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# Downregulation of Cdc6 and pre-replication complexes in response to methionine stress in breast cancer cells

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Abbreviations: SAM, S-adenosylmethionine; Met, Methionine; Hcy, homocysteine; MAT, methionine adenosyl transferase; BrdU, 5-bromo-2'-deoxyuridine; SRB, sulforhodamine B; Cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor

Methionine and homocysteine are metabolites in the transmethylation pathway leading to synthesis of the methyldonor S-adenosylmethionine (SAM). Most cancer cells stop proliferating during methionine stress conditions, when methionine is replaced in the growth media by its immediate metabolic precursor homocysteine (Met-Hcy+). Nontransformed cells proliferate in Met-Hcy+ media, making the methionine metabolic requirement of cancer cells an attractive target for therapy, yet there is relatively little known about the molecular mechanisms governing the methionine stress response in cancer cells. To study this phenomenon in breast cancer cells, we selected methionineindependent-resistant cell lines derived from MDAMB468 breast cancer cells. Resistant cells grew normally in Met-Hcy+ media, whereas their parental MDAMB468 cells rapidly arrest in the G<sub>1</sub> phase. Remarkably, supplementing Met-Hcy+ growth media with S-adenosylmethionine suppressed the cell proliferation defects, indicating that methionine stress is a consequence of SAM limitation rather than low amino acid concentrations. Accordingly, mTORC1 activity, the primary effector responding to amino acid limitation, remained high. However, we found that levels of the replication factor Cdc6 decreased and pre-replication complexes were destabilized in methionine-stressed MDAMB468 but not resistant cells. Our study characterizes metabolite requirements and cell cycle responses that occur during methionine stress in breast cancer cells and helps explain the metabolic uniqueness of cancer cells.

#### Introduction

Cancer cell metabolism is significantly altered compared with metabolism of non-transformed cells. For example, the nearly universal cancer cell characteristic of aerobic glycolysis, known as the Warburg effect, allows cancer cells to evade apoptosis and thrive in regions of intratumoral hypoxic stress.<sup>1</sup> Importantly, the same peculiarities of cancer cell metabolism that promote growth and survival also distinguish them from normal cells and therefore represent a potentially fertile field for therapeutic development. Past research focused on the increased glycolysis commonly observed in tumors, yet there are numerous other aspects of altered cancer cell metabolism possessing the potential for therapeutic development. Indeed, recent attention highlights the need to expand the knowledge of cancer cell metabolism beyond the Warburg effect, exploring other metabolic pathways altered in cancer.<sup>2-4</sup>

Cancer cell methionine metabolism is one such area ripe for investigation. Specifically, cancer cells originating from a variety

\*Correspondence to: Peter Kaiser; Email: pkaiser@uci.edu Submitted: 07/16/12; Revised: 10/19/12; Accepted: 11/03/12 http://dx.doi.org/10.4161/cc.22767 of different tissue types are unable to grow when methionine is restricted from the culture media. Methionine is an essential amino acid required by all cells, but in the case of non-transformed cells, providing the immediate metabolic precursor to methionine, homocysteine, is sufficient for growth. By contrast, availability of homocysteine does not rescue cancer cells from their methionine dependence.<sup>5-8</sup> Importantly, remethylation of homocysteine to generate methionine appears to be unaffected in cancer cells.<sup>9</sup>

Cancer cell methionine dependency is well-documented. When a mixture of breast carcinoma cells and normal breast epithelial cells grow in a standard medium (Met+Hcy-), breast cancer cells rapidly outgrow normal cells. When the same cells are cultured in methionine stress conditions, methionine-free media supplemented with homocysteine (Met-Hcy+), after 1 wk only normal cells exist in the culture dish.<sup>6</sup> In vitro studies of prostate cancer cells indicate that they suffer a specific cell cycle arrest and eventually undergo apoptosis when cultured in Met-Hcy+ media.<sup>10</sup> Consistent with a specific effect of methionine restriction on cell cycle regulation, microarray analysis using central nervous system tumor cell lines revealed upregulation of defined cell cycle checkpoints and apoptotic pathways while inhibition of known survival pathways was observed.<sup>11</sup> Cancer cell methionine dependency is also evident for tumors in vivo. Lowering the plasma methionine level concomitant with homocysteine supplementation in animal models causes regression of tumors, inhibition of metastasis and blocks growth of solid tumors and leukemia, but has no harmful effects on normal tissues.<sup>12-14</sup> The apparent hunger of cancer for methionine is also exploited by clinicians in tumor detection using <sup>11</sup>C-methionine as an alternative tracer for the glucose analog <sup>18</sup>F-FDG in positron emission tomography.<sup>15</sup>

