Mapping metabolic events in the cancer cell cycle reveals arginine catabolism in the committed SG2M phase

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Summary

Alterations in cell cycle regulation and cellular metabolism are associated with cancer transformation, and enzymes active in the committed cell cycle phase may represent vulnerabilities of cancer cells. Here, we map metabolic events in the G1 and SG2M phases by combining cell sorting with mass spectrometry-based isotope tracing, revealing hundreds of cell cycle-associated metabolites. In particular, arginine uptake and ornithine synthesis was active during SG2M in transformed but not in normal cells, with the mitochondrial arginase2 (ARG2) enzyme as a potential mechanism. While cancer cells exclusively used ARG2, normal epithelial cells synthesized ornithine via ornithine aminotransferase (OAT). Knockdown of ARG2 markedly reduced cancer cell growth and caused G2M arrest, while not inducing compensation via OAT. In human tumors, ARG2 was highly expressed in specific tumor types, including basal-like breast tumors. This study sheds light on the interplay between metabolism and cell cycle, and identifies ARG2 as a potential metabolic target.

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Data and Software availability

All primary LC-MS data will be public available via the authors’ website. mzAccess is publicly available at mzaccess.org
Keywords
isotope tracing; mass spectrometry; cancer metabolism; ornithine; polyamines; arginase 2; basal-like breast cancer

Introduction

The cell cycle is of fundamental importance in cell biology and in biomedicine, as cancer, inflammation and autoimmune disorders all involve cell proliferation. Mechanisms in the “committed” SG2M phase of the cell cycle, between onset of DNA synthesis (S) and mitosis (M), are commonly targeted by antiproliferative drugs (Chabner & Roberts 2005), which are highly effective but generally cause adverse side effects in normal proliferating tissues. On the other hand, cancer transformation is associated with altered metabolism (Hammoudi et al. 2011) and expression of metabolic enzymes not commonly found in normal cells (Mazurek et al. 2005), which might be targeted with better specificity (Vander Heiden 2011). A combination of these two approaches, seeking cancer-associated metabolic enzymes active in the committed SG2M phase of the cell cycle, might identify new antiproliferative drug targets that are both effective and specific.

Proliferating cells must synthesize a multitude of cellular components while simultaneously catabolizing nutrients to obtain energy, all while maintaining essential cell functions. How cells orchestrate these complex metabolic processes while progressing through the cell cycle is still poorly understood. In the yeast *S. cerevisiae*, respiration and redox state can undergo spontaneous cycles (Tu et al. 2005) that can be coupled to the cell cycle (Papagiannakis et al. 2017), and carbohydrate utilization occurs during the SG2M phase (Ewald et al. 2016). In mammalian cells, data obtained using synchronization methods indicate that central metabolic processes like glycolysis (Colombo et al. 2011), glycogen utilization (Favaro et al. 2012), glutamine catabolism (Ahn et al. 2017) and polyamine synthesis (Bettuzzi et al. 1999) are cyclic. However, synchronization methods are problematic in that they block cell cycle progression by inhibiting metabolic processes such as thymidine or mevalonate synthesis (Whitfield et al. 2002; Keyomarsi 1996), and hence may disturb metabolism. Moreover, most mammalian cells are difficult to synchronize (Cooper 2004), and consequently most data have been gathered from a few transformed cell lines that are amenable to synchronization, including HeLa and mouse 3T3 cells. This is problematic, since cell cycle regulation clearly differs across cell types (Kung et al. 1990), and it is of particular interest to study differences between normal and transformed cells. Clearly, systematic studies of metabolic events in the cell cycle across multiple cell types, including normal cells, are needed.

To address this problem, we here report a systematic mapping of metabolic events in the G1 and SG2M stages in transformed and normal human cells, utilizing a combination of cell sorting and mass spectrometry-based isotope tracing. This approach does not require synchronization, and is potentially applicable to any cell type. In the present study, this technique revealed that ornithine synthesis by mitochondrial arginase 2 (ARG2) is important...
Results and Discussion

Cell sorting allows metabolism measurements in pure G\textsubscript{1} and SG\textsubscript{2}M subpopulations

To enable the study of metabolic events in the cell cycle, we sought a method for separating cells into the G\textsubscript{1} and “committed” SG\textsubscript{2}M phases that is applicable to any cell type and has minimal disturbance on cellular metabolism. We chose to sort cells by DNA content into 2n and 4n fractions, corresponding to the G\textsubscript{1} and SG\textsubscript{2}M phases respectively, using the DNA-binding fluorescent molecule Hoechst-34580 (Figure 1A). This method provided robust quantification of DNA content in live cells with only 15min of incubation, and yet was nontoxic for up to 17h in most cell types (Figure S1A–G). To obtain pure populations, we excluded cells in a window between 2n and 4n that contained G\textsubscript{1}/S-transition cells, as demonstrated by Hoechst-34580 vs. geminin expression (Figure 1B). With this strategy, the markers Cdt1 for G\textsubscript{1} and cyclin A for SG\textsubscript{2}M were exclusively found in the 2n and 4n fractions respectively (Figure 1C), indicating high purity of the sorted fractions. We then analyzed 80,000 cells from the 2n and 4n fractions using liquid chromatography-mass spectrometry (LC-MS). Although cell sorting has been reported to distort cell redox state in astrocytes (Llufrio et al. 2018), and we cannot entirely exclude such effects, we found that redox couples were not altered in a concerted manner (Figure S1H–J). Also, to reduce artefacts caused by the sorting procedure, we throughout compare peak areas in a paired fashion, between fractions sorted from the same sample, which have been affected in the same way. Reassuringly, we observed that dNTPs, which are synthesized only during S phase (Bray & Brent 1972; Skoog et al. 1973), were exclusively found in the 4n fraction (Figure 1D). Hence, cell sorting coupled with LC-MS analysis can reliably detect cellular metabolites present in specific cell cycle phases.

