
33. Wiese AH, Auer J, Lassmann S, Nahrig J, Rosenberg R, Hofler H, et al. Identification of gene signatures for invasive colorectal tumor cells. Cancer Detect Prev. 2007; 31:282–95. [PubMed: 17936523]
34. Lin HM, Chatterjee A, Lin YH, Anjomshoaa A, Fukuzawa R, McCall JL, et al. Genome wide expression profiling identifies genes associated with colorectal liver metastasis. Oncol Rep. 2007; 17:1541–9. [PubMed: 17487416]
35. Li M, Lin YM, Hasegawa S, Shimokawa T, Murata K, Kameyama M, et al. Genes associated with liver metastasis of colon cancer, identified by genome-wide cDNA microarray. Int J Oncol. 2004; 24:305–12. [PubMed: 14719106]
36. Kawakami K, Enokida H, Tachiwada T, Gotanda T, Tsuneyoshi K, Kubo H, et al. Identification of differentially expressed genes in human bladder cancer through genome-wide gene expression profiling. Oncol Rep. 2006; 16:521–31. [PubMed: 16865252]
37. van't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002; 415:530–6. [PubMed: 11823860]
38. Liberal V, Martinsson-Ahlzen HS, Liberal J, Spruck CH, Widschwendter M, McGowan CH, et al. Cyclin-dependent kinase subunit (Cks) 1 or Cks2 overexpression overrides the DNA damage response barrier triggered by activated oncoproteins. Proc Natl Acad Sci USA. 2012; 109:2754–9. [PubMed: 21697511]
39. Bourne Y, Watson MH, Hickey MJ, Holmes W, Rocque W, Reed SI, et al. Crystal structure and mutational analysis of the human CDK2 kinase complex with cell cycle-regulatory protein CksHs1. Cell. 1996; 84:863–74. [PubMed: 8601310]
40. Krude T. Mimosine arrests proliferating human cells before onset of DNA replication in a dose-dependent manner. Exp Cell Res. 1999; 247:148–159. [PubMed: 10047457]
41. Bartek J, Lukas C, Lukas J. Checking on DNA damage in S phase. Nat Rev Mol Cell Biol. 2004; 5:792–804. [PubMed: 15459660]
42. Wang CX, Tian J, Tian LL, Wu HL, Meng AM, Ma TH, et al. Role of Cks1 amplification and overexpression in breast cancer. Biochem Biophys Res Commun. 2009; 379:1107–13. [PubMed: 19161979]
43. Bashir T, Dorrello NV, Amador V, Guardavaccaro D, Pagano M. Control of SCF(Skp2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase. Nature. 2004; 428:190–3. [PubMed: 15014502]

44. Kunz C, Focke F, Saito Y, Schuermann D, Lettieri T, Selfridge J, et al. Base excision by thymine DNA glycosylase mediates DNA-directed cytotoxicity of 5-fluorouracil. PLoS Biol. 2009; 7:e1000091.

