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ORIGINAL ARTICLE

Arginine methylation in yeast proteins during stationary-phase growth and heat shock

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Abstract Arginine methyltransferases (RMTs) catalyze the methylation of arginine residues on proteins. We examined the effects of log-phase growth, stationary-phase growth, and heat shock on the formation of methylarginines on yeast proteins to determine if the conditions favor a particular type of methylation. Utilizing linear ion trap mass spectrometry, we identify methylarginines in wild-type and *RMT* deletion yeast strains using secondary product ion scans (MS^3), and quantify the methylarginines using multiple reaction monitoring (MRM). Employing MS^3 and isotopic incorporation, we demonstrate for the first time that *N*η1, *N*η2-dimethylarginine (sDMA) is present on yeast proteins, and make a detailed structural determination of the fragment ions from the spectra. *N*η-monomethylarginine (ηMMA), *N*δ-monomethylarginine (δMMA), *N*η1, *N*η1-dimethylarginine (aDMA), and sDMA were detected in *RMT* deletion yeast using MS^3 and MRM with and without isotopic incorporation, suggesting that additional RMT enzymes remain to be discovered in yeast. The concentrations of ηMMA and δMMA decreased by half during heat shock and stationary phase compared to log-phase growth of wild-type yeast, whereas sDMA increased by as much as sevenfold and aDMA decreased by 11-fold. Therefore, upon entering stressful conditions like heat shock or stationary-phase growth, there is a net increase in sDMA and decreases in aDMA, ηMMA, and δMMA on yeast proteins.

Keywords ADMA · sDMA · Yeast · Mass spectrometry · Protein methylation · Delta-monomethylarginine · Heat shock · Stationary phase · Log phase · MRM^3

Abbreviations

CE	Collision energy
CPS	Counts per second
CXP	Collision cell exit potential
DP	Declustering potential
MRM	Multiple reaction monitoring
MRM^3	Multiple reaction monitoring cubed
MS^2	Primary product ion spectrum
MS^3	Secondary product ion spectrum
MS	Mass spectrometry
UHPLC	Ultra-high performance liquid chromatography
YEPD	Yeast extract peptone dextrose

Introduction

The enzymes that catalyze the post-translational modification of arginine methylation are called protein arginine

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N-methyltransferases (PRMTs) in humans, and arginine methyltransferases (RMTs) in the yeast *Saccharomyces cerevisiae.* Both families of enzymes utilize *S*-adenosyl-L-methionine (AdoMet) as a source of methyl groups, which are transferred to the guanidino nitrogen atoms of arginine residues within proteins. The three RMT enzymes that have been identified in yeast are Rmt1p (also known as Hmt1p) (Gary et al. 1996; Henry and Silver 1996), histone synthetic lethal 7 (Hsl7p) (Lee et al. 2000), and Rmt2p (Zobel-Thropp et al. 1998). However, many uncharacterized genes containing putative AdoMet-dependent methyltransferase domains have been identified in yeast and some may possess arginine methyltransferase activity (Niewmierzycka and Clarke 1999; Low and Wilkins 2012). A recently discovered example is the SPOUT methyltransferase encoded by the gene YOR021C (Sfm1p), which can produce methylarginines on yeast proteins (Low and Wilkins 2012; Young et al. 2012). However, it is not structurally related to known RMTs, and is therefore not part of this study (Young et al. 2012).

RMTs have been shown to produce three different methylarginines in yeast (Fig. 1). Common to all eukaryotes, *N*η-monomethylarginine (ηMMA) and asymmetric *N*η1, *N*η1-dimethylarginine (aDMA) are produced by Rmt1p (Gary et al. 1996). Rmt2p catalyzes the formation of *N*δ-monomethylarginine (δMMA), a modification present exclusively in yeast (Zobel-Thropp et al. 1998; Niewmierzycka and Clarke 1999). Although most eukaryotic organisms also produce *N*η1, *N*η2-dimethylarginine (sDMA) on select proteins, previous studies have failed to show that proteins derived from yeast contain sDMA (Miranda et al. 2006; Sayegh and Clarke 2008). Despite this, the yeast enzyme Hsl7p can catalyze the formation of ηMMA and sDMA on calf thymus histones with prolonged in vitro incubations (Sayegh and Clarke 2008).

