Nic1 Inactivation Enables Stable Isotope Labeling with $^{13}$C$_6^{15}$N$_4$-Arginine in Schizosaccharomyces pombe

Alejandro Carpy†¶, Avinash Patel§¶, Ye Dee Tay§, Iain M. Hagan§, and Boris Macek‡¶

Stable Isotope Labeling by Amino Acids (SILAC) is a commonly used method in quantitative proteomics. Because of compatibility with trypsin digestion, arginine and lysine are the most widely used amino acids for SILAC labeling. We observed that Schizosaccharomyces pombe (fission yeast) cannot be labeled with a specific form of arginine, $^{13}$C$_6^{15}$N$_4$-arginine (Arg-10), which limits the exploitation of SILAC technology in this model organism. We hypothesized that in the fission yeast the guanidinium group of $^{13}$C$_6^{15}$N$_4$-arginine is catalyzed by arginase and urease activity to $^{15}$N$_1$-labeled ammonia that is used as a precursor for general amino acid biosynthesis. We show that disruption of Ni$_2^+$-dependent urease activity, through deletion of the sole Ni$_2^+$ transporter Nic1, blocks this recycling in ammonium-supplemented EMMG medium to enable $^{13}$C$_6^{15}$N$_4$-arginine labeling for SILAC strategies in S. pombe. Finally, we employed Arg-10 in a triple-SILAC experiment to perform quantitative comparison of G1 + S, M, and G2 cell cycle phases in S. pombe. Molecular & Cellular Proteomics 14: 10.1074/mcp.O114.045302, 243–250, 2015.

Stable Isotope Labeling by Amino acids in Cell culture (SILAC) is one of the most widely used methods in quantitative proteomics (1). It involves in vivo metabolic labeling of cell cultures (or small organisms) with different versions of stable isotope-labeled amino acids (2). To maximize the number of peptides that can be quantified after proteome digestion with trypsin, proteins are usually differentially labeled with different forms of lysine and arginine (3): L-lysine (Lys-0) and L-arginine (Arg-0); 2H$_4$-lysine (Lys-4) and 13C$_6$-arginine (Arg-6); or 13C$_6$-15N$_2$-lysine (Lys-8) and 13C$_6$-15N$_4$-arginine (Arg-10). The availability of multiple forms of labeled lysine and arginine support the application of SILAC in duplex (comparison of two states) or triplex (comparison of three states) formats. Efficient anabolic pathways mean that lysine and arginine are not essential for growth of wild type yeast cells. Auxotrophic mutants that are defective in these pathways can be used to switch yeast to an absolute dependence upon the provision of these amino acids in the external medium. Consequently, mutations in arginine and lysine biosynthesis pathways can be used to drive the complete labeling of all tryptic peptides with specific forms of these amino acids (4, 5). SILAC has been used in quantitative proteomics in several yeast species, but most widely in Saccharomyces cerevisiae (6) (budding yeast) and Schizosaccharomyces pombe (fission yeast). S. pombe is extensively exploited to study cell cycle control (7), heterochromatin (8), and differentiation (9) and is increasingly the subject of large-scale quantitative proteomic studies (10, 11).

A major challenge that is faced when using SILAC in fission yeast, is metabolic conversion of arginine to other amino acids such as proline, glutamine, and lysine (5). This partial labeling of additional amino acids after the conversion event produces spectra with complex isotope clusters that makes the downstream analysis challenging and error-prone. Inactivation of the “arginine conversion pathway” by removal of the orthinine transferase, Car2, effectively overcomes this problem to support the use of arginine labeling in SILAC-based experiments (5). Although this exploitation of the car2Δ mutation now enables SILAC technology in fission yeast, the choice of amino acids that can be employed remains limited. Only one form of heavy arginine (R6) is currently used alongside three forms of heavy lysine (Lys-4, Lys-6, and Lys-8) (5, 12). Surprisingly, we could not find any studies that use arginine (Arg-10) in fission yeast, even though this is a widely exploited reagent for labeling other cell types (1).

Here, we show that labeling of fission yeast with Arg-10 leads to a general misincorporation of the stable isotope label that prevents the identification of labeled peptides. We hypothesize that successive arginase and urease activities catalyze the guanidinium group of Arg-10 to $^{15}$N$_1$-labeled ammonia. This labeled ammonium is then used as a general...
precursor for amino acid biosynthesis. Disruption of Ni²⁺-dependent urease activity through deletion of Ni²⁺ transporter Nic1 in ammonium-supplemented medium, blocked this recycling to support \( ^{15}N_{\alpha} \) -arginine labeling SILAC strategies. As a proof of principle we employ Arg-10 in a triple-SILAC experiment to perform quantitative comparison of G1 + S, M, and G2 cell cycle phases in \( S. \) pombe.