Molecular mechanisms describing how methionine dependency induces cell cycle arrest and apoptosis are unknown; however, the diverse ways in which a cell uses methionine are well-understood. Beyond protein synthesis, methionine and ATP are joined by the enzyme methionine adenosyl transferase (MAT) to produce S-adenosylmethionine (SAM or AdoMet). SAM represents the primary methyl donor for the vast majority of cellular methylation reactions, including transfer of methyl groups to proteins, nucleic acids and phospholipids. In addition, its propyl amine moiety is used for reactions that synthesize polyamines. The remaining methylthioadenosine can be salvaged and used as an alternative purine source for nucleotide biosynthesis. Finally, homocysteine and the transsulfuration branch intermediates of methionine metabolism are used to synthesize glutathione, the cell's primary defense against reactive oxygen species. The methionine-derived SAM is of particular interest, because cancer cells are especially sensitive to SAM limitation. Targeted inhibition of the regulatory subunit of MAT reduced SAM levels and strongly induced apoptosis and growth inhibition of leukemic cells.<sup>16,17</sup> Given such high demand for methionine and SAM by cancer cells, it is conceivable that replacement of methionine by homocysteine curtails flux through methyl group metabolism reducing methionine to a level insufficient for the high methionine requirement of cancer cells, thus affecting a cell cycle arrest and apoptosis.

In order to gain mechanistic understanding of the changes seen in cancer cells in response to methionine stress, we monitored the cell cycle in breast cancer cell lines during metabolic shift to homocysteine medium. Methionine stress induced a  $G_1$ phase cell cycle arrest, downregulated the replication factor Cdc6 and prevented pre-replication complex formation. Furthermore, we demonstrate that methionine stress can be suppressed through addition of SAM suggesting that SAM limitation contributes to the metabolic phenomenon of cancer cell methionine dependence.

#### **Results**

S-adenosylmethionine supplementation suppresses methionine stress in MDAMB468 breast cancer cells. Methionine dependency of cancer cells refers to the phenomenon that supplementation of methionine-free growth medium with the junction metabolite homocysteine (Fig. 1A), the immediate metabolic precursor of methionine, is sufficient for the proliferation of non-transformed cells but leads to cell proliferation block in many cancer cells.<sup>6,7,10,11</sup> Relatively little was known about methionine dependency of breast cancer cells. We therefore analyzed growth of various breast cancer cell lines in growth medium with (Met+) or without (Met-Hcy+) methionine (**Fig. 1B**). Breast cancer cell lines showed methionine dependency and, similar to results obtained with prostate cancer cell lines, the methionine-dependent phenotype did not correlate with the status of the tumor suppressors p53, Rb or p16<sup>1NK4A</sup>. We chose to focus further studies on the breast cancer cell line MDAMB468.

Knowledge of the phenomenon that cancer cell proliferation is selectively blocked in medium lacking methionine but supplemented with homocysteine dates back more than 30 y.6 However, whether growth arrest is directly attributable to methionine limitation or a limiting metabolite connected to methionine is not known. It is interesting to note that in transformed cells, the rate of methionine synthesis from homocysteine is comparable to that of non-transformed cells,9 suggesting that methionine dependence is a manifestation of limiting availability of methionine-derived metabolites rather than limitation of methionine. Regardless, there was a clear dose-dependent decrease of cell proliferation with reduced methionine concentration (Fig. 1C). In the model system, yeast, a regulatory pathway connecting sulfur-containing metabolites, most notably methionine, with cell cycle regulation, has been suggested to respond primarily to S-adenosylmethionine (SAM) levels.<sup>18-20</sup> Furthermore, earlier studies showed that reducing SAM synthesis blocks proliferation of leukemic cells.<sup>16,17</sup> Therefore, we hypothesized that methionine dependence is a reflection of limiting SAM availability caused by reduced flux through the methionine metabolic pathway in Met-Hcy+ conditions. Consistent with our hypothesis, SAM supplementation restored proliferation of MDAMB468 cells in Met-Hcy+ medium (Fig. 1D). These results suggest that methionine dependence of MDAMB468 breast cancer cells is caused by SAM limitation.