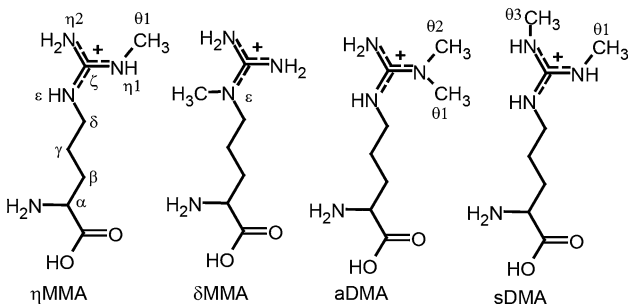

Fig. 1 Common methylarginines. Displayed are the methylarginines ηMMA, δMMA, aDMA, and sDMA. All atoms are designated with Greek letters according to IUPAC conventions. According to these conventions, δMMA should actually be called εMMA, however, as δMMA is in wide use we use this designation. Collectively, aDMA and sDMA are dimethylarginines (DMA), and ηMMA and δMMA are monomethylarginines (MMA)

Proteomic analysis has shown that at least 31 yeast proteins contain methylarginines, including 20 sites with MMA and 18 sites with dimethylarginine (Pang et al. 2010). Many of the targets are involved in protein synthesis, gene expression, and responses to heat shock or nutrient deprivation.

The growth of yeast depends upon the availability of nutrients and environmental stress such as heat shock. In media containing glucose, yeast grow through several phases based on the availability of nutrients. They initially grow exponentially in the log phase until glucose is consumed, then switch to the post-diauxic growth period in which ethanol produced during the log phase is consumed. As the availability of carbon sources become scarce, yeast enter the stationary phase of growth in which the total cell number does not increase and they become trapped in the G1 phase of the cell cycle (Herman 2002). The heat-shock response is characterized by adaption to heat stresses and repair of damaged proteins through increased heat-shock protein expression. Heat shock results in protein denaturation, misfolding, and aggregation. Therefore, many heat-shock proteins protect proteins from damage, prevent aggregation and unfolding, target unfolded proteins to the proteasome for recycling, and refold proteins (Verghese et al. 2012). Like stationary-phase growth, yeast cells become transiently arrested in the G1 phase during heat shock (Herman 2002; Verghese et al. 2012).

To investigate changes in arginine methylation abundance associated with changes in growth conditions, we measured methylarginines under log phase, stationary phase, and heat-shock conditions. We examined methylarginines from wild-type (WT), *rmt1-*, *hsl7-*, and *rmt1-/hsl7-* yeast strains utilizing mass spectrometry (MS) to determine and quantify the types of methylarginines present. Using multiple reaction monitoring (MRM), primary (MS^2) and secondary product ion spectra (MS^3), and isotopic incorporation we show for the first time that sDMA is produced on yeast proteins, and determine the structures of all fragment ions in the MS^2 and MS^3 spectra for sDMA and aDMA. The concentrations of ηMMA, δMMA, and aDMA decreased during heat shock and stationary phase compared to log-phase growth, whereas sDMA increased. Such changes may be a marker for stressful conditions. We also add to the growing case for the presence of undiscovered *RMT* genes in yeast because all methylarginine species were detected in knockout yeast strains.

Experimental

Yeast strains were analyzed for the presence of methylarginines by acid hydrolysis of total yeast protein into amino

acids followed by liquid chromatography, and qualitative and quantitative mass spectral analysis of free methylarginines as described in the supplemental material. An MRM assay was used for the simultaneous quantification of aDMA, [Cθ1D_3, Cθ2D_3]aDMA, sDMA, [Cθ1D_3, Cθ3D_3]sDMA, histidine, and MMA (total) (Supplemental Table 1). The methods for detection of undeuterated methylarginines and histidine were described previously (Lakowski and Frankel 2009; Pak et al. 2011). The method of quantification of δMMA and ηMMA using MRM^3 is described elsewhere (Lakowski et al. 2013).

Product ion spectra

MS^2 were recorded for aDMA and sDMA, selecting the precursor ion 203.1 *m/z* scanning from 30 to 200 *m/z* with a 40-V declustering potential (DP) and a 20-eV collision energy (CE). MS^3 spectra were obtained for aDMA and sDMA selecting the precursor ion 203.1 *m/z*, primary product ions 158.2 and 172.2 *m/z* scanning from 50 to 170 *m/z*, with a 40-V DP and a 20-eV CE. To detect deuterated sDMA and aDMA, MS^2 were recorded with the precursor ion 209.1 *m/z* and scanned from 30 to 200 *m/z* with a 40-V DP and a 20-eV CE. MS^3 spectra of deuterated δMMA and ηMMA were recorded for the precursor ion 192.1 *m/z* and the primary product ions 175.2 and 147.0 *m/z* scanning from 50 to 170 *m/z*. MS^3 spectra of deuterated sDMA and aDMA were recorded for the precursor ion 209.1 *m/z* and the primary product ions 164.2 and 175.2 *m/z* scanning from 50 to 170 *m/z*. DP and CE values for deuterated methylarginines were the same as those used with natural abundance. All spectra were recorded in positive ion mode and qualitative so no attempt was made to optimize the parameters.