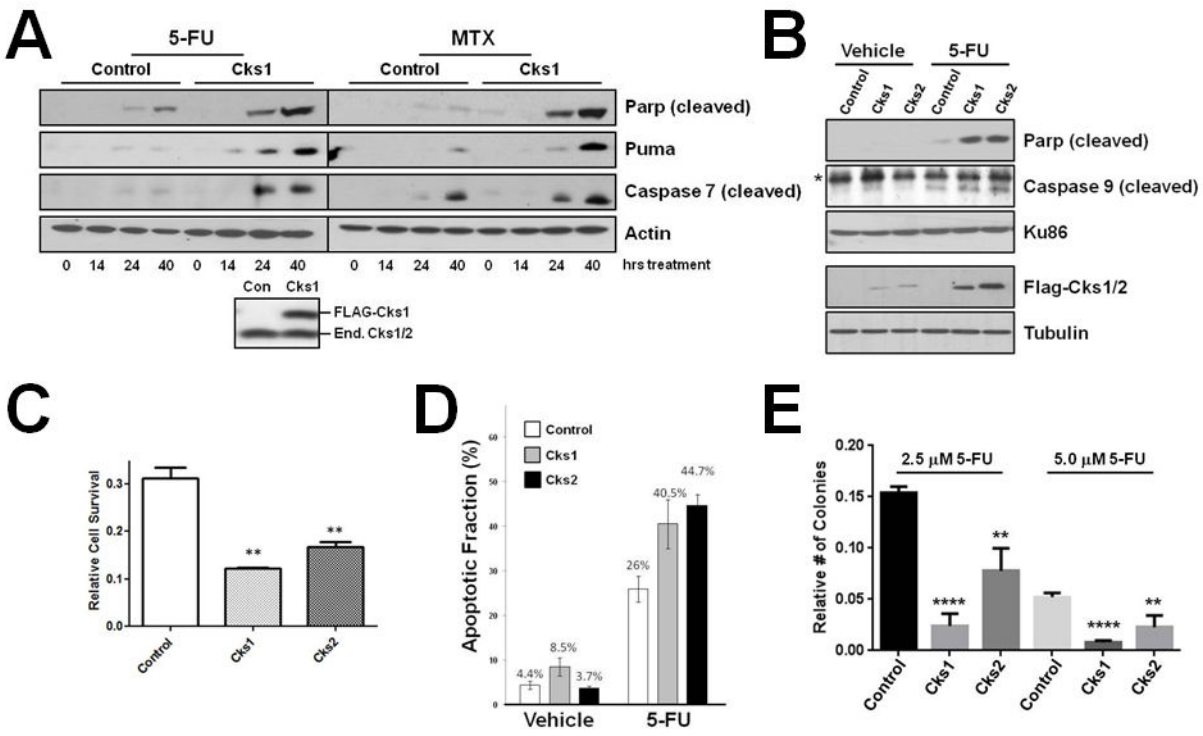

Figure 1. Cks1/2 overexpression sensitizes breast cancer cells to replication stress-inducing chemotherapies

(**a**) Increased apoptotic markers cleaved Parp, Puma, and cleaved caspase 7 in stable Cks1 overexpressing MCF-7 cells treated with 5-FU or MTX for the indicated times. Figure below shows Flag-Cks1 expression relative to endogenous Cks1/2 protein level. An antibody that detects both Cks1 and Cks2 was used. (**b**) Enhanced apoptosis markers cleaved Parp and cleaved caspase 9 in MCF-7 cell populations transduced with Cks1 or Cks2 expressing lentiviruses and treated with 5-FU for 24 hrs. Asterisk indicates background band detected with the anti-cleaved caspase 9 antibody. (**c**) Growth suppression of Cks1 and Cks2 overexpressing MCF-7 cell populations treated with 5-FU for 48 hrs ($p<0.01$ for both). Cells were counted 72 hrs post-treatment. (**d**) Increased pyknosis in Cks1 and Cks2 overexpressing MCF-7 cell populations treated with 5-FU for 24 hrs. (**e**) Decreased colony formation of Cks1 and Cks2 overexpressing MCF-7 cell populations treated with 2.5 or 5 μM 5-FU for 72 hrs ($p<0.01$ and $p<0.0001$, respectively). Colonies were stained 21 days post-plating.