Methionine stress-resistant derivatives of MDAMB468 cells. To further explore the mechanism that selectively blocks cancer cell proliferation in Met-Hcy+ medium, it was important to directly compare methionine-dependent cells with methionine-independent cells. Most studies analyzing methionine stress in cancer cells have used cell pairs of different origin for this purpose.<sup>6,7,10,11</sup> Consequently, earlier studies generally compared cells with very different genetic makeup. In addition, growth rates of cell lines can differ significantly, and it is thus difficult to exclude slower proliferation rates and consequently lower demand for nutrients as cause for methionine independence. This is particularly evident when methionine-independent nontransformed cells are compared with methionine-dependent cancer cells. We wanted to compare the methionine stress response of MDAMB468 cells with a methionine-independent cell line that had a comparable proliferation rate and similar genetic background, so we used a strategy described by Hoffman and colleagues to generate methionine-independent MDAMB468derived cells.<sup>21,22</sup> Hereafter, the isolated methionine-independent cells are referred to as resistant MDAMB468 cells to signify the fact that they are resistant to methionine stress in contrast to



**Figure 1.** MDAMB468 breast cancer cell lines are dependent upon methionine and S-adenosylmethionine (SAM) for proliferation. (**A**) Schematic representation of methionine metabolism. (**B**) Cells were seeded into 96-well plates and their ability to proliferate in Met+Hcy- or Met-Hcy+ media was monitored using an SRB assay. (**C and D**) MDAMB468 cells were incubated in Met-Hcy+ media and supplemented with different concentrations ( $\mu$ M) of either methionine (**C**) or S-adenosylmethionine (**D**) as listed, and proliferation was monitored using an SRB assay.

their methionine stress-sensitive MDAMB468 parental cell line. The proliferation rate of both the MDAMB468 and the isolated R8 clone of resistant MDAMB468 cells in Met+ medium was similar, but only the resistant cells maintained the ability to proliferate in Met-Hcy+ medium (**Fig. 2A**).

One possibility as to how the resistant cells overcame methionine stress is that by limiting flux through the transsulfuration pathway for cysteine synthesis, cells can maintain sufficient SAM synthesis (**Fig. 1A**). We therefore asked whether R8 cells became methionine-independent by this proposed mechanism and grew MDAMB468 and R8 cells in cysteine-free medium to test for possible cysteine dependence. Neither MDAMB468 nor R8 cells were cysteine-dependent as long as the junction metabolite homocysteine was supplied (**Fig. 2B**). Furthermore, homocysteine was sufficient to support proliferation of R8 cells in the absence of both methionine and cysteine (**Fig. 2B**).

Methionine stress induces  $G_1$  phase cell cycle arrest in MDAMB468 cells. To determine in which cell cycle phase breast cancer cells arrest in response to methionine stress, we cultured MDAMB468 and R8 cells in Met+ medium, shifted them

to Met-Hcy+ medium and analyzed the DNA content by flow cytometry. The number of MDAMB468 cells in G<sub>1</sub> increased and remained high during the time course. In contrast, the cell cycle profile of R8 cells was largely unaffected (Fig. 2C). Similar results were obtained with MCF7 breast cancer cells (data not shown). Given that the total number of MDAMB468 cells did not increase after cells were shifted to Met-Hcy+ medium (for example, see Figs. 1B-D and 2A), the increase in the G<sub>1</sub> population suggests that methionine stress induced a G<sub>1</sub> arrest (Fig. 2C). To more directly investigate the possible  $G_1$  arrest, we determined the fraction of cells entering S-phase after cells were shifted to Met-Hcy+ medium using BrdU incorporation (Fig. 2D). Consistent with our hypothesis that methionine stress blocks entry into S-phase, only a very small fraction of MDAMB468 cells incorporated BrdU after shifting to Met-Hcy+ medium (Fig. 2D).

Induction of G<sub>1</sub> cell cycle arrest by methionine depletion or SAM limitation is analogous to the G<sub>1</sub> arrest induced by the SAMcheckpoint in yeast cells.<sup>19</sup> By comparison, previous reports using cells from human prostate cancer cell lines and Yoshida sarcoma



**Figure 2.** Methionine stress causes cell cycle arrest of MDAMB468 but not resistant cells. (**A**) MDAMB468 and the R8 clone of methionine stressresistant cells were assessed for their proliferative potential in both Met+Hcy- and Met-Hcy+ growth media using the SRB assay. (**B**) MDAMB468 and R8 cells were assessed for their ability to proliferate either without cysteine (Met+Hcy+Cys-), without homocysteine and cysteine (Met+Hcy-Cys-) or without methionine and cysteine (Met-Hcy+Cys-). Proliferation was measured using the SRB assay. (**C**) MDAMB468 and R8 cells were grown in Met-Hcy+ media, labeled with propidium iodide and DNA content was analyzed by flow cytometry at the times indicated. (**D**) MDAMB468 and R8 cells were grown in Met-Hcy+ media for 3 d and labeled with BrdU for 24 h. (**E**) MDAMB468 cells were grown in Met+Hcy- media and pulsed with BrdU for 1 h then immediately shifted to Met-Hcy+ conditions. DNA content and cell cycle distribution of BrdU-labeled cells was measured by flow cytometry.