Yeast strains and growth

Isogenic *S. cerevisiae* strains *rmt1*-, *hsl*7-, *rmt1-/hsl7*-, and WT were gifts from Professor Steven Clarke at UCLA and are described in the supplemental material (Miranda et al. 2006). A single colony was selected for each strain and used to inoculate YEPD media and grown at 30 °C until the OD_{600} reached 0.3–0.6. Yeast strains were also grown in minimal media (Treco et al. 2001).

Isotopic incorporation

Incorporation of deuterated (CD_3) methyl groups onto arginine residues was performed in minimal media similar to previous studies except [CεD_3]methionine (DLM-431-1 Cambridge Isotope Laboratories) was used as the methionine source (Ong et al. 2004). Minimal media was inoculated with yeast, and grown to an OD_{600} of 0.2–0.4 at 30 °C. Cultures were pelleted and divided into matching groups with additional minimal media and grown to an OD_{600} of 0.2–0.4. Yeast were centrifuged and rinsed twice with water, resuspended in 50 mL of minimal media with [CεD_3]methionine or natural abundance methionine (control group). All groups, prepared in duplicate, were incubated at 30 °C until the OD_{600} reached 0.6–0.8 and harvested by centrifugation.

Heat shock and stationary phase studies

Minimal media was inoculated with single colonies from WT, *rmt1*-, *hsl*7-, and *rmt1-/hsl*7- yeast and grown at 30 °C until the OD_{600} reached 0.3–0.6. The cells were pelleted, washed in water, divided into two groups for heat shock and stationary-phase experiments, and resuspended in minimal media. The stationary phase group was incubated again at 30 °C for 8 days to ensure exit from the log and post-diauxic growth phases and entry into the stationary growth phase (Herman 2002). The heat-shock group was incubated at 30 °C until the OD_{600} reached 0.3-0.5. The cells were pelleted, divided into 1.5- and 3-h heat-shock groups, resuspended in minimal media, and incubated at 30 °C overnight. Cells were then pelleted, resuspended in minimal media, incubated for 3 h at 30 °C, and transferred to an incubator at 37 °C for 1.5 h or 3 h. Heat shock was performed at temperatures less than 40 °C according to previous studies and to ensure synthesis of heat-shock proteins (Ribeiro et al. 1997). All samples were prepared in duplicate.

Preparation of yeast protein amino acids for MS analysis

Yeast proteins were hydrolyzed producing free δMMA, ηMMA, aDMA, and sDMA, which were quantified using MS. Yeast cells were lysed, total protein was precipitated, hydrolyzed, and purified according to previous protocols [(Lakowski et al. 2013) and Supplemental Material]. Hydrolyzed samples were purified according to previously described methods (Teerlink et al. 2002; Teerlink 2007). Dried purified samples were resuspended in 100 μL of mobile phase A (Supplemental Material), and 5 μL of each sample was injected for analysis described above.

Standards aDMA, sDMA, ηMMA, and histidine were purchased from Sigma, and δMMA was prepared according to previous methods (Schade et al. 2008). Standards were prepared at concentrations of 5–5000 nM except for histidine, which was prepared at 1000 to 20,000 nM. To normalize all hydrolyzed yeast protein extracts, the measured histidine peak area for each sample was normalized to the peak area for an equal volume injection of a 20,000 nM standard of histidine. In addition, tenfold dilutions of these

samples were also used to confirm the ratios and to normalize all analyte samples. Normalization by histidine was compared to normalization by OD_{600} of the original cultures, the corresponding wet pellet weight, and the precipitated dry protein weight; the differences in values varied by less than 15 % among normalization methods.