To compare the cell sorting approach with commonly used synchronization methods, we generated LC-MS data from HeLa cells synchronized in S phase using the double thymidine block (DTB) technique (Whitfield et al. 2002), and in G\textsubscript{1} phase by lovastatin (Keyomarsi 1996). Although HeLa cells are among the easiest to synchronize, perfect synchrony is never attained, and in this case 20% of DTB cells were not in S phase, while 22% of lovastatin-treated cells were not in G\textsubscript{1} (Figure S1K). Accordingly, we detected dNTPs also in lovastatin-synchronized cells (Figure 1E). Such cross-contaminations suggest that metabolite fold changes will be underestimated from synchronized populations, while the higher purity attained by cell sorting should give more power to detect cycling metabolites. We also noted a clear increase in the DNA damage marker ADP-ribose (Berger 1985) in DTB-synchronized cells, but not in sorted SG\textsubscript{2}M cells (Figure 1F), consistent with reports that DTB can cause DNA damage (Kurose et al. 2006). In addition, ribose-5-phosphate (Figure 1G) and the pentose phosphate pathway metabolite sedoheptulose-7-phosphate (Figure 1H) were markedly elevated in DTB-synchronized, but not in sorted SG\textsubscript{2}M cells, possibly indicating a disturbance in ribose metabolism. Taken together, these data indicate that our approach reliably detects cellular metabolites present in specific cell cycle phases.
Isotope tracing identifies cell cycle-associated metabolic events

To determine activities of enzymes and pathways in the G1 and S/G2M phases, cells were pulse-labeled with a medium where glucose and all amino acids were fully $^{13}$C (Grankvist et al. 2018), followed by cell sorting as above (Figure 2A). Since metabolites in any given cell are $^{13}$C-labeled according to its metabolic activities during the $^{13}$C pulse, this design reveals cell cycle-associated metabolic events as they occurred in the undisturbed culture, prior to cell sorting, and also reduces the impact of disturbances from the sorting procedure (Roci et al. 2016). To minimize cases where cells are in G1 phase during $^{13}$C-labeling but transition to S phase before sorting, we used a short (3 hour) pulse in combination with the gating scheme described above (Figure 2A and Figure 1B). We performed such isotope tracing experiments in normal human mammary epithelial cells (HMECs), H-rasV12 transformed HMECs (HMEC Ras) (Elenbaas et al. 2001) and HeLa cells. Typically, several hundred putative metabolites exhibited $^{13}$C labeling (Table S1). For example, in HeLa cells, 921 high-quality LC-MS peaks were annotated with a putative metabolite identity (see Methods), and of these, 546 (59%) had detectable $^{13}$C enrichment, indicating that they were synthesized by cells within the 3 hour $^{13}$C pulse (Figure S2A–B). A total of 179 of these peaks (33%) reproducibly differed between G1 and SG2M cells by at least 5% in one or more mass isotopomer (MI) fractions (Figure 2B); a complete list is provided in Table S1. For example, metabolites of the pentose phosphate pathway (PPP), including 6-phosphogluconate and sedoheptulose-7-phosphate, were preferentially labeled in G1 (Figure 2C), suggesting increased activity of both the oxidative and non-oxidative PPP branches in this phase. These differences between G1 and SG2M were also seen when data was expressed as MI fractions, indicating that they are not due to changes in metabolite pool sizes (Figure S2C–F). These data may reflect PPP activity at the G1/S transition (Vizán et al. 2009), since our gating scheme excludes early S phase cells from the SG2M fraction. As a whole, these data indicate that a variety of metabolic differences exist between the SG2M and G1 phases in human cells. Beyond detecting synthesis of metabolites, mass isotopomers provided detailed information on the coordination of metabolic processes with the cell cycle. For example, UDP exhibited $^{13}$C5, $^{13}$C7 and $^{13}$C8 MIs typical of pyrimidine de novo synthesis in both G1 and SG2M cells (Figure 2D), indicating that de novo synthesis occurs throughout the cell cycle, while $^{13}$C dTMP was mainly formed in SG2M cells, as expected (Figure 2E). Moreover, most dTMP formed in SG2M was $^{13}$C-labeled on the methyl group within 3 hours (indicated by a +1 shift of dTMP MIs compared to UDP), showing that both the dTMP pool and the upstream folate-bound one-carbon pool turns over rapidly in SG2M. Similarly, S-adenosylmethionine (SAM) was mostly $^{13}$C after 3 hours, but most of this pool was $^{13}$C5, indicating that only the methionine group was labeled (Figure 2F), which shows that the SAM cycle turnover is much faster than de novo purine synthesis. In contrast to dTMP, formation of $^{13}$C5 SAM appears to be constant across the cell cycle phases. Hence, the folate- and SAM-driven methylation systems are differently coordinated with the cell cycle.