cells from tumors grown in nude mice showed cell cycle arrests in all phases, with significant increases in the  $G_2/M$  population.<sup>10,13</sup> To further analyze the cell cycle defect, we used a BrdU pulselabeling protocol (**Fig. 2E**). We added BrdU to cells growing in Met+ medium for 1 h to label a population of S-phase cells so we could follow a synchronized population of cells moving through the cell cycle. Cells were then shifted to Met-Hcy+ medium without BrdU and analyzed by flow cytometry (**Fig. 2E**). At no point after the switch to methionine-free medium did the BrdU-labeled cell population re-enter S-phase, supporting a robust arrest at the  $G_1$  to S-phase transition. In addition, a significant population of cells did delay passage through  $G_2$  or M phase. Taken together, flow cytometric analysis indicated that MDAMB468 cells initiate a  $G_1$  arrest and delayed passage through  $G_2/M$  in response to methionine stress. Intriguingly, this is reminiscent of the SAM-checkpoint in budding yeast, which induces a robust  $G_1$  arrest and a significant delay of M-phase.<sup>19</sup>

Methionine stress does not inhibit Akt or mTORC1 growth factor signaling pathways. Methionine is an important nutrient, and nutrient limitation often leads to downregulation of mTORC1 signaling and induction of cell cycle arrest through repression of  $G_1$  cyclin production and induction of cyclindependent kinase inhibitors.<sup>23,24</sup> Environmental cues that influence mTORC1 activity are processed through plasma membrane receptor tyrosine kinases that transduce signals to secondary messengers such as the Ser/Thr protein kinase Akt. To test for possible methionine stress-induced changes in growth factor response pathway signaling upstream from mTORC1, we monitored the phosphorylation status of Akt at Ser<sup>473</sup>. Immunoblot analysis of both MDAMB468 and R8 cells revealed no noticeable change in the phosphorylation status of Akt<sup>Ser473</sup> during methionine stress, nor was there any change in total Akt protein levels in either cell line (Fig. 3A).

We next asked whether mTORC1 activity was affected by methionine stress. MDAMB468 and R8 cells were shifted to Met-Hcy+ medium and mTORC1 activity was monitored by analyzing phosphorylation of the mTORC1 substrate S6K (p70) (Fig. 3A).<sup>24</sup> S6K phosphorylation was not inhibited during methionine stress. Surprisingly, S6K phosphorylation increased starting from 3 d of methionine stress in MDAMB468 as compared with R8 cells, suggesting compensatory hyperactivation of mTORC1 (Fig. 3A). These results argue against a role for the mTORC1 pathway in methionine stress-induced cell cycle arrest. Consistent with these findings, phosphorylation of another mTORC1 substrate, 4EBP, was also not inhibited during methionine stress (Fig. 3B).

Methionine stress does not affect levels of G<sub>1</sub> cell cycle regulatory proteins. MDAMB468 cells arrested primarily in  $G_1$  in response to methionine stress (Fig. 2C-E). In normal cells, the G<sub>1</sub>-cyclin/Cdk complexes control G<sub>1</sub>/S transition in large part through phosphorylation and inhibition of the retinoblastoma protein Rb, which prevents S-phase.<sup>25</sup> MDAMB468 cells lack the Rb protein,26 which we confirmed by immunoblotting. To evaluate the status of the major G<sub>1</sub>/S transition regulators, we cultured MDAMB468 and R8 cells in Met+ medium, shifted them to Met-Hcy+ medium and analyzed samples from various time points as indicated by immunoblot analysis (Fig. 3C). No changes in the levels of either cyclin D or E nor their binding partners, Cdk4 or Cdk2, were observed. Analysis of the Cdk inhibitors (CKI) p21<sup>CIP1</sup> and p27<sup>Kip1</sup> showed an increase in their protein levels beginning after 72 h of methionine stress, which was long after MDAMB468 cells arrested in G<sub>1</sub>, indicating that the G<sub>1</sub> cell cycle arrest was independent of the increased levels of p21<sup>CIP1</sup> and p27<sup>Kip1</sup> (Fig. 3C). The increase in CKI levels coincided with the timing of apoptosis induction rather than cell cycle arrest (manuscript in preparation). The notion that methionine stress induced expression of the CKIs p21<sup>CIP1</sup> and p27<sup>Kip1</sup> in MDAMB468 is not directly related to cell cycle arrest is further supported by the observation that MCF7 cells did not show any increase in p21<sup>CIP1</sup> or p27<sup>Kip1</sup> levels during methionine stress, yet they showed a robust G<sub>1</sub>/S arrest (Fig. 1B and data not shown).