**EXPERIMENTAL PROCEDURES**

Yeast Strains—

Standard Yeast Culture—Strains used in this used study are listed in Table 1. Cell culture and maintenance were according to Moreno et al. (13). The amino acid supplements were uracil, adenine, leucine, and histidine at a concentration of 200 μg/ml. Yeast strains were streaked onto YES plates (Solid Yeast Extract agar with supplements) from frozen glycerol stocks at ~80 °C, and grown at the permissive temperature (25 °C) until single colonies formed. One single colony was used to start each liquid culture. For all physiological experiments, asynchronous cultures of cells were grown in variants of Edinburgh minimal media 2 (EMM2), with appropriate supplements from log phase starter cultures (1 × 10⁶ cells/ml). In EMMG, the 5g/L ammonium chloride of EMM2 is replaced with 5g/L monosodium glutamate (14). In EMMGn, EMMG is supplemented with 12 mm ammonium chloride.

**S. pombe Transformation Using Lithium Acetate—** Cultures that had been grown in YES (nutrient rich) to mid-log phase (4 × 10⁸ – 8 × 10⁹ cells/ml) were pelleted by centrifugation at 3400 × g for 2 min, washed once in water followed by resuspension in appropriate volume of lithium acetate (0.1M, pH 4.9) to a final density of 2 × 10⁶ cells/ml. After 1 h at 25 °C 10–15 μg of DNA was added to 100 μl of competent cells along side 2.9 volumes of PEG 4000 (50% (w/v) in 0.1 M Lithium acetate (0.1M, pH 4.9)). After 1 h at 25 °C and 15 min at 42 °C cells were washed once in water and plated onto selective minimal media.

**SILAC labeling—** L-lysine:2HCl (4,4,5,5-D₄, 96–98%, Cambridge Isotopes, cat no. CLM-2265-H-0.5) were used as medium isotopic labels; L-lysine:2HCl (U-13C₆, 99%; U-15N₂, 99%, Cambridge Isotopes, cat no. CNLM-539-H-0.5) were used as heavy isotopic labels.

**Cell Culture—** EMMG media is equivalent to EMM2 except that 5 g/L ammonium chloride is replaced with 5 g/L monosodium glutamate (14). EMM2 media was also prepared with a range of ammonium chloride concentrations up to 96 mm. EMMGn is EMMG containing 12 mm NH₄Cl. To label the cells with heavy or medium isotopes of Arginine (R) and Lysine (K), starter cultures were prepared by inoculation into EMMG/EMMG supplemented with 37.5 mM NH₄Cl. To label the cells with heavy or medium isotopes of Arginine (R) and Lysine (K), starter cultures were prepared by inoculation into EMMG/EMMG supplemented with 37.5 mM NH₄Cl. After 4000 rpm for 2 min in screw cap 1.5 ml microfuge tubes. Cell pellets were washed once in ice-cold STOP buffer (10 mM EDTA, 50 mM NaF, 150 mM NaCl, and 1 mM Na₃VO₄) before being flash frozen in liquid nitrogen and stored at −80 °C. Proteins were extracted by breaking the cells with 200 μl acid washed glass beads in the presence of 200 μl of denaturation buffer (6 M Urea, 2 M Thio-urea in 10 mM Tris buffer, pH 8.0) in a ribolaster (Fast prep FP120-BIO101, ThermoSavant, Carlsbad, CA) used for 30 s at 6.5 rpm. 200 μl of denaturation buffer was added to the glass beads before incubation on a rotating rocker at RT for 30 min. A hole was made in the bottom of each tube before each was placed on the top of an empty 1.5 ml microfuge tube and spun at 4800 rpm for 2 min at RT. The insoluble debris was separated by centrifugation at 13,000 rpm for 10 min at RT. The supernatant was collected in a fresh 1.5 ml microfuge tube to be processed for LC-MS/MS.

**SILAC Experiment to perform quantitative comparison of G1 + S, M, and G2 cell cycle phases in S. pombe.**
arated into 12 fractions using 13 cm Immobiline DryStrips with a pH 3–10 gradient (GE Healthcare) with the 3100 Off-Gel fractionator (Agilent, Santa Clara, CA). A maximum of 50 μA were applied until 20 kVH were reached. Peptide fractions were acidified with 10 μL of 30% ACN, 5% acetic acid, and 10% TFA in water.