Arginine metabolism is active during the SG2M phase in transformed cells

Among the hundreds of cell cycle-associated metabolites, we noted a group consisting of arginine (Figure 3A) and several of its downstream metabolites (Figure 3D–G), which acquired $^{13}$C label preferentially in the SG2M phase. Since arginine is essential for most
cultured cells, increased $^{13}$C$_6$ arginine in SG$_2$M likely reflects increased uptake, which commonly occurs via the SLC7A5 transporter that also carries lysine (Schnorr et al. 2003). Consistent with this mechanism, we also observed higher $^{13}$C$_6$ lysine in the SG$_2$M phase (Figure 3B). Importantly, $^{13}$C$_6$ arginine was low in non-transformed HMEC cells and did not increase in SG$_2$M in this cell type (Figure 3A, Figure S3A–B). Although HMEC cells were cultured in a mammary epithelial cell medium (MCDB-170) with lower arginine concentration, transformed HMEC-Ras cells grown in MCDB-170 also showed high $^{13}$C$_6$ arginine abundance, which again increased in SG$_2$M (Figure 3A). This indicates that arginine uptake increases in the committed SG$_2$M phase, and is associated with transformation in the HMEC model.

Besides being required for protein synthesis, arginine has several metabolic fates (Figure 3C), including nitric oxide (NO), creatine and polyamines (Morris 2007). To identify reactions that might be important in the SG$_2$M phase specifically for transformed cells, we compared $^{13}$C labeling of arginine metabolites between HeLa, HMEC and HMEC-Ras cells. We did not observe $^{13}$C$_6$ citrulline, indicating that NO synthase activity is minor (data not shown). $^{13}$C$_4$ creatine was observed in HeLa cells (Figure 3D) but not in other cell types (data not shown), and was therefore not considered further. Polyamines were not measurable on our LC-MS system, but $^{13}$C$_4$ acetyl-putrescine, a breakdown product of polyamines, was present and increased in the SG$_2$M phase of all cell types (Figure 3E), consistent with synthesis and turnover of polyamines in SG$_2$M in both transformed and non-transformed cells. In contrast, we observed increased $^{13}$C$_5$ ornithine in SG$_2$M in HeLa and HMEC Ras cells, but not in HMEC cells (Figure 3F), and these differences were also evident when viewed as MI fractions, indicating that they are not merely due to changes in metabolite pool size (Figure S3B). Because ornithine is the only known substrate for polyamine synthesis, which is required for cell proliferation (Landau et al. 2012), and since it was formed from arginine specifically in transformed cells, we chose to focus on this step of arginine metabolism.

To determine more accurately the cell cycle phase where arginine uptake and ornithine synthesis occurs, we analyzed cells harboring fluorophore-tagged endogenous Cyclin A2, whose expression allows separating cells into G$_1$, S and G$_2$M phases (Cascales et al. 2017). Both arginine and ornithine were highest in G$_2$M phase cells (Figure 3G), suggesting that uptake and catabolism of arginine occurs in this phase. These findings agree with previous reports that ornithine and putrescine are important for progression through the S and G$_2$ phases (Aneshus et al. 1984). In addition, arginase activity in cell lysates as well as cellular urea content were increased in SG$_2$M phase cells obtained by DTB synchronization (Figure S3C–F).

Given that ornithine is the only known source of polyamines in human cells (Coleman et al. 2004), it was puzzling that ornithine did not acquire $^{13}$C label in non-transformed HMEC cells. We hypothesized that HMEC cells might synthesize ornithine through a slower reaction, which was not detected during the 3-hour $^{13}$C pulse used in our sorting experiments; possibly from glutamine via ornithine aminotransferase (OAT) (Figure 3C). To address this possibility, we performed separate tracing experiments with $^{13}$C$_5$-glutamine and $^{13}$C$_6$-arginine tracers for 48 hours in unsynchronized cultures of several cell types. In these
conditions, both HMEC cells and non-transformed BJ fibroblasts exhibited ornithine (Figure 3H) as well as downstream acetyl-putrescine (Figure 3I) labeling from $^{13}$C$_5$-glutamine, indicating that OAT is active in these cell types. At this time point, we also detected $^{13}$C ornithine and acetyl-putrescine from $^{13}$C$_6$-arginine in all cell types tested (Figures 3HI). This indicates that some arginase activity is present in all cell types, but the enzyme is most active in SG$_{2M}$ phase in HeLa and HMEC-Ras cells, as measured by $^{13}$C pulse labeling (Figure 3H). Importantly, we did not observe any ornithine or acetyl-putrescine labeling from $^{13}$C$_5$-glutamine in the tumor-derived HeLa and MDA-MB-231 cell lines (Figure 3HI, Figure S3G), indicating that there is no ornithine synthesis from glutamine via OAT in these cancer cells. Therefore, HeLa and MDA-MB-231 should be dependent on arginase in the SG$_{2M}$ phase.