Loss of Cdc6 and Mcm proteins from chromatin during methionine stress in MDAMB468 cells. Analyses of cell cycle regulators showed no significant changes that correlated with the onset of methionine stress-induced G<sub>1</sub> arrest (Fig. 3C). In budding yeast, methionine stress triggers the SAM-checkpoint and destabilizes pre-replication complexes as measured by loss of Mcm proteins at replication origins.<sup>19</sup> Therefore, we explored the



**Figure 3.** Growth factor pathway and G<sub>1</sub> cell cycle regulatory protein levels in methionine-stressed MDAMB468 and R8 cells. (**A–C**) Extracts prepared from MDAMB468 and R8 cells grown in Met+Hcy- media then shifted to Met-Hcy+ media for the time period indicated were analyzed for potential changes in the levels of phospho-p70 S6 kinase (Thr389), phospho-Akt (Ser473), total p70 S6 kinase and total Akt (**A**), or 4EBP1, phospho-4EBP1 and tubulin (**B**), or cyclin D, cyclin E, p21, p27, CDK2 and CDK4 (**C**).

possibility that pre-replication complexes are similarly destabilized in methionine-stressed MDAMB468 cells. We found that levels of the three Mcm proteins investigated, Mcm2, Mcm3 and Mcm7, decreased in the chromatin-bound but not in the cytosolic fractions or total-cell extracts compared with tubulin and Orc3 controls during methionine stress conditions (Fig. 4A). Reduced Mcm levels exclusively in the chromatin-bound fraction suggest either increased disassembly or reduced assembly of origins as a result of methionine stress.

Cdc6 is an important loading factor for Mcm proteins during pre-replication complex assembly.<sup>27</sup> Interestingly, Cdc6 levels decreased in the chromatin fractions over the same time frame, but unlike the Mcm proteins, Cdc6 levels also decreased in totalcell extracts (Fig. 4A). Closer analysis revealed that Cdc6 loss occurred starting just 6 h after the switch to methionine stress growth conditions (Fig. 4B). By comparison, no such loss was observed in R8 cells (Fig. 4B), suggesting loss of Cdc6 as a primary reason for the inability to form pre-replication complexes and cause cell cycle arrest.

Cdc6 is an unstable protein with low intracellular concentrations during early  $G_1$ , only increasing as cells approach S-phase.<sup>28</sup> Therefore, it was possible that the observed methionine stressinduced cell cycle arrest occurred at a point during  $G_1$  when Cdc6 was not efficiently expressed. To determine whether Cdc6 loss is coincident with cell cycle arrest or is instead a direct result of the methionine stress, we synchronized both MDAMB468 and R8 cells in early S-phase with the DNA polymerase inhibitor aphidicolin before shifting cells to Met-Hcy+ conditions (**Fig. 4C**). Cells were kept in aphidicolin during the entire time course to exclude cell cycle position effects. As expected, Cdc6 levels increased as cells were synchronized in S-phase and remained high in cells cultured in Met+ medium. Importantly, Cdc6 levels dropped



**Figure 4.** Methionine stress downregulates Cdc6 in cancer cells. (**A**) MDAMB468 cells were grown in Met-Hcy+ for the time period indicated and separated into fractions containing either whole-cell (WCE), cytosolic or chromatin-bound extracts as described in the methods. Efficient fractionation was determined by comparing the separation of tubulin, for cytosolic, from Orc3, for chromatin, in the preparations. The levels of pre-replication complex proteins Mcm2, 3, 7 and Cdc6 in each fraction were determined by immunoblot. Protein abundance was determined using a Fuji LAS-4000 imaging system and is plotted normalized to Orc3 levels (**B**) Cdc6 and Orc3 protein levels were analyzed by immunoblot in extracts from MDAMB468 and R8 cells incubated with Met-Hcy+ media for the time period indicated. (**C**) MDAMB468 and R8 cells were synchronized in S-phase by the addition of 5 µg/mL aphidicolin for 16 h, and protein levels of Cdc6 and tubulin were determined by immunoblot using extracts prepared from cells incubated in either Met+Hcy- or Met-Hcy+ for the times indicated. Both media conditions were supplemented with aphidicolin to maintain S-phase synchronization. Cell cycle arrest in early S-phase was confirmed by flow cytometry (data not shown). (**D**) Cdc6 levels for cell lines incubated under methionine stress conditions for the time period indicated were determined by immunoblot. Protein levels for all panels were determined on a Fuji LAS-4000 imaging system.

significantly when MDAMB468 cells were shifted to Met-Hcy+ medium (Fig. 4C). By contrast, levels of Cdc6 remained high in R8 cells grown in either methionine-rich or methionine stress conditions. The rate at which the Met-Hcy+-grown R8 cells' Cdc6 levels increased was slower than that of R8 cells grown in Met+ conditions, indicating that R8 cells have not completely lost their response to methionine stress. Nevertheless, Cdc6 levels remained well above starting levels (time point 0 in Fig. 4C). These results exclude a cell cycle position effect as the cause of Cdc6 loss in MDAMB468 cells in response to methionine stress and suggest Cdc6 downregulation as a physiological response to methionine stress.