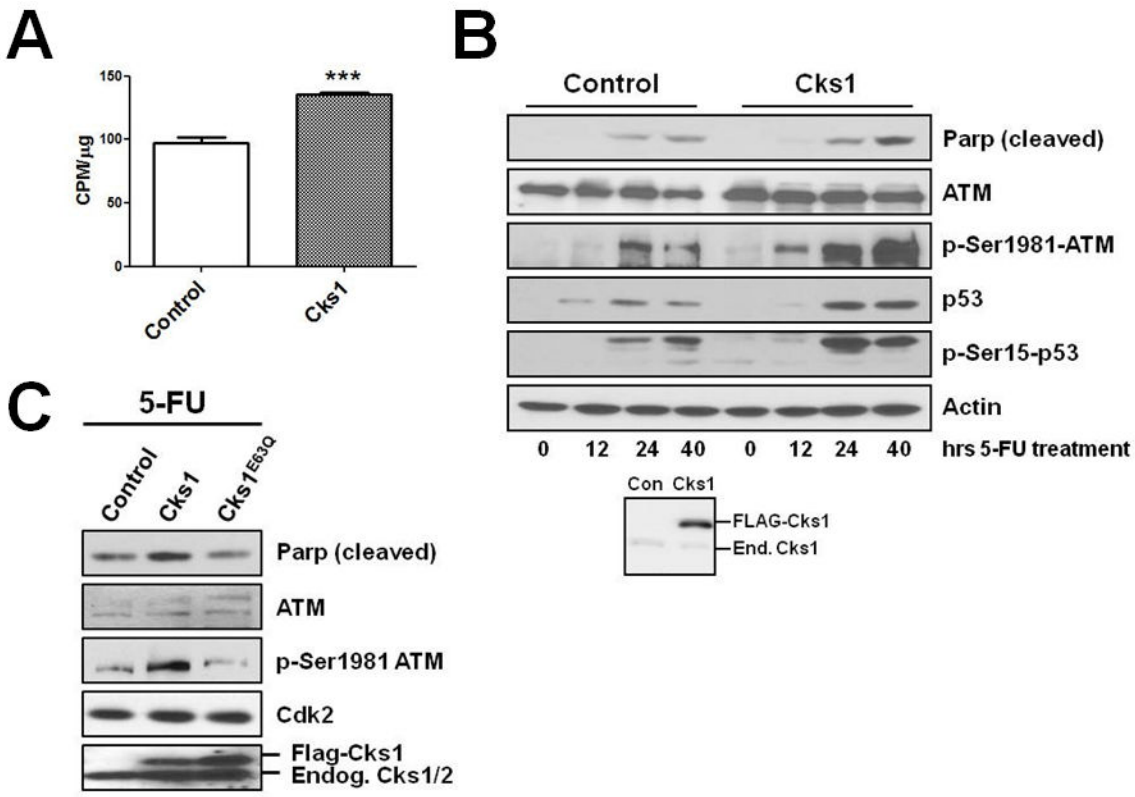

Figure 2. Enhanced 5-FU sensitivity of Cks1 overexpressing cells is associated with increased 5-FU incorporation and DNA damage response, and is dependent on Cdk binding

(**a**) Increased incorporation of 2-^{14}C-5-FU derivatives into the genomic DNA of stable Cks1 overexpressing MCF-7 cells compared to control cells ($p < 0.001$). (**b**) Increased DNA damage markers phospho-Ser1981-ATM and phospho-Ser15-p53 in stable Cks1 overexpressing MCF-7 cells treated with 5-FU for the indicated times. Western blot below shows expression of Flag-Cks1 relative to endogenous Cks proteins. (**c**) Cks1 sensitivity to 5-FU is dependent on Cks1 binding Cdks. MCF-7 cells were transiently transduced with adenoviruses that express control (β-gal), Cks1, or Cks1^{E63Q}, a mutant defective in Cdk-binding. Cells were then treated with 5-FU for 48 hrs and apoptosis markers analyzed by Western blotting. Cdk2 is shown as a loading control.

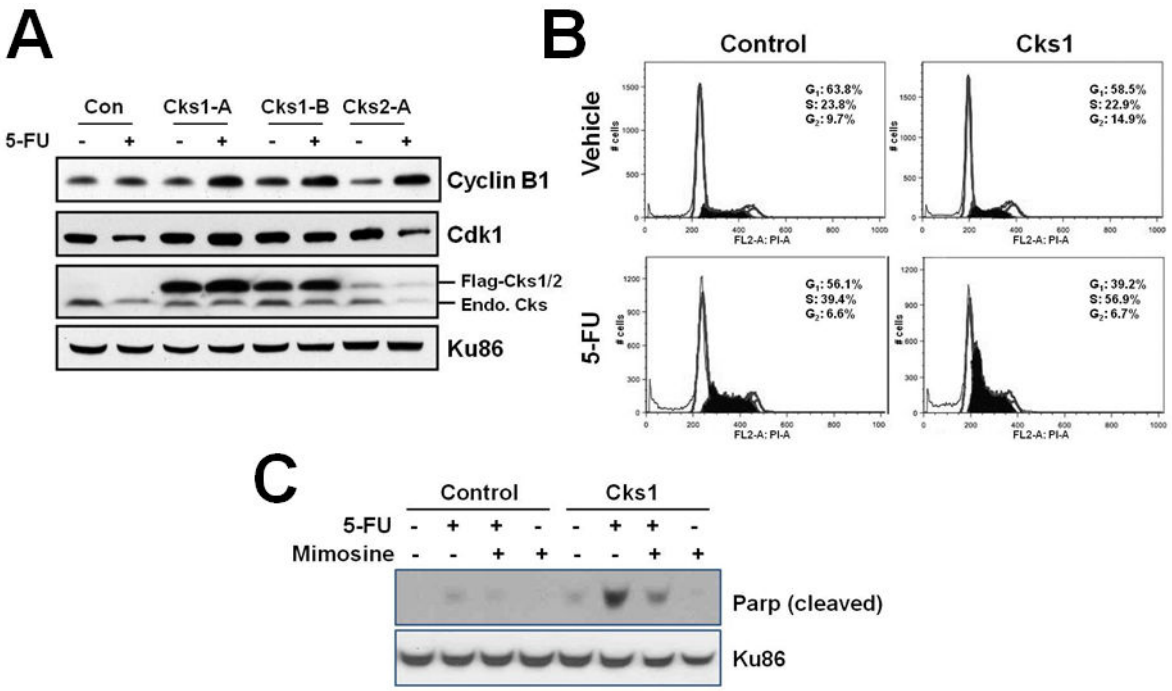

Figure 3. Cks1 overexpressing cells fail to arrest cell cycle progression in response to 5-FU