**Arginase 2 is required during the SG$_{2M}$ phase**

To identify specific enzymes or transporters that might be responsible for the observed metabolic events, we integrated our isotope tracing data with data on cell cycle gene expression profiles (Kagawa et al. 2013; Grant et al. 2013; Sadasivam et al. 2012; Peña-Díaz et al. 2013) and RNAi screens for cell cycle phenotypes (Kittler et al. 2007; Björklund et al. 2006; Mukherji et al. 2006), using the Recon 2.2 human metabolic network model (Swainston et al. 2016) to map metabolites to enzymes. This analysis revealed 202 enzyme-metabolite pairs where the enzyme exhibited cyclic expression and/or RNAi phenotypes, and the related metabolite differed by cell cycle phase in our tracing experiments (Table S2), suggesting cell cycle-regulated metabolic reactions. In particular, we found that both the arginine/lysine transporter SLC7A2 and the mitochondrial arginase 2 (ARG2) showed RNAi cell cycle phenotypes, suggesting that they may underlie arginine metabolism in SG$_{2M}$.

Also, ARG2 was clearly present in the cell lines studied (Figure 4A), and ARG2 protein as well as enzymatic activity was increased by oncogene transformation in both HMEC cells and fibroblasts (Figures 4A–B). In contrast, ARG1 is known to be expressed mainly in liver (Uhlen et al. 2010). We therefore focused on ARG2 as a likely candidate for the high arginase activity in SG$_{2M}$.

Since ornithine synthesis from arginine was consistently found in the committed SG$_{2M}$ phase in transformed cells, we reasoned that suppressing ARG2 might prevent cancer cell proliferation. Transient siRNA knockdown of ARG2 (siARG2) reduced ARG2 protein by >95% in HeLa, MDA-MB-231 and MDA-MB-468 cells at 70h (Figure S4A–C), and also reduced $^{13}$C$_5$ ornithine, indicating that the ARG2 reaction was suppressed (Figure 4C). At 70 hours, after ~1 day of ARG2 suppression, cell number was consistently reduced by up to 50%, indicating that the ARG2 protein is required for proliferation in multiple transformed cell types (Figures 4D–G). Since the duration of ARG2 suppression was roughly equal to the doubling time of these cells, a 50% reduction in cell number corresponds to nearly complete growth suppression. Moreover, the decrease in cell number was correlated with the degree of ARG2 knockdown (Figures 4D–G, S4A-C). Interestingly, siARG2 cell cultures had fewer G$_1$/G$_0$ cells, and approximately 30% more cells at the G$_2$/M stage (Figure 4H, Figure S4D), consistent with ornithine synthesis occurring mainly in G$_2$ (Figure 3F). Moreover, an analysis of the transcriptional response to ARG2 knockdown based on the Connectivity Map dataset (Subramanian et al. 2017) revealed Cyclin B2-dependent mechanisms as the top...
differentially expressed gene set (Figure 4I). This data suggests that ARG2 inhibition does not render cells quiescent, a source of resistance to many antiproliferative drugs (Jedema et al. 2003), but rather interferes with G2/M phase progression. In contrast, arginine deprivation arrested cells in G1/G0 (Figure 4J), consistent with reports that arginine is sensed by mTOR-dependent mechanisms that arrest cells in this phase (Brown et al. 2015). Hence, ARG2 inhibition causes a phenotype distinct from arginine deprivation. To investigate whether cancer cells can compensate for loss of ARG2 by activating the OAT reaction, we next performed tracing with U-13C-glutamine in siARG2 cells. Neither ornithine nor acetylputrescine acquired label from 13C5-glutamine in ARG2 knockdown HeLa cells even at 70 hours (Figure 4K), indicating that these cells are unable to compensate for loss of ARG2 activity using OAT. Moreover, siARG2 cells were depleted of 13C2 (acetyl-labeled) acetylputrescine (Figure 4L), suggesting defective polyamine synthesis downstream of ARG2. These results suggest that ornithine synthesis by ARG2 in the committed phase of the cell cycle might be a vulnerability of cancer cells (Figure 4M).

To assess the role of ARG2 in human cancer, we analyzed its expression pattern in over 2,000 human breast tumors from multiple patient studies (see Methods). We found ARG2 to be highly expressed (Figure 4N, Figure S4E) and associated with poor patient survival (Figure S4F) in tumors with low estrogen receptor α (ER-α), but not in breast tumors overall. Hence, ARG2 is likely not a typical proliferation-associated gene that is broadly overexpressed in a wide variety of cancers (Selfors et al. 2017), but may be important in particular cell types. Accordingly, a recent study showed that in clear-cell renal carcinomas, ARG2 expression is lower than in normal kidney, and in this context over-expression of ARG2 decreased cancer cell proliferation (Ochocki et al. 2018). Hence, it is instrumental to identify specific cancer types that depend on ARG2 for growth. Towards this goal, we stratified breast tumors into normal-like, basal-like, Her2-positive, Luminal A and Luminal B subtypes using the PAM50 method (Parker et al. 2009). We observed high ARG2 expression specifically in basal-like tumors, which are considered aggressive and difficult to treat with existing drugs (Rakha et al. 2008), and occasionally in Her2-positive tumors (Figure 4O, Figure S4G). This was likely not due to infiltration of fibroblasts or macrophages, since we found no correlation between ARG2 expression and expression of markers for these cell types in the tumor samples analyzed (Table S3). In a meta-analysis (Figure 4P), high ARG2 expression was also correlated with poor survival of patients specifically within basal-like breast cancer. Interestingly, the breast cancer cell lines for which we observed decreased proliferation upon ARG2 knockdown (Figure 4FG) were derived from basal-like tumors as well. These results suggest that ARG2 should be considered a potential target for specific tumor types.