Given that methionine-sensitive MDAMB468 cells, but not MDAMB468-derived R8 cells, showed a loss in total levels of Cdc6 protein following methionine stress, we wanted to determine if Cdc6 loss is a common response of cancer cells to methionine stress. We prepared lysates from the breast cancer cell lines MCF7 and MDAMB361, and prostate cancer cell lines DU145 and PC-3, grown in Met-Hcy+ medium, and then analyzed Cdc6 levels. Like MDAMB468 cells, levels of the Cdc6 protein fell markedly in each cell line tested (**Fig. 4D**), indicating that Cdc6 levels are directly responsive to methionine stress and may play an important role in triggering the methionine dependence of cancer cells. Interestingly, DU145 cells, which are only moderately sensitive to methionine stress,<sup>10,29</sup> showed a relatively small Cdc6 decrease.

Loss of activating phosphoThr160 on Cdk2 in methioninesensitive cancer cells. Recent studies show that Cdc6 plays a role in activating Cdk2/cyclinE complexes. Specifically, chromatinassociated Cdc6 facilitates the activating phosphorylation at Thr160 of Cdk2 and so establishes a tight link between functional pre-replication complexes and Cdk2 activity.<sup>30</sup> We have shown loss of Cdc6 levels and pre-replication complex destabilization during methionine stress in cancer cells (Fig. 4) and, therefore, hypothesized that the robust block in S-phase entry observed in breast cancer cells during methionine stress is reinforced by a corresponding loss of phosphorylation at Thr160 of Cdk2. We probed lysates from MDAMB468 and R8 cells incubated in Met-Hcy+ medium using a phospho-specific antibody that recognizes phosphoThr160 on Cdk2. Phosphorylation of Thr160 on Cdk2 was significantly reduced in MDAMB468 cells during methionine stress (Fig. 5A). Importantly, there was very little change in the amount of phosphoThr160 on Cdk2 from R8 cells (Fig. 5A). In accordance with a loss in the activating phosphorylation on Thr160 of Cdk2 in MDAMB468 cells, we found a substantial decline in Cdk2 activity following methionine stress (Fig. 5B). By contrast, methionine-stressed R8 cells showed an initial drop in kinase activity similar to that seen for MDAMB468 cells, but kinase activity did not drop any further after the initial loss, indicating that Cdk2 remains active in Met-Hcy+ medium. Methionine stress had no or very little effect on total Cdk2 levels in breast cancer cells or the R8 derivatives (Fig. 5C).

Reduced phosphorylation of Thr160 on Cdk2 appears to be a general response of breast cancer cells to methionine stress, for the breast cancer cell lines MCF7 and MDAMB361 also showed a substantial drop in the amount of phosphoThr160 on Cdk2 when shifted to Met-Hcy+ medium (**Fig. 5D**). The loss of activating Cdk2 phosphorylation in MCF7, MDAMB361 and MDAMB468 cells coincides with the loss of Cdc6 in each cell line and suggests that methionine stress blocks S-phase initiation by destabilization of pre-replication complexes and, consequently, loss of activating phosphorylation of Thr160 on Cdk2.

#### Discussion

In an update to their seminal paper enumerating the six shared hallmarks of all cancer cells, Hanahan and Weinberg now list "reprogramming energy metabolism" as an emerging hallmark important to the fundamental

understanding of cancer.<sup>2</sup> Indeed, others have also noted that altered metabolism accompanies transformation and have proposed exploiting those peculiarities for therapeutic development. Cancer cell methionine dependency fits within this paradigm. The fact that cancer cells possess the ability to metabolize seemingly adequate amounts of methionine during methionine stress conditions (Met-Hcy+) suggests that cancer cell methionine dependency is the result of a deficiency in a metabolite related to methionine rather than methionine itself.<sup>9</sup> Our data demonstrate that the methionine-derived and ubiquitous methyldonor S-adenosylmethionine (SAM) is likely the key metabolite