Results

In yeast proteins, sDMA has not been detected (Sayegh and Clarke 2008), and both sDMA and aDMA have identical molecular weights. Therefore, control MS^2 and MS^3 spectra for aDMA and sDMA standards were prepared to differentiate them and to qualitatively identify sDMA in yeast. The MS^2 spectra show the fragmentation of aDMA (Fig. 2a) and sDMA (Fig. 2b) standards (precursor ions), into characteristic fragment structures (primary product ions). Such spectra differentiate sDMA from aDMA. The MS^3 spectra show the further fragmentation of primary product ions into secondary product ions. Figure 2a displays the MS^2 of aDMA and the resulting MS^3 of the primary product ion 158.1 *m/z*. The proposed structures of the primary and secondary product ions are displayed in Fig. 2c, and d, corresponding to the spectra in Fig. 2a, and b, respectively. Methylated and unmethylated structures of the ion 158.1 *m/z* are proposed (Fig. 2c). This is supported by isotope incorporation studies with yeast (Supplemental Figure S1), showing that the ion 158.1 *m/z* from natural abundance aDMA appears as unmethylated

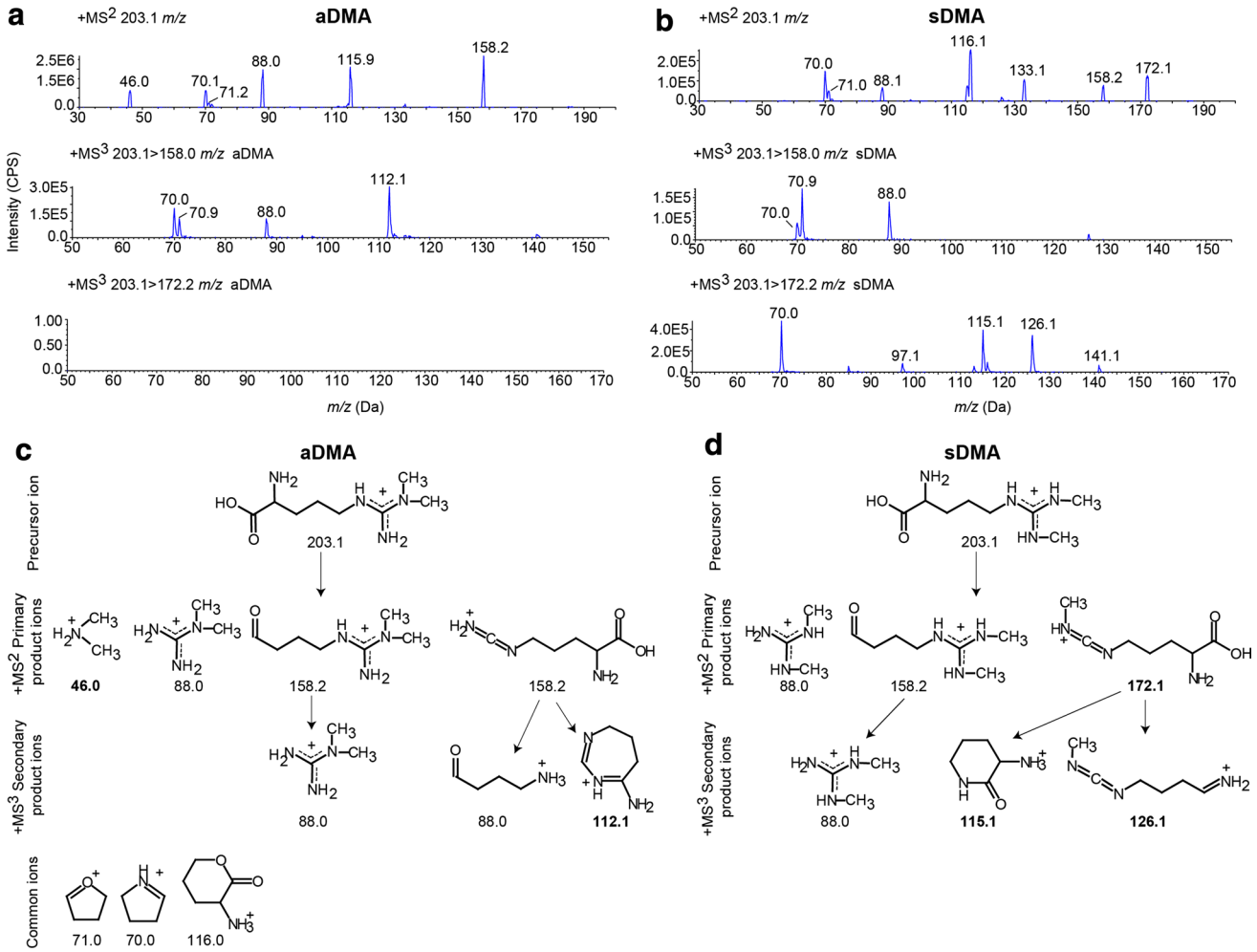

Fig. 2 MS^2 and MS^3 spectra of aDMA and sDMA standards. The MS^2 (*top*), MS^3 spectra for the primary product ions 158.0 *m/z* (*middle*) and 172.2 *m/z* (*bottom*) for aDMA (**a**) and sDMA (**b**) are shown. The ions from the MS^2 that were selected for MS^3 are displayed at the top of the MS^3 spectra. As aDMA does not produce a primary product ion 172.2 *m/z*, no signal in the MS^3 for aDMA is seen for this ion. The precursor and primary product ion values are displayed above each spectrum (precursor > primary product) and the intensity is in counts per second (CPS). The proposed structures of the primary and secondary product ions are displayed for aDMA (**c**) and sDMA (**d**). *Arrows point* from primary product ions to the resultant secondary product ions. The structures of the common ions 70, 71, and 116 *m/z* are displayed at the *bottom*. The *m/z* of diagnostic primary or secondary product ions are *bolded*