(**a**) Western blot analysis showing late S/G_2/M phase marker cyclin B1 in Cks1 and Cks2 overexpressing MCF-7 cell populations treated with 5-FU for 48 hrs. Cks blot used an anti-Cks1 antibody that favors detection of Cks1 over Cks2. Ku86 is shown as loading control. (**b**) Flow cytometry analysis demonstrating progression into S phase for stable Cks1 overexpressing MCF-7 cells treated with 5-FU for 24 hrs. S phase cells (which include the shoulder of the G_1 peak) are shaded in black. (**c**) Arrest of Cks1 overexpressing MCF-7 cells in late G_1 phase abrogates 5-FU sensitivity. Stable Cks1 overexpressing cells were incubated with mimosine for 16 hrs prior to treatment with 5-FU for 24 hrs and apoptosis marker cleaved Parp analyzed by Western blotting.

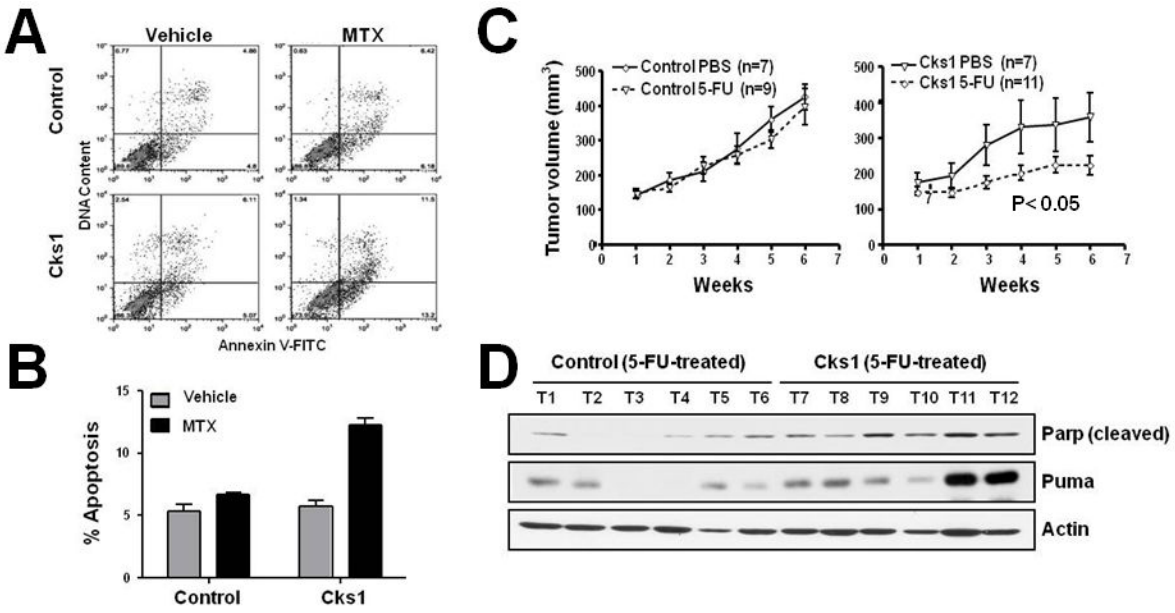

Figure 4. Cks overexpression can re-sensitize MTX-resistant cancer cells and promotes favorable response to 5-FU in an orthotopic breast cancer mouse model

(**a**) Cks1 overexpression in MTX-resistant MDA-MB-231 cells re-sensitizes them to MTX treatment. Flow cytometry analysis of Annexin V staining vs. DNA content (PI staining). Early apoptotic cells (lower right quadrant) and late-stage apoptotic cells (upper right quadrant) are indicated. (**b**) Quantification of apoptotic cells in A. (**c**) Cks1 overexpression sensitizes breast cancers to 5-FU treatment *in vivo*. Nude mice were implanted with control (T1-6) or stable Cks1 overexpressing (T7-12) MCF-7 cells into their mammary fat pads and treated with 5-FU after tumor volume reached 100 mm^3. (**d**) Western blot analysis of apoptosis markers cleaved Parp and Puma of resultant breast tumors described in C. Quantifications of cleaved Parp were normalized to actin for statistical comparisons.