Figure 5. Cdk2 activation is inhibited by methionine stress. (A) Lysates were prepared from MDAMB468 and R8 cells incubated in Met-Hcy+ media for the time period indicated, and levels of Cdk2 and phospho-Thr160 on Cdk2 were measured via immunoblot. Protein levels were determined using a Fuji LAS-400 imaging system. Quantification represents the mean of three experiments +/- standard error. (B) Cdk2 was immunopurified from MDAMB468 and R8 cells incubated in Met-Hcy+ conditions for the time period indicated, and kinase activity was measured in an in vitro kinase activity assay using histone H1 as substrate. Kinase activity was measured by phosphoimaging. (C) Total Cdk2 levels in MDAMB468, R8, MCF7 and MDAMB361 cells were compared between cells grown in methionine medium ("+") and after cells were cultured in Met-Hcy+ media for 3 d ("-"). Protein concentrations of duplicate samples were determined using a Fuji LAS-400 imaging system and the average of the two samples was plotted. (D) Cdk2 and phospho-Thr160-Cdk2 levels were determined via immunoblot for extracts taken from MCF7 and MDAMB361 breast cancer cells incubated in Met-Hcy+ media for the time period indicated. Protein levels were determined using a Fuji LAS-400 imaging system.

triggering breast cancer cell methionine dependency (Fig. 1D). Because a cell cycle checkpoint monitoring SAM concentrations has been described in yeast,<sup>18,19</sup> it is tempting to speculate that a similar SAM-checkpoint blocks proliferation of cancer cells.

In yeast, the SAM checkpoint-induced cell cycle arrest correlates with inefficient assembly of the replication factors Mcm4, Mcm7 and Cdc45 at replication origins. In striking agreement with the previous findings in the yeast model, our data show that methionine stress leads to destabilization of pre-replication complexes in MDAMB468 cells, as evidenced by the loss of replication factors Mcm2, Mcm3, Mcm7 and Cdc6 within the chromatin-bound cellular fractions (Fig. 4A). Downregulation of the Mcm loading factor Cdc6 was observed in several breast cancer cell lines as well as prostate cancer cells (Fig. 4), suggesting that Cdc6 and destabilization of pre-replication complexes are targets of methionine stress in cancer cells. Collectively, these results suggest that SAM checkpoint activation is a conserved mechanism from yeast to humans and provides new insight as to the mechanism of cancer cell cycle arrest caused by methionine stress.

The reason why Cdc6 levels dropped in response to methionine stress is yet to be determined. Reduced Cdc6 levels in direct response to methionine stress could be due to a number of different contributing factors, including targeted destruction of the Cdc6 protein as a result of increased proteasome-mediated turnover, downregulation of Cdc6 mRNA expression or downregulation of Cdc6 mRNA polypeptide synthesis at the level of translation. Future studies will have to address the specific mechanism of Cdc6 loss.

It has only recently become appreciated that Cdc6 is necessary for the activating T-loop phosphorylation at threonine160 of Cdk2.<sup>30,31</sup> The methionine stress-induced drop in Cdc6 predicted a corresponding loss in phosphorylation of Thr160 on Cdk2, resulting in lowered kinase activity, which we observed. Our data confirms Cdk2 kinase inactivation during methionine stress in MDAMB468 cells, and suggests that loss of Cdk2 activity might contribute to the observed G1/S cell cycle arrest. Our study does not definitively determine whether or not Cdk2 inactivation alone is sufficient for cell cycle arrest, but sustained cell proliferation in Cdk2-knockout mice suggests that additional effectors need to be regulated to achieve a robust G<sub>1</sub> arrest as observed in response to methionine stress.<sup>32</sup> It is most probable that G<sub>1</sub>/S cell cycle arrest during methionine stress is due to a combination of factors, including destabilization of pre-replication complexes, inactivation of Cdk2 and, perhaps, other as-yetunidentified mechanisms of stress-induced growth inhibition.

We used spontaneous mutants derived from MDAMB468 cells that were resistant to methionine stress to compare molecular differences in the response to methionine stress. R8 cells do not show any of the cell cycle defects displayed by the methionine stress-sensitive cancer cells and are able to maintain their proliferation potential, Cdc6 levels, assemble pre-replication complexes and activate Cdk2. These methionine stress-resistant cells are valuable tools for further studies of methionine dependency of cancer cells, because their proliferation rate is identical to that of the methionine-dependent parental cancer cells. Interpretation of cancer-specific metabolic dependencies are frequently complicated by the slow proliferation rate of non-cancer cells, which can significantly affect metabolic needs. Methionine stress-resistant cell lines developed in this study eliminate these difficulties, and altered proliferation rates can be excluded as the cause for the reported molecular responses to methionine stress. However, the molecular basis for reversion to methionine independence is unknown. Generating resistant colonies is a rare event, and it is unclear whether genetic or epigenetic changes are responsible. It is interesting to note that SAM is the critical co-factor for chromatin methylation, and it is possible that resistance to

the methionine-dependent phenotype is caused by epigenetic changes.