**MS Analysis**—Desalted peptide fractions were separated using a nano-LC (Proxeon Biosystems) coupled to either an Orbitrap Elite or a LTQ Orbitrap XL (Thermo Fisher Scientific) mass spectrometer (MS). Peptides were separated on a 15 cm PicoTip fused silica emitter with a 75 μm ID (New Objective) packed, in-house, with ReproSil-Pur C18-AQ 3 μm resin (Dr. Maisch GmbH). Peptides were loaded on the column in Buffer A (0.5% acetic acid) at 700 nL/min using a maximum pressure of 280 Bar. Peptides were then eluted using a 57 or 87 min segmented gradient of 5–50% Buffer B (80% ACN in 0.5% acetic acid) at a flow rate of 200 nL/min. The mass spectrometers were operated in the positive ion mode. Spectra were recorded with data-dependent mode using a dynamic exclusion of 90 s (LTQ Orbitrap XL) or 60 s (Orbitrap Elite). Resolution was set at 60,000 (LTQ Orbitrap XL) or 120,000 (Orbitrap Elite) with an accumulation target value of 1E6 charges. For peptide analysis the 10 (LTQ Orbitrap XL) or 20 (Orbitrap Elite) most intense peaks were selected from the survey scan (300–2000 Thompson) and fragmented with collision induced dissociation at a target value of 5000 charges and recorded in the linear ion trap.

**MS Data Processing**—The MS data were analyzed with the MaxQuant Software Package version 1.3.0.5 (19) and searched using the Andromeda peptide search engine (20). The MS/MS spectra were searched against a database containing the proteome sequence of *S. pombe* downloaded from PomBase (03.01.2013). The database contained 5145 entries from the *S. pombe* proteome, 248 frequently observed contaminants (downloaded from http://www.maxquant.org/contaminants.zip), as well as the reverse version of all sequences. Mass tolerance was set to 6 ppm at the MS and 0.5 Da at the MS/MS level. A minimal length of seven amino acids and full tryptic enzyme specificity were required. Two enzyme missed cleavages were allowed. Oxidation of methionine and protein N-terminal acetylation were defined as variable modifications and carbamidomethylation of cysteines was used as fixed modification. The false discovery rate was set to 1% on the peptide and protein level. In quantitative measurements, SILAC amino acids Lys6, Lys8, Arg6, and/or Arg10 were defined as fixed modifications. Significantly regulated protein groups were defined as those with calculated “Significance B” lower than 0.01.

**Immunofluorescence and FACs Analysis**—FACS analysis and Immunofluorescence microscopy were performed as described by Tay et al. (21). The Sad1 (spindle pole body component) and TAT1 (Trypanosome Alfa Tubulin) (22) primary antibodies were diluted 1:25 and 1:80 respectively. FITC anti-mouse antibody (Sigma Aldrich) and Cy3 anti-rabbit antibody (Sigma Aldrich) were used in PEMBAL as secondary antibodies for Sad1 and TAT1 at dilutions of 1:100 and 1:2500 respectively. Cytox green (Invitrogen, Carlsbad, CA) was used in FACS analysis.

**RESULTS AND DISCUSSION**

To assess the feasibility of using arginine (Arg-10) for quantitative proteomics of fission yeast, we performed SILAC labeling of *S. pombe* cells as described by Bicho et al. (5). We used arg3D4 lys3.131 car2::natMX6 background and refer to this genotype as “SILAC” in the following text. Consistent with previous observations (5), the SILAC strain grows poorly in the EMM2 medium that uses ammonium as a nitrogen source (Fig. 1A). Ammonium ions inhibit one of two arginine uptake pathways, to severely compromise the growth of arginine auxotrophs such as the SILAC strain (23). Substitution of ammonium for monosodium glutamate in the medium EMMG enables both arginine import pathways to be used. Mid-log phase growth of the SILAC strain in EMMG was comparable to wild type growth in the same medium (Fig. 1A, 1B). We therefore grew cells in EMMG rather than the reduced ammonium EMM2 used by Bicho et al. EMMG is widely used throughout the fission yeast community and, beyond an impact upon TOR growth controls (24), no differences from growth on EMM2 have been observed. However, as an additional check we asked whether the inclusion of the SILAC mutations affected the expression of mitotic phenotypes arising from incapacitation of both polo kinase and the kinesin 5 motor protein? Shifting a *plo1ts41 cut7.24* SILAC strain to 36°C (25, 26) led to an efficient mitotic arrest, indicating that neither the switch of nitrogen source, nor the incorporation of SILAC mutations, had any impact upon the degree of mitotic arrest invoked by these mutations (Fig. 1C).