Conclusion

In this paper, we have presented a large-scale investigation of metabolic events in the “committed” G2/M phase of the cell cycle, using a combination of cell sorting with mass spectrometry-based isotope tracing. A number of metabolites and enzymes were associated with cell cycle phase, and should be of great interest for future studies. In particular, we find that the mitochondrial ARG2 is active in the G2/M phase of cancer cells, but not in normal epithelial cells. Importantly, ARG2 appears to be the only source of ornithine in transformed
cells, while normal cells can obtain ornithine via the OAT enzyme. This suggests that targeting ARG2 may disrupt polyamine synthesis specifically in transformed cells, which would be a major improvement on current strategies for targeting polyamine synthesis. Our results indicate that ARG2 suppression indeed reduces cell growth, without compensatory induction of OAT. However, it is important to note that ARG2 is expressed only in specific tumor types, including basal-like breast tumors, and additional studies are needed to investigate how specific tumor or cell types would respond to ARG2 inhibition.

Contact for Reagent and Resource Sharing

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Roland Nilsson (roland.nilsson@ki.se).

Experimental Model and Subject Details

Cell culture

The cell lines used were cultured at 37°C in the media specified below, with 1% Penicillin/Streptomycin added in all cases. The list of cell lines and culture media are: HeLa, cultured in RPMI-1640 (Gibco) + 5% heat-inactivated FBS (Gibco); Human Mammary Epithelial Cells, hTERT immortalized (HMEC), cultured in MCDB-170 (USBiological) + 5% mammary epithelium growth supplement (MEGS) (Invitrogen); Human Mammary Epithelial Cells, ras-transformed (HMEC-ras), MCDB-170 + 5% MEGS; MDA-MB-231, RPMI-1640 + 10% FBS; MDA-MB-468, RPMI-1640 + 10% FBS; Retinal Pigment Epithelium, hTERT immortalized (RPE), DMEM + 5%.

Method Details

Hoechst staining

Before sorting into G1 and SG2M, cells were cultured for 15 minutes in medium containing 10μL Hoechst-34580 and 20μL Verapamil. Cells were then rinsed with Hank’s balanced salt solution (HBSS) and detached with warm trypsin, blocked with FBS for HeLa cells or trypsin neutralizing solution for HMEC and HMEC-ras cells, and centrifuged at 300g for 3 min. The supernatant was aspirated and the cell pellet was resuspended in HBSS + 5% dialyzed FBS + 1mM EDTA at a cell concentration of 1–2 × 106 per mL.

Fluorescence Activated Cell Sorting

Cells in suspension were filtered using 40um strainers to avoid clumps of cells. Cells were sorted using a BD Influx (inFlux v7 Sorter) with a 100um nozzle at ~1000 events/sec. Hoechst-34580 staining was detected with a 405nm laser and 460/50nm filter. Cells were sorted based on the Hoechst-34580 signal into G1 and SG2M, applying the gating for 2n vs 4n shown in Figure 1B. 500,000 cells were sorted into 5mL tubes containing 2mL HBSS + 5% dialyzed FBS + 1mM EDTA and used for metabolite extraction.
Metabolite extraction

After sorting, cells were centrifuged at 300g for 3min, the supernatant was aspirated, and the cell pellet was resuspended in 10μL dH₂O. Immediately, 500μL of −80°C methanol (kept on dry ice) was added for metabolite extraction.

Metabolite extraction of cells from the culture dish

Culture medium was aspirated, cells were rinsed with PBS, and −80°C methanol was added to the culture dish for metabolite extraction. Then cells were scraped with a plastic cell scaper, and the resulting extracts were carefully transferred to a new tube.

Isotope tracing

For isotope tracing experiments, culture media (RPMI-1640, MCDB-170) were prepared according to the concentrations specified in the corresponding commercial medium. Nutrients (as indicated in the text) were replaced with ¹³C labeled counterparts, while the rest of the components remained the same. Dialyzed FBS for isotope tracing experiments was prepared by dialysis overnight in Snakeskin dialysis tubing against 0.15M NaCl, as described by the manufacturer. Cells were cultured for 3h or 48h, as indicated in the text. For the 3h tracing experiments, cells were pre-cultured for 45h, then medium was replaced with labeled medium containing the tracer of interest, and further experiments (flow cytometry or metabolite extraction) were performed at the end of this incubation time.

Transient knockdown

For knockdown experiments, 70 000 cells were seeded in a 6-well plate. At day one, the medium was replaced and the transfection mixture was added dropwise into the culture dish. The transfection mixture contained 200μL Optimem, a volume of 2mM siRNA solution and 4μL Interferin per well. The volume of siRNA stock added was calculated to achieve the final concentration of siRNA indicated in the text. The experiment was ended after 70h.

For arginine or glutamine tracing experiments in ARG2 knockdown cells, 70 000 cells were seeded in a 6-well plate. At day one, unlabeled medium was replaced and the transfection mixture was added dropwise into the culture dish as described above. At day two, the media was replaced with U-¹³C-arginine or U-¹³C-glutamine containing medium, and the transfection procedure was repeated with same conditions as in day one. The experiment was ended after 70h to perform cell counting and metabolite extraction.