(158.1 *m/z*) and deuterated dimethylated (164.1 *m/z*) ions upon deuterium incorporation. Accordingly, the MS^3 of aDMA (Fig. 2a) and the structural analysis (Fig. 2c) show that the ion 158.1 *m/z* can be explained with unmethyated and dimethylated structures and each can give rise to corresponding unmethylated 112.1 and 88.0 *m/z*, and dimethylated 88.0 *m/z* secondary product ions (also confirmed below in Fig. 3). The secondary product ion 112.1 *m/z* is unique to aDMA and is diagnostic for its presence. Although the MS^2 for aDMA does not have a peak at 172.1 *m/z*, an MS^3 with the primary product ion 172.1 *m/z* was recorded to confirm that no signal from aDMA can be detected (Fig. 2a, bottom). The absence of a signal from such a spectrum suggests aDMA, while the presence of a signal spectrum suggests sDMA. Figure 2b displays the MS^2 of sDMA (top) and the MS^3 derived from the primary product ions 158.1 *m/z* (middle) and 172.0 *m/z* (bottom). As observed previously, the MS^2 for sDMA and aDMA are similar except for the unique product ions 46 *m/z* for aDMA and 172.1 *m/z* for sDMA (Rappsilber et al. 2003; Brame et al. 2004; Gehrig et al. 2004; Lakowski and Frankel 2009; Lakowski et al. 2010b; Pak et al. 2011). The MS^3 spectrum of the primary product ion 172.2 *m/z* is diagnostic for sDMA (Fig. 2b). The structures in Fig. 2c, and d are supported by our current and previous work (Lakowski and Frankel 2009; Lakowski et al. 2010a; Lakowski et al. 2013; Thomas et al. 2014) and by other studies (Shek et al. 2006). The structures of dimethylated primary product ions derived from aDMA and sDMA and having 158.1 *m/z* are proposed based on primary product ion spectra of arginine that showed a 130.0 *m/z* product ion with a similar structure but no methyl groups (Shek et al. 2006).