In summary, our study provides insight into the molecular responses of breast cancer cells following methionine stress and suggests pre-replication complexes as a likely molecular target for induction of a checkpoint-like cell cycle arrest at the G<sub>1</sub>/S transition. Control of pre-replication complex stability has previously been suggested as a critical component in regulation of S-phase initiation during SAM-limitation in budding yeast<sup>19</sup> and in response to UV-irradiation in fission yeast.<sup>33</sup> Pre-replication complex assembly might thus represent an underappreciated cell cycle checkpoint target that could be particularly important to link cell proliferation with cellular metabolites. Our results suggest that SAM is the critical mediator of cancer cell methionine dependency, and it is tempting to speculate that the pathway we describe represents a cell cycle checkpoint that ensures epigenetic stability by preventing S-phase during limited SAM availability. Why cancer cells are hypersensitive to this checkpoint is unknown, but their greater demand for SAM may be a contributing factor. Clearly our results bolster the case for further exploration into the causes and eventual development of therapeutic strategies designed to exploit cancer cell methionine dependency.

#### **Materials and Methods**

Cell lines and growth conditions. All cells were passaged in DMEM (Sigma-Aldrich<sup>®</sup>), supplemented with 10% dialyzed FBS (Gemini Bio-Products), 1.5  $\mu$ M vitamin B12, 4 mM L-glutamine, 100  $\mu$ M l-cysteine (Fisher Scientific) and 100  $\mu$ M l-methionine (Sigma-Aldrich<sup>®</sup>). In the case of methionine-free media, 200  $\mu$ M DL-homocysteine (Sigma-Aldrich<sup>®</sup>) was added. Resistant cell lines were isolated according to the method of Hoffman et al.<sup>22</sup> Cell proliferation was measured as previously described.<sup>34</sup> Each assay was run in quadruplicate. Error bars represent +/– SD of the mean. To arrest cells in S-phase, aphidicolin (Fisher Bioreagents) was added to cells at a concentration of 5  $\mu$ g/mL. Flow cytometry was performed according to the method of reference 35.

Protein analysis. Cells were lysed in 8 M urea buffer (8 M urea, 200 mM NaCl, 100 mM Tris, pH 7.5, 0.2% SDS, 10 mM Na-pyrophosphate, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 0.1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 1  $\mu$ g/ml each of aprotenin, leupeptin and pepstatin). For immunoblot analysis, protein samples were normalized, diluted to 4 M urea using 2x SDS buffer (4% SDS, 125 mM Tris-Hcl pH 6.8, 20% glycerol, 0.002% bromophenol blue and 0.4 M DTT), separated by SDS-PAGE and then transferred to an Immobilon-P membrane (Millipore). We detected proteins using antibodies against phospho-p70 S6 kinase<sup>Thr389</sup> (9206), p70 S6 kinase (9202), CDK4 (2906), CDK2 (2546), phospho-Akt<sup>Ser473</sup> (9271), MCM2 (4007), MCM3 (4012), MCM7 (4018), 4E-BP1 (9452), phospho-4E-BP1 (9459), 4E-BP1 (9452) (Cell Signaling Technology<sup>®</sup>), cyclin E (554182 - BD Biosciences), cyclin D1 (ab16663 - Abcam), p21 (sc-397), p27 (sc-1641), Akt1 (sc-5298), Cdc6 (sc-9964), Orc3 (sc-23888) (Santa Cruz Biotechnology) and histone H3 (06-755 - Millipore), Cdk2 Phospho-Thr160

(A7039) (Assay Biotechnology Company). Signals were imaged using the Fuji LAS-4000 imaging system. Protein quantification was performed using Multi Gauge Image Analysis software.

Cellular fractionation. Chromatin was isolated from totalcell extracts according to Mendez and Stillman.<sup>36</sup> In short, cells were lysed by resuspending in a non-ionic detergent on ice for 5 min. Following centrifugation at  $1,300 \times \text{g}$  for 4 min, chromatincontaining pellets were washed once and then resuspended in a no-salt buffer for 30 min on ice. Chromatin-enriched fractions were collected following centrifugation for 4 min at  $1,700 \times \text{g}$ .

Cdk2 activity. Cdk2 complexes were immunopurified (antibody sc-163, Santa Cruz Biotechnology) and kinase activity was analyzed as described<sup>37</sup> and quantified using phosphoimaging and the Bio Rad Quantity One software.

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#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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