Western blot

To prepare for western blot, cells were rinsed with PBS and lysed in RIPA buffer. Lysates were kept in 4°C while agitating, then centrifuged for 10min at 13,000g and 4°C. Supernatants were transferred to a new tube. 10μL of each supernatant was used for BCA assay to measure total protein content, while the remainder was mixed with loading buffer, and incubated at 95°C for 5 minutes. 10μg of each sample was loaded to each well of a PAGE gel, which was run at 100mV. Meanwhile, PVDF membrane was wetted with methanol for 1min, and filter papers soaked in the transfer buffer for 5min. After completed electrophoresis, the gel was blotted using a Thermo Scientific Pierce G2 Fast Blotter, as
described by the manufacturer. The membrane was incubated in 5% milk + PBST (PBS + 0.5% Tween20) for 1h at room temperature, then incubated overnight with primary antibody in 5% milk + PBST at 4°C, washed with PBST 3 times, and finally incubated with secondary antibody in 5% milk + PBST at 4°C. The membrane was washed 3 times with PBST, incubated with the developing reagents (Thermo Fisher, 34096), and imaged using a Vilber Lourmat Fusion Solo chemoluminescence camera.

**Propidium Iodide (PI) staining - Flow cytometry analysis**

For DNA staining with PI, cells in culture were detached using trypsin (as described above), then the pellet was resuspended in 1mL PBS. The cell mix was added dropwise to 9mL 70% ethanol at 4°C for fixation. Cells were kept in ice for 30min or in 4°C overnight. Then, tubes were centrifuged at 300g for 3min, and the supernatant was discarded. The cell pellet was washed once with PBS and re-suspended in 100μL PBS. 400μL of propidium iodide (PI) staining solution was added to the cell suspension, and incubated for 30min at 37°C in the dark. After incubation, cells were centrifuged, the supernatant was aspirated, and cells were resuspended in PBS at a concentration of 1–2 × 10⁶ cells/mL and filtered through 40μm strainers. Flow cytometry was performed on a Gallios flow cytometer instrument. PI was detected using 535nm laser and the 617nm filter. Data analysis was performed using the FlowJo analysis software. PI staining was analyzed for the single cell subpopulation, after gating out debris and doublets. For preparation of 500μL PI staining solution, 467μL PBS, 2μL EDTA 1M, 25μL PI 1mg/mL and 6μL RNase A 1mg/mL were mixed.

For viability assessment with PI staining of live cells, ~1×10⁶ cells in culture were detached using trypsin (as described above), then the pellet was resuspended in 1mL PBS solution containing PI at a final concentration of 0.6μM. Cells suspension was incubated at RT for 15min and analyzed in FACS. PI staining was analyzed for the single cell subpopulation, after gating out debris and doublets.

**Urea assay**

For urea assay cells were lysed and treated as described by the manufacturer. Since the samples reached saturation at the recommended 30min, the incubation time was decreased to 15min.

**Arginase enzyme activity assay**

For arginase activity assay cells were lysed and treated as described by the manufacturer.

**Metabolomics**

Cell and media samples were analyzed by liquid chromatography-mass spectrometry (LC-MS). To ensure complete cell lysis, the resuspended cell pellets underwent three freeze-thaw cycles in which the samples were alternated between 37°C and −80°C liquid baths in 60 second intervals. This was followed by sonication for 2 minutes and a 30 second vortexing step. Samples were then centrifuged at 14,000g at 4°C for 10min and the supernatant was transferred to a clean microcentrifuge tube and dried down in vacuo using a vacuum concentrator. The dried cell samples were resuspended in 80:20 methanol:water and transferred to LCMS vials containing a 200μL glass inserts. The media samples were...
prepared by taking 50 μL of media and adding 200μL of ice cold methanol. Samples were vortexed for 30 seconds and placed in the −80°C freezer for 30min to allow for protein precipitation. Thereafter, the samples were vortexed again for 30 seconds and centrifuged at 14,000g at 4°C for 10 minutes. The media supernatant was then transferred to LCMS vials containing 200 μL glass inserts.

The extracted cell and media samples were injected on a Thermo Vanquish UPLC system coupled to a Thermo QExactive orbitrap mass spectrometer which was operated in positive and negative ion mode using a heated electrospray ionization (HESI) source. Data was collected over a mass range of m/z 67–1000 using an auxiliary gas flow rate of 20 units, a sheath gas flow rate of 40 units, sweep gas flow rate of 2 units, spray voltage of 3.5 and 2.5 kV for positive and negative ion modes (respectively), capillary inlet temperature of 275°C, auxiliary gas heater temperature of 350°C and a S-lens RF level of 45. The mass spectrometer was operated at 35,000 resolution with a 100ms ion trap time for MS1 and 17,500 resolution with a 50ms ion trap time for MS2 collection. For MS2 collection, MS1 ions were isolated using a 1.0 m/z window and fragmented using a normalized collision energy of 35. Fragmented ions were placed on dynamic exclusion for 30 seconds before being allowed to be fragmented again. Chromatographic separation of metabolites was achieved using a Millipore (Sequant) Zic-pHILIC 2.1×150mm 5μm column maintained at 25°C. Compounds were eluted via a 19-minute linear gradient beginning with 90:10 acetonitrile:20mM ammonium bicarbonate and ending with 45:55 acetonitrile:20mM ammonium bicarbonate.