While performing a standard triple SILAC experiment using “light” (Lys-0 and Arg-0), “medium” (Lys-4 and Arg-6), and “heavy” (Lys-8 and Arg-10) amino acids in EMMG we noticed that almost all heavy (Lys-8 and Arg-10)-labeled peptides had complex isotope clusters that effectively prevented identification of heavy peptides and proteins. The presence both arginine- and lysine-containing peptides in these complex clusters (Fig. 2A) is indicative of a global conversion of the stable isotope label of either, the heavy arginine and/or lysine. To characterize the labeling problem in greater depth, we analyzed the light, medium-heavy, and heavy labeled samples separately. The identification rate for light and medium-heavy labeled samples was normal, whereas it was significantly reduced in samples from heavy labeled cells. To pinpoint the heavy amino acid responsible for the conversion, we measured samples labeled with a single heavy label (Arg-0/Lys-8 or Arg-10/Lys-0). Only the sample containing heavy arginine (Arg-10/Lys-0) presented the complex isotope clusters that reduced identification rates at both protein and peptide level. Thus, Arg-10 is incompatible with SILAC labeling approaches currently used for fission yeast analyses (Fig. 2B; a summary of the proteins identified in each experiment is presented in supplementary Tables S1 to S15).

We reasoned that this extensive mislabeling of multiple amino acids probably arose from catabolism of guanidine group of heavy arginine (Arg-10) to heavy urea. Catalysis by Car1 and Ara1 would convert the guanidine group into urea that could be further catabolized by ureases into ammonia (Fig. 1D) (5). The 15N1-ammonia derived from 15N2-urea would then be incorporated into multiple amino acids, each containing different numbers of 15N atoms to alter the isotope distributions of all peptides. Unlike the medium-heavy 15C6-arginine (Arg-6), only heavy 15N416C8-arginine (Arg-10) has isotopically labeled nitrogen and so only samples labeled with this form of arginine (Arg-10) are vulnerable to this urease catabolism problem. Consistently, conversion was not observed in measurements of...
samples containing only medium-heavy amino acids arginine (Arg-6) and lysine (Lys-4), (Fig. 2B).

Ureases require Ni\(^{2+}\) as the major cofactor for their catalytic activity. S. pombe encodes one characterized urease (Ure1) (27) alongside several other proteins that are predicted to exercise urease activity. Nic1 is the sole plasma membrane Ni\(^{2+}\) transporter and supports urease activity of fission yeast (28). The observations of Eitinger et al. (28) led us to reason that the global reduction of the urease activity, arising from abolition of Nic1 function, would reduce the amino acid mislabeling we observed following growth of the SILAC strain on heavy arginine (Arg-10). We therefore deleted nic1/H11001 in the SILAC strain to generate "SILACn," before repeating the SILAC labeling as described above. As reported previously, nic1/H11001 ablation had no major impact upon the growth rate of otherwise wild type strains (Fig. 1B). Most importantly, the growth rate of SILAC and SILACn strains were identical (Fig. 1B), indicating that removal of nic1\(^+\) is no more detrimental to cell physiology than the established mutations that are currently exploited for SILAC labeling in fission yeast (5).

Introduction of nic1.\(\Delta\) increased the identification of arginine (Arg-10)-labeled peptides, suggesting that urease-dependent arginine catabolism was indeed a key factor in arginine (Arg-10) mislabeling (supplemental Fig. S1). However, the number of peptides that could be identified in samples labeled with heavy amino acids was below that obtained in samples containing light amino acids. Furthermore, the isotopic distribution of peptides was unusual, suggesting a partial incorporation of \(^{15}\)N atoms. This incomplete suppression suggested either, that residual traces of Ni\(^{2+}\) persist in nic1/H9004 cells, or that the urea is catabolized to ammonium ions by Ni\(^{2+}\)-independent urease/pathways.