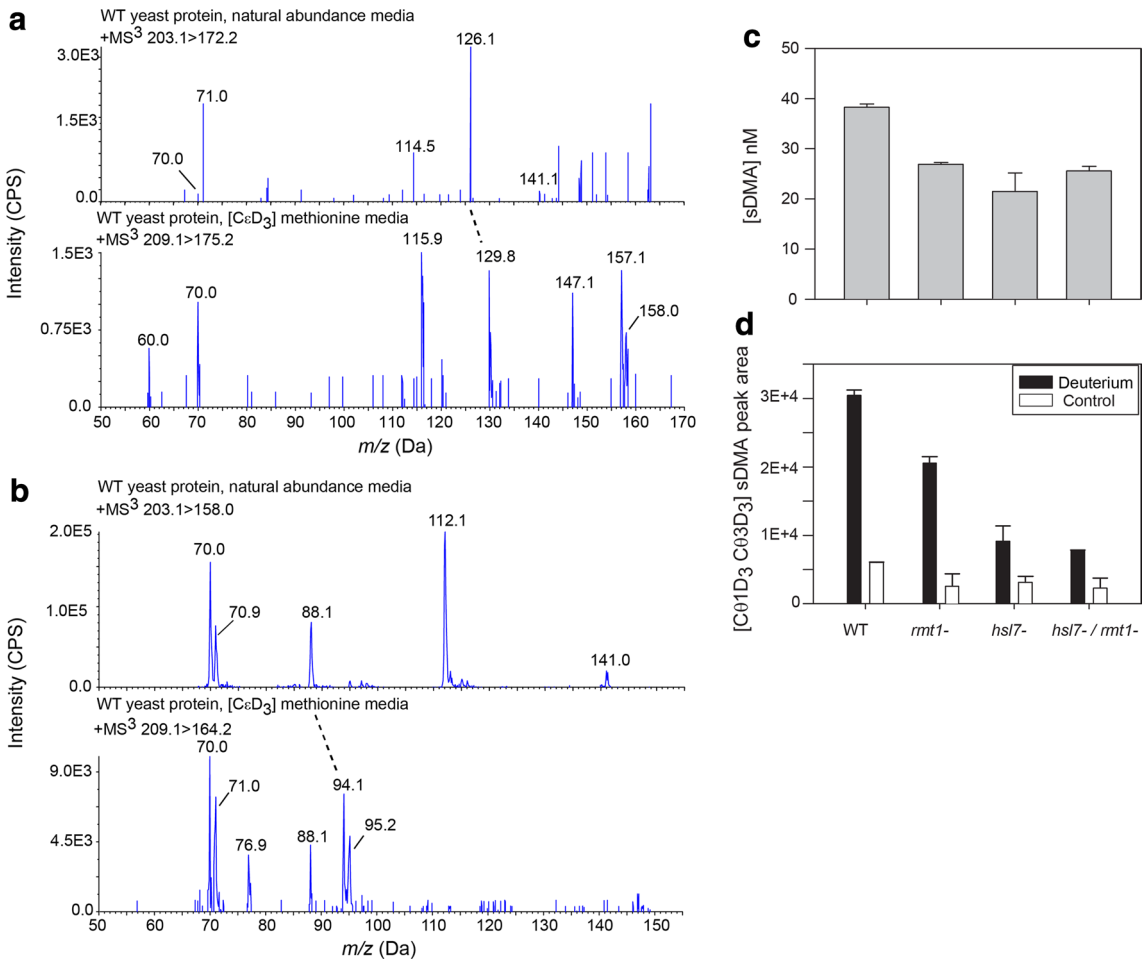

Fig. 3 Natural abundance and deuterated sDMA derived from yeast protein. Displayed are secondary product ions produced from the primary product ions, 172.2 *m/z* corresponding to sDMA (**a**) and 158.0 *m/z* corresponding to both sDMA and aDMA (**b**) derived from yeast grown in natural abundance and [$C\varepsilon D_3$]methionine minimal media. Peaks with a single or two deuterated methyl group(s) are 3 or 6 Da heavier than those that have the same number of natural abundance methyl groups, respectively. The quantification of sDMA from yeast grown in YEPD under log-phase conditions is displayed (**c**). Incorporation of deuterated methyl groups into sDMA as measured by peak area derived from yeast grown in minimal media with [$C\varepsilon D_3$]methionine and natural abundance methionine is displayed in **d**. Concentrations depicted are mean and standard deviation for two samples, and are normalized to account for differences in total protein

sDMA is formed on yeast proteins

Studies have failed to demonstrate sDMA on yeast proteins in vivo even though Hsl7p is capable of producing ηMMA and sDMA in vitro (Miranda et al. 2006; Sayegh and Clarke 2008). To determine if yeast produce sDMA in vivo, we performed MS^3 on hydrolyzed proteins derived from WT yeast grown in the log phase in minimal media with natural abundance methionine or [$C\varepsilon D_3$]methionine. The MS^3 produced from the primary product ion 172.2 *m/z* and its deuterated equivalent (175.2 *m/z*) are displayed in Fig. 3a. The acquisition of these spectra confirms the presence of sDMA since aDMA does not produce these ions or spectra. Figure 3a shows the characteristic MS^3 peaks 70 and 126.1 *m/z* consistent with the presence of sDMA. The spectrum for the deuterated equivalent of sDMA derived from yeast grown in [$C\varepsilon D_3$]methionine minimal media possess characteristic peaks at 70 and 129.8 *m/z*, the latter corresponding to the ion 126.1 *m/z* in the natural abundance spectrum. These data corroborate the structures in Fig. 2c, and d, demonstrating that the ion 126.1 *m/z* from sDMA possesses a single methyl group. The MS^3 from the primary product ion 158.0 *m/z* and its deuterated equivalent (164.2 *m/z* Da) produced by sDMA and aDMA are displayed in Fig. 3b. The peak at 112 *m/z* in Fig. 3b, which is diagnostic for aDMA, was not observed in the corresponding spectrum with deuterium incorporation. This likely occurred because the primary product ion 158 *m/z* from the more abundant aDMA results from two isomeric fragments and only one is dimethylated (Supplemental Figure S1 and Fig. 2c). Therefore, Fig. 3b (bottom) represents the fragmentation pattern from the deuterated, dimethylated ion (i.e., 158 + 6 Da). This is also consistent with the finding that the characteristic fragment ion 112 *m/z* for aDMA is not methylated (Fig. 2c). We have previously demonstrated that the dimethyl-guanidine ion at 88.1 *m/z* corresponding to 94.1 *m/z* in the deuterated spectrum (Fig. 3b, bottom) results from incorporation of two deuterated methyl groups (i.e., a 6-Da increase) (Lakowski and Frankel 2009).