Quantification and Statistical Analysis

The data access layer mzAccess (Lyutvinskiy et al. 2017) was used to retrieve LC-MS chromatograms at retention time and mz coординates of interest, and mathematica scripts were used to calculate peak areas and build plots. There was no derivatization of molecules, and hence no correction for natural abundances. Peaks were annotated with HMDB metabolite identifiers by matching the observed peak m/z to the ideal m/z. Additional metabolite annotations were obtained by matching MS/MS data to publicly available libraries using the GNPS online tool (Wang et al. 2016). We estimated from forward scatter cytometry measurements that SG2M HeLa cells were on average only 1.16 times larger than G1 cells, and therefore did not correct peak areas for this factor.

Mass isotopomer (MI) fraction was defined as peak area of the MI divided by the total peak area for all MIs. 13C enrichment was defined as $\sum_{x=0}^{n} x \cdot x \cdot \text{MI fraction of } x / n$, where n is the total number of carbons in the metabolite. MI differences (Table S1) were calculated for each MI as the mean of difference in MI fraction between the paired replicates (SG2M minus G1). This was done for each peak.

Gene expression analysis

Expression data for breast cancer datasets from the studies by Miller et al. 2005; Pawitan et al. 2005; Kao et al. 2011; Hu et al. 2009; Desmedt et al. 2007; Terunuma et al. 2014; Wang et al. 2005; van de Vijver et al. 2002; Anders et al. 2011; and Weigman et al. 2012 was
obtained from NCBI GEO database (Edgar 2002). Data was analyzed in the form provided by the original authors using Mathematica, as follows.

**Preprocessing: Collapsing to gene level data.**—For Affymetrix data sets, for each gene we chose the probeset with highest average expression across all samples. For Agilent and other cDNA two-color arrays (van de Vijver et al. 2002) we averaged values for all probes matching a given gene. Missing values were discarded in all calculations.

**PAM50 classification**—Following ref no (Parker et al. 2009), we normalized each gene to its median across samples, and for each sample calculated the Spearman rank correlation between the measured PAM50 genes and the centroids defined by Parker et al (Parker et al. 2009) for the five PAM50 subtypes (normal-like, luminal A, luminal B, HER2 and basal).

**Connectivity Map analysis**—Expression data from the Connectivity Map (CMap) project in Level 5 (signature) GCTX format was obtained from GEO (accession GSE92742), and z-score data for 12,328 measured genes for nine cell lines treated with three distinct shRNA hairpins against ARG2 (27 samples total) was extracted using the cmapR package ([https://github.com/cmap/cmapR](https://github.com/cmap/cmapR)). The z-scores were condensed into a final z-score vector by taking the 0.8-quantile of absolute z-scores across the 27 samples (capturing genes that differed in either direction in at least 5 experiments), and this vector was used for enrichment analysis using the GSEA-P statistic. The genesets examined were downloaded from ConsensusPathDB collection ([http://cpdb.molgen.mpg.de/](http://cpdb.molgen.mpg.de/)). False discovery rates were calculated based on 1,000 gene permutations.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgements**

This work was supported by grants from the Strategic Research Programme in Cancer at Karolinska Institutet (I.R., R.N.); and the Foundation for Strategic Research (FFL12–0220: I.R., R.N.); Robert Lundberg Memorial Foundation (2017–00516: IR); the UC San Diego Frontiers of Innovation Scholars Program (K.L.); Tobacco-Related Disease Research Program (#24RT-0032: M.J., #24FT-0010: J.D.W.), National Institutes of Health (R01ES027595, R03HL133720: M.J., K01DK116917: J.D.W.), Swedish Research Council and Swedish Cancer Society (AL) and Swiss National Science Foundation (PZHP3–161692: LL).

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Figure 1. A cell sorting approach to studying metabolism in the G₁ and SG₂M phases.

(A) Design of cell sorting and LC-MS experiments. (B) Gating strategy for isolating G₁ and SG₂M cells, illustrated by Hoechst staining vs. Geminin probe expression in HeLa cells. Blue and red highlighted regions indicate gates for 2n (G₁) and 4n (SG₂M), respectively. (C) Western blot of cell cycle phase markers Cdt1 (G₁) and Cyclin A (SG₂M) in sorted HeLa cell populations. (D–E) Relative abundance (LC-MS peak area) of deoxythymidine triphosphate (dTTP) and deoxyadenosine triphosphate (dATP) in sorted G₁ and SG₂M phase HeLa cells (D), and in unsynchronized, double thymidine block (DTB) and lovastatin (LOV)
synchronized HeLa cells (E). (F–H) Relative abundance of ADP-ribose (F), ribose/ribulose-5-phosphate (G), and sedoheptulose-7-phosphate (H), in synchronized and sorted HeLa cells, normalized to the mean of LOV and G1 samples, respectively. See also Figure S1.
Figure 2. Pulse $^{13}$C labeling of sorted cells identifies cell cycle-regulated pathways.