The increased identification rate of (Arg-10) labeled peptides in the SILACn background indicated that, although \(^{15}\)N-ammonium has not been completely eliminated by removal of Nic1, its levels had been severely reduced. We therefore asked whether we could out compete the ability of the cells to incorporate these low levels of \(^{15}\)N-ammonium ions in amino acid anabolic pathways, by supplementing the EMMG growth medium with levels of unlabeled \(^{14}\)N-ammonium ions that are tolerated by SILAC strains (5). The SILACn strain grew with a doubling time of 5.5 h in EMMG media that contained 12 mM NH\(_4\)Cl (EMMGn) supplemented with standard "light" forms of both lysine and arginine. This doubling rate is close to the 5 h of the SILAC strain and 4.5 h of wild type prototrophs in the same medium. When stable isotope-labeled amino acids were used as the sole source of lysine and arginine the mid-log phase, the doubling rate increased to 7 h at 25 °C. For reasons that are not clear, the original SILAC strain (nic1\(^+\)) grew very poorly in EMMGn (supplemental Fig. S2). Impor-
Fig. 2. **Assessment of arginine conversion using MS.** A, Isotopic distribution of peptides from a triple-SILAC experiment for an arginine and a lysine containing peptide using the SILAC strain (top) and an arginine and a lysine containing peptide using the SILACn strain (bottom). B, Protein groups (blue bars) and peptide sequences (orange bars) identified in individual experiments using different isotopic versions of lysine and arginine. The lines on the top indicate the media used and the colored background indicates the strain used.
SILAC in Fission Yeast

### Table I

**Overview of the cdc25.22 arrest-release datasets**

| Replicate 1 | Proteins Identified | Proteins Quantified (H/L) | Proteins Quantified (M/L) |
|-------------|---------------------|---------------------------|---------------------------|
| G2          | M                   | 3446                      | 2672                      | 2672                      |
| K4 R8       | K6 R10              |                           |                           |                           |
| K0 R0       | G1S                 | 3462                      | 2833                      | 2833                      |
| K4 R8       | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |
| K4 R8       | M                   |                           |                           |                           |
| K0 R0       | M                   |                           |                           |                           |
| K6 R10      | G1S                 |                           |                           |                           |
| K0 R0       | G2                  |                           |                           |                           |

**Yeast strains**

| Strain No. | Genotype | Source |
|------------|----------|--------|
| IH 1540    | h<sup>+</sup> lys1.131 | Lab Stock |
| IH 5224    | h<sup>+</sup> pku70::kanMX6 ura4.d18 leu1.32 ade6.704 | Lab Stock |
| IH 6113    | pku80::ura4<sup>+</sup> ura4.d18 leu1.32 | Lab Stock |
| IH 6297    | h<sup>+</sup> arg3.D4 | Lab Stock |
| IH 8987    | h<sup>+</sup> car2::NatMX6 | This study |
| IH 8988    | h<sup>+</sup> car2::NatMX6 | This study |
| IH 9091    | h<sup>+</sup> car2::NatMX6 lys1.131 arg3.D4 ura4.d18 leu1.32 | This study |
| IH 9092    | h<sup>+</sup> car2::NatMX6 lys1.131 arg3.D4 ura4.d18 leu1.32 | This study |
| IH 9281    | plo1-ts41:ura4<sup>+</sup> cut7.24 lys1.131 arg3.D4 car2::NatMX6 ura4.d18 leu1.32 | This study |
| IH 8439    | cut7.24 ura4.d18 | This study |
| IH 11849   | h<sup>+</sup> nic1::kanMX6 car2::NatMX6 lys1.131 arg3.D4 | This study |
| IH 13324   | cut7.24 nic1::kanMX6 car2::HphX6 arg3.D4 lys1.131 | This study |
| IH 13302   | cdc25.22 nic1::kanMX6 car2::HphX6 arg3.D4 lys1.131 | This study |
| IH 13303   | cdc10.v50 nic1::kanMX6 car2::HphX6 arg3.D4 lys1.131 | This study |

**TABLE I**

| Yeast strains | Source |
|---------------|--------|
| IH 9281       | This study |
| IH 8439       | This study |
| IH 11849      | This study |
| IH 13324      | This study |
| IH 13302      | This study |
| IH 13303      | This study |

**Molecular & Cellular Proteomics 14.1**

---

**Note:**

Tantantly, however, the regular isotope pattern of the heavy-labeled peaks established that growth of SILAC<sup>n</sup> cells in EMMGn successfully resolved the mislabeling problem (compare Fig. 2A top spectra with bottom spectra). This improved detection rate enabled us to identify comparable numbers of heavy labeled peptides to those that are routinely detected in cells labeled with light or medium amino acids (Fig. 2B).