An MRM assay was used to quantify sDMA derived from hydrolyzed protein from yeast strains grown in YEPD in the log phase (Fig. 3c). The amount of sDMA produced is modestly dependent on the presence of either Hsl7p or Rmt1p because *rmt1*-, *hsl7*-, and *hsl7-/rmt1*- strains produce almost half the sDMA seen in WT yeast. The production of sDMA in yeast was confirmed by detecting deuterated sDMA from extracted proteins from yeast strains grown in minimal media with natural abundance or [$C\varepsilon D_3$]methionine (Fig. 3d). As a control, we attempted to detect deuterated sDMA in yeast grown with natural abundance media and there was a very low intensity background signal. In all groups, the deuterated signal was higher. Deuterated sDMA, though present in all yeast strains, is produced to a lesser extent in *hsl7*- yeast. Taken together, these data demonstrate for the first time that we have confirmed the presence of sDMA derived from yeast proteins. Although it has been suggested that Hsl7p is capable of producing sDMA, it was only shown to do so in vitro after extended incubation with calf thymus histones (Sayegh and Clarke 2008). The natural abundance and deuterated experiments confirm that *hsl7*- yeast produce less sDMA than WT, suggesting that Hsl7p contributes to production of sDMA in vivo.

We searched for methylarginine species within yeast extract to rule it out as a contaminating source in our analyses. YEPD media and yeast extract were tested and all methylarginines could be detected (Supplemental Figure S2). It is unknown if free methylarginines in the media can be incorporated into new yeast proteins. It has been shown that ηMMA can form ηMMA-tRNA and be inefficiently incorporated into proteins, but it is unclear if tRNA acylation is possible with other methylarginines or if any significant incorporation occurs when unmodified arginine is present to compete (Hartman et al. 2007). To mitigate the potential for contamination of free or protein-containing methylarginines, yeast cell pellets in this study were washed prior to cell lysis and protein isolates were precipitated so that all methylarginines measured derived only from proteins. The concentrations of methylarginines within cells grown in YEPD vary widely among the yeast strains, suggesting that the results are due to yeast RMT rather than contamination with methylarginines from the media. Isotopic incorporation and minimal media experiments that do not contain yeast extract also suggest enzymatic formation of methylarginine residues on yeast proteins rather than contamination.

Changes in methylarginine production during log phase, stationary phase, and heat shock

To determine if changes in the concentrations of ηMMA, δMMA, aDMA, and sDMA accompany changes in environment and stress, we subjected yeast strains grown in minimal media to log and stationary phase growth conditions, as well as heat shock. Figure 4 depicts the normalized concentrations of methylarginines from yeast proteins. The concentrations of ηMMA and δMMA produced during stationary phase conditions are consistent with those under log-phase growth (Fig. 4a, and b) with *hsl7*- and *rmt1-/hsl7*- groups. However, stationary phase production of ηMMA and δMMA in WT yeast is almost threefold lower than that produced under log-phase growth. After 1.5 and 3 h of heat shock, the production of ηMMA and δMMA is almost half that of stationary phase conditions, but there are few differences among strains.