(A) Left, experimental design of pulse labeling followed by cell sorting and LC-HRMS. Asterisk (*) denotes $^{13}$C isotopes. Right, cell cycle diagram indicating gating for 2n (G$_1$) cells (blue arc), and 4n (SG$_2$M) cells (red arc) based on Hoechststaining. Grey circle sections represent example cell cycle trajectories during a 3 hour incubation with $^{13}$C labeled medium, designed to minimize cross-contamination (see text).

(B) Distribution of difference in mass isotopomer (MI) fraction between SG$_2$M and G$_1$ cells. Solid black line denotes mean of biological triplicates, gray denotes standard deviation. Highlighted
metabolites, see text. (C) Selected MI peak areas for pentose phosphate pathway metabolites in G_1 (blue) and SG_2M (red) cells. Error bars denote standard deviation of biological triplicates. (D–E) Above, MI peak areas of uridine-diphosphate (UDP) (D) and dTMP (E) in G_1 (blue) and SG_2M (red) cells. Paired data from three independent sorting experiments are shown, with adjacent bars from the same experiment. Below, UDP and dTMP structures with origin of observed MIs indicated. dTMP derives from UDP, with one methyl group added from CH_2-folate by thymidylate synthase (TYMS). (F) MI peak areas and structure of S-adenosyl methionine (SAM), as in (D,E). See also Figure S2 and Table S1.
Figure 3. Arginine metabolism and ornithine synthesis occurs preferentially in SG2M. 
(A–B) LC-MS peak areas of $^{13}$C$_6$ arginine (A) and $^{13}$C$_6$ lysine (B). Paired data from three independent sorting experiments are shown, with adjacent bars from the same experiment. 
(C) Diagram of metabolic reactions downstream of arginine. Metabolites that were not observed are shown in gray. Black and white circles indicate $^{13}$C and $^{12}$C atoms, respectively, as observed in (A–F). 
(D–F) LC-MS peak areas of, $^{13}$C$_4$ creatine (D), $^{13}$C$_6$ acetyl-putrescine (E) and $^{13}$C$_5$ ornithine (F), in G$_1$ (blue) and SG2M (red) phase HeLa, HMEC and HMEC Ras cells. 
(G) Abundance of arginine (arg), lysine (lys), ornithine (orn) and acetyl-putrescine (aptrc) in S and G$_2$M cells, relative to G$_1$. 
(H–I) $^{13}$C$_5$ ornithine (H) and $^{13}$C$_4$ acetylputrescine (I) MI fractions from $^{13}$C$_6$-arginine and $^{13}$C$_5$-glutamine tracing experiments at 48 hours, in indicated cell lines. Biological triplicates are shown for each sample in (H–I); error bars represent standard deviation. See also Figure S3.
Figure 4. ARG2 is important for proliferation of transformed cells.
(A) Western blot of ARG2 protein (size 39 kDa) in lysates of indicated cell types. Beta tubulin (TUBB gene product, size 50kDa), was used as a loading control. (B) Arginase activity in lysates of indicated cell types as measured by absorbance. Two independent experiments are shown for each sample. (C) Relative peak areas of $^{13}$C$_6$ arginine and $^{13}$C$_5$ ornithine in ARG2 knockdown samples. Data is presented as ratio over siCtrl samples. Two independent experiments are shown for each sample. (D–G), Relative cell numbers at 70 hours in cultures of untreated cells (Ctrl), cells treated with scrambled siRNA (siCtrl) or...
with siRNAs against ARG2 (siA, siB, si2, si3), in HeLa (D), HeLa Fucci (E), MDA-MB-231 (F) and MDA-MB-468 cells (G), in all cases normalized to Ctrl. (H) Fractions of HeLa cells in the G1, S and G2M phases as determined by DNA analysis, in cultures treated with siCtrl or siB. (I) Gene set analysis of mRNAs responding to ARG2 knockdown, from the ConnectivityMap dataset. Left, enrichment score vs. false discovery rate (FDR) for 3,582 gene sets, top gene set indicated. Right, individual gene ranks for the top gene set. (J) Fraction of G1 population in HCT116 (left) and MCF7 (right) cells grown in control (grey bars) and arginine deprived (black bars) medium. Two independent experiments are shown for each sample. (K) 13C5 ornithine and 13C4 acetyl-putrescine MI fractions in Ctrl and siB treated HeLa cells, labeled from 13C6-arginine (arg) or 13C5-glutamine (gln) tracers, as indicated. (L) LC-MS chromatogram of 13C2 (acetyl-labeled) acetyl-putrescine in Ctrl and siB treated HeLa cells labeled from 13C5-glutamine. (M) Model of arginine metabolism based on the experimental results. (N) ARG2 expression in the Miller et al. (GSE3494) breast tumor dataset, stratified by above-median (high) or below-median (low) estrogen receptor α (ESR1) expression. Affymetrix probe set accession number is indicated; p-value indicates one-sided permutation test. (O) ARG2 expression in breast cancer subtypes based on PAM50 classification, as in (H). (P) Meta-analysis of ARG2 expression association with patient survival in basal breast cancers, from indicated data sets. Error bars in A–C and E denote standard deviation across three experiments. Biological triplicates are shown for each sample in (B–J) and (K); error bars represent standard deviation. See also Figures S4 and Table S2.