Fission yeast is widely exploited for analysis of the cell division cycle. We therefore asked whether the combination of the SILAC<sup>n</sup> background and EMMGn medium would impact upon the ability to use some well characterized mutations to synchronize progression through the cell division cycle. Removal of the function of the START transcription factor Cdc10 through the cdc10.v50 mutation reversibly arrests cell cycle progression in G1, because cells are unable to transcribe genes encoding DNA replication factors (29). As S phase is initiated immediately after anaphase chromosome segregation, the vast majority of cells in an asynchronous cdc10.v50 culture complete cytokinesis before accumulating at the G1 execution point (30). Transient cell cycle arrest at the cdc10.v50 execution point is therefore a commonly employed approach to synchronize progression through S phase, immediately after the block to Cdc10 function that is triggered by the return to the permissive temperature. We asked whether this cdc10.v50 “arrest release” approach could be used to synchronize S phase progression in a SILAC<sup>n</sup> background. An early log phase cdc10.v50 SILAC<sup>n</sup> culture was shifted to 36 °C before returning to 25 °C. FACs profiles indicated a proficient accumulation of 1N cells and an equally proficient reappearance of 2N cells following return to 25 °C (supplemental Fig. S3A). Temperature dependent inactivation of Kinesin 5 through temperature shift of the cut7.24 mutation removes the ability of the two half spindles to interdigitate. Consequently, the bipolar chromosome attachments that are required to silence the spindle assembly checkpoint (SAC) surveillance system are not established. This results in a persistent activation of the spindle assembly checkpoint that blocks cell cycle progression and so causes cells to accumulate in mitosis with monopolar spindles (26). The cut7.24 mutation completely blocked spindle formation in EMMGn, irrespective of the presence of the SILAC<sup>n</sup> mutations (supplemental Fig. S3B). Finally, we tested the combined impact of the SILAC<sup>n</sup> mutations and growth in EMMGn upon the widely exploited ability of the cdc25.22 mutation to reversibly arrest cell cycle progression at the G2/M boundary. Such cdc25.22 “arrest release” cultures are highly synchronized with respect to mitotic progression (5, 21). cdc25.22 SILAC<sup>n</sup> cells were grown to early log phase (1 × 10<sup>6</sup>) in EMMGn at 25 °C before a transient shift to 36 °C for 4.25 h. The absence of the septa that identify cells undergoing cytokinesis indicated an efficient cell cycle arrest at the G2/M boundary (data not shown). The transient appearance of replication and 4n peaks in FACS analysis of DNA content 90–150 min after return to the permissive temperature of 25 °C indicated an equally efficient return to cycle after release (supplemental Fig. S4). We conclude that neither the inclusion of the SILAC<sup>n</sup> mutations nor growth in EMMGn has a deleterious impact upon the ability to manipulate cell cycle progression to generate populations at discrete cell cycle or mitotic stages with the cut7.24, cdc10.v50, or cdc25.22 mutations. The slightly slower growth rate of SILAC<sup>n</sup> strains compared with WT is common among yeast auxotrophs. This reduced growth rate will have no major impact upon the interpretation of triple SILAC<sup>n</sup> experiments because all three strains under query will harbor the same SILAC<sup>n</sup> genetic background.

To validate this approach we performed a proof-of-principle triplex-SILAC measurement based on the duplex-SILAC cdc25.22 arrest release experiment performed by Bicho et al.
In this experiment, we grew cdc25.22 SILACn cells in either EMMGn supplemented with arginine and lysine for “light,” “medium-heavy,” or “heavy” labeling. Cells were transiently arrested at the G2/M boundary before return to 25 °C, to release the arrest. FACs analysis revealed equivalent efficiencies of cell cycle progression following release in all cultures. Because the inclusion of the nic1./H9004 mutation had generated the option of conducting triple-SILAC labeling, we supplemented the sampling at G2 and G1/S, performed by Bicho et al., with a third time point in M phase (see Supplemental Fig. S4 for the time at which each sample was taken).

In the first experiment, samples were labeled as follows: G2 (Lys-0, Arg-0), M (Lys-4, Arg-6), and G1S (Lys-8, Arg-10), in the second experiment we did a label swap: M (Lys-0, Arg-0), G1S (Lys-4, Arg-6), and G2 (Lys-8, Arg-10). An overview of the experiment can be found in Table I.

In 24 LC-MS runs, we recorded 747,658 MS/MS spectra that identified a total of 25,548 nonredundant peptides from 3606 S. pombe proteins, at estimated false discovery rate (FDR) of 1.30% at the protein level (supplemental Table S14).

As shown in Table I, the use of Arg-10 did not influence peptide identification and the number of quantified M/L and H/L pairs were very similar, demonstrating that Arg-10 can be routinely used in SILAC measurements in the cdc25.22 SILACn strain. Of the 3075 quantified proteins, 2430 (79%) were quantified in both replicates and correlation was high (R² = 0.89), in spite of the fact that the labels were inverted in replicate analyses (supplemental Fig. S5A, S5B). We next compared our dataset to the data published by Bicho et al. (5) and observed good correlation (R² = 0.59) between proteins that were significantly regulated in both datasets. Importantly, this correlation was confirmed in the replicate measurement with inverted SILAC labels (Supplemental Fig. S5C, S5D). Thus, Arg-6 and Arg-10 labeling was robust. Despite a relative paucity of significantly regulated proteins in both datasets, we could confirm several findings observed by Bicho et al. (5, 31).

In addition to several differentially regulated metabolic enzymes, we confirmed significant changes in the levels of polo kinase Plo1, condensin subunit Cnd2, and the HIRA histone chaperone complex hip3 in comparisons between the G2/ G1 + S datasets. We also detected significant regulation of the Cdc14 related cell cycle phosphatase Clp1/Fip1, the membrane anchor for the Tea1 cell morphogenesis complex, Mod5, and the Greatwall kinase family member Ppk18, in both the M/G2 and G2/G1 + S datasets (Fig. 3).

We conclude that removal of Nic1 from cells grown in EMMGn finally renders fission yeast fully compatible with SILAC labeling, to greatly expand the options for interrogation of the S. pombe proteome. The ability to routinely conduct triple SILAC analysis in this genetically malleable model organism will rapidly advance our quantitative understanding of many core principles of molecular cell biology.

Acknowledgments—We thank M. Mann and S. Hauf for helpful comments on the manuscript, as well as K. Krug and C. Täumer for help with preparation of figures. We thank K. Gull (Oxford University) for TAT1 antibody.

* Our work is supported by funding from the Juniorprofessoren-Programm of the BW-Stiftung, FP7 PRIME-XS Consortium and SFB 766 of the German Research Council (DFG) to B.M. and by Cancer Research UK (CRUK) C147/A6058 to I.H., A.P., and Y.D.T.; A.P. was also supported by an EMBO STF.

This article contains supplemental Tables S1 to S15 and Figs. S1 to S5.

Accession codes: The mass spectrometry data from this publication have been submitted to PeptideAtlas (www.peptideatlas.org) and assigned the accession number PASS00437. The fission yeast strain h-nic1::kanMX6 arg3-D4 lys1-131 car2::NatMX6 has been submitted to Yeast Genetic Resource Center (http://yeast.lab.nig.ac.jp) and assigned accession number FY24007.

To whom correspondence should be addressed: Proteome Center Tuebingen, Interfaculty Institute for Cell Biology, University of Tuebin-
REFERENCES

1. Ong, S. E., and Mann, M. (2005) Mass spectrometry-based proteomics turns quantitative. Nat. Chem. Biol. 1, 252–262
2. Ong, S. E., Blagoev, B., Kratchmarova, I., Kristensen, D. B., Steen, H., Pandey, A., and Mann, M. (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics 1, 376–386
3. Olsen, J. V., Blagoev, B., Grad, F., Macak, B., Kumar, C., Mortensen, P., and Mann, M. (2006) Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648
4. de Godoy, L. M., Olsen, J. V., de Souza, G. A., Li, G. Q., Mortensen, P., and Mann, M. (2006) Status of complete proteome analysis by mass spectrometry: SILAC labeled yeast as a model system. Genome Biol. 7, R50
5. Bicho, C. C., Alves, F. D., Chen, Z. A., Rappsilber, J., and Sawin, K. E. (2010) A genetic engineering solution to the “arginine conversion problem” in Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC). Mol. Cell. Proteomics 9, 1567–1577
6. de Godoy, L. M., Olsen, J. V., Cox, J., Nielsen, M. L., Hubner, N. C., Frohlich, F., Wellner, T. C., and Mann, M. (2008) Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast. Nature 455, 1251–1254
7. Eledge, S. J. (1996) Cell cycle checkpoints: preventing an identity crisis. Science 274, 1664–1672
8. White, S. A., and Allshire, R. C. (2008) RNAi-mediated chromatin silencing in fission yeast. Curr. Top. Microbiol. 320, 157–183
9. Harigaya, Y., and Yamamoto, M. (2007) Molecular mechanisms underlying the mitosis–meiosis decision. Chromosome Res. 15, 523–537
10. Marguerat, S., Schmidt, A., Codlin, S., Chen, W., Aebersold, R., and Bahler, J. (2012) Quantitative analysis of fission yeast transcriptomes and proteomes in proliferating and quiescent cells. Cell 151, 671–683
11. Gunaratne, J., Schmidt, A., Quandt, A., Neo, S. P., Sarac, O. S., Gracia, T., Loguercio, S., Ahme, E., Xia, R. L., Tan, K. H., Lossner, C., Bahler, J., Beyer, A., Blackstock, W., and Aebersold, R. (2013) Extensive mass spectrometry-based analysis of the fission yeast proteome: the Schizosaccharomyces pombe PeptideAtlas. Mol. Cell. Proteomics 12, 1741–1751
12. Koch, A., Krug, K., Pengelly, S., Macak, B., and Haff, S. (2011) Mitotic substrates of the kinase aurora with roles in chromatin regulation identified through quantitative phosphoproteomics of fission yeast. Sci. Signal. 4, ra6
13. Moreno, S., Klar, A., and Nurse, P. (1991) Molecular genetic analysis of fission yeast Schizosaccharomyces pombe. Methods Enzymol. 194, 795–823
14. Fantes, P., and Nurse, P. (1977) Control of cell size at division in fission yeast by a growth-modulated size control over nuclear division. Exp. Cell Res. 107, 377–386
15. Nurse, P. (1975) Genetic control of cell size at cell division in yeast. Nature 256, 547–551
16. Hentges, P., Van Driessche, B., Tafforeau, L., Vandenhauwe, J., and Carr, A. M. (2005) Three novel antibiotic marker cassettes for gene disruption and marker switching in Schizosaccharomyces pombe. Yeast 22, 1013–1019
17. Krug, K., Carpy, A., Behrends, G., Matic, K., Soares, N. C., and Macek, B. (2013) Deep coverage of the Escherichia coli proteome enables the assessment of false discovery rates in simple proteogenomic experiments. Mol. Cell. Proteomics 12, 3420–3430
18. Rappsilber, J., Mann, M., and Ishihama, Y. (2007) Protocol for micro-purification, enrichment, prefractionation, and storage of peptides for proteomics using StageTips. Nat. Protoc. 2, 1896–1906
19. Cox, J., and Mann, M. (2008) MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies, and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372
20. Cox, J., Neuhauer, N., Michalski, A., Schelter, R. A., Olsen, J. V., and Mann, M. (2011) Andromeda: a peptide search engine integrated into the maxquant environment. J. Proteome Res. 10, 1794–1805
21. Tay, Y. D., Patel, A., Kaemena, D. F., and Hagan, I. M. (2013) Mutation of a conserved residue enhances the sensitivity of analog-sensitized kinases to generate a novel approach to the study of mitosis in fission yeast. J. Cell Sci. 126, 5052–5061
22. Woods, A., Sherwin, T., Sasse, R., MacRae, T. H., Baines, A. J., and Gull, K. (1989) Definition of individual components within the cytoskeleton of Trypanosoma brucei by a library of monoclonal antibodies. J. Cell Sci. 93, 491–500
23. Fantes, P. A., and Creanor, J. (1984) Canavanine resistance and the mechanism of arginine uptake in fission yeast Schizosaccharomyces pombe. J. Gen. Microbiol. 130, 3265–3273
24. Petersen, J. (2009) TOR signaling regulates mitotic commitment through sterol-activated MAPK and Polo kinase in response to nutrient stress. Biochem. Soc. T. 37, 273–277
25. Grallert, A., Patel, A., Tallada, V. A., Chan, K. Y., Bagley, S., Krapp, A., Simanis, V., and Hagan, I. M. (2013) Centrosomal MPF triggers the mitotic and morphogenetic switches of fission yeast. Nat. Cell Biol. 15, 88–95
26. Hagan, I., and Yanagida, M. (1990) Novel potential mitotic motor encoded by the fission yeast cut7 gene. Nature 347, 563–566
27. Lubbers, M. W., Rodriguez, S. B., Honey, N. K., and Thornton, R. J. (1996) Novel potential mitotic motor protein in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol. Cell. Proteomics 15, 320–332
28. Eitinger, T., Degen, O., Bohnke, U., and Muller, M. (2000) Nic1p, a relative of bacterial transition metal permeases in Schizosaccharomyces pombe. Can. J. Microbiol. 42, 132–140
29. Elledge, S. J. (1996) Cell cycle checkpoints: preventing an identity crisis. Science 274, 1664–1672
30. Nurse, P., Thuriaux, P., and Nasmyth, K. (1976) Genetic control of the cell division cycle in the fission yeast Schizosaccharomyces pombe. Science 194, 376–383
31. Carpy, A., Krug, K., Grat, S., Koch, A., Popic, S., Haff, S., and Macek, B. (2014) Absolute proteome and phosphoproteome dynamics during the cell cycle of fission yeast. Mol. Cell. Proteomics 13, 1925–1936