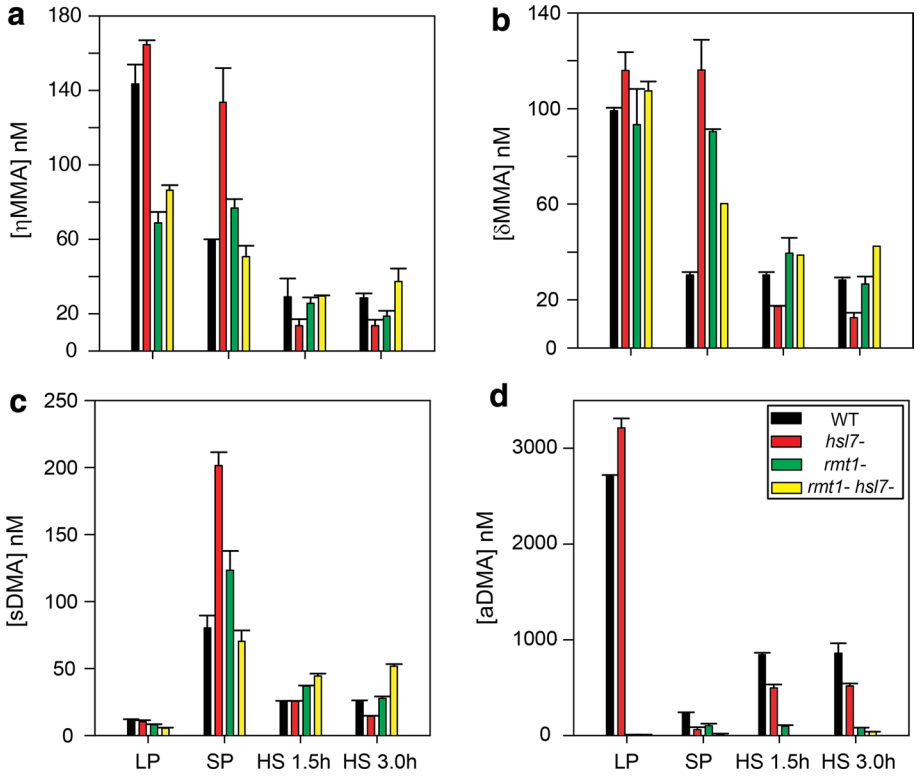

Fig. 4 Quantification of methylarginines in yeast strains grown under log phase, stationary phase, and heat shock conditions. Depicted are the concentrations of ηMMA (**a**), δMMA (**b**), sDMA (**c**), aDMA (**d**) from hydrolyzed proteins derived from yeast. The yeast strains WT, *rmt1-*, *hsl7-*, and *rmt1-/hsl7-* are grown under log phase (LP), stationary phase (SP), and 1.5 h (HS 1.5 h) and 3 h of heat shock (HS 3.0 h) in minimal media. Concentrations depicted are mean and standard deviation for two samples, and are normalized to account for differences in total protein

During stationary phase growth, WT yeast produce at least sevenfold more sDMA on proteins than under log phase and twofold more sDMA under heat-shock conditions in minimal media (Fig. 4c). For *hsl7-* yeast, the production of sDMA on proteins under stationary phase growth is 20-fold higher than log phase. All yeast strains exhibit higher concentrations of sDMA in heat shock and stationary phase compared to log-phase growth. In contrast, the production of aDMA on WT yeast proteins under stationary phase conditions (Fig. 4d) is more than 11-fold lower than that produced in minimal media under log-phase growth, and fourfold lower than under heat-shock conditions. Similar but more extreme effects were observed with the *hsl7-* mutant.

aDMA is produced in yeast lacking RMT1

MS^3 spectra were used to test for the presence of aDMA in hydrolyzed protein from yeast mutant strains *rmt1-* and *rmt1-/hsl7-* grown in the log phase in minimal media. Both mutants lack Rmt1p, which is the only characterized RMT in yeast known to form aDMA (Gary et al. 1996). Figure 5 displays the MS^3 from these strains. The secondary product ion 112 *m/z* is present in *rmt1-/hsl7-* (Fig. 5a) and *rmt1-* (Fig. 5b) strains. A comparison with the control spectrum (Fig. 2) demonstrates that the secondary product ion 112 *m/z* derived from the primary product ion 158 *m/z* is diagnostic for aDMA. These data demonstrate the presence of aDMA in protein samples derived from yeast lacking Rmt1p.

An MS assay was used to quantify aDMA produced by yeast strains. All strains produced some aDMA in YEPD during log-phase growth (Fig. 5c). As expected, both *rmt1-* and *rmt1-/hsl7-* strains have much lower aDMA concentrations than WT and *hsl7-* owing to the absence of Rmt1p. These results, corroborating data presented in Fig. 5a, and b, indicate that some aDMA is produced by *rmt1-* and *rmt1-/hsl7-* strains.

To confirm that *rmt1-* and *rmt1-/hsl7-* strains produce aDMA, we detected deuterated aDMA formed enzymatically on yeast proteins. Yeast strains were grown in minimal media with natural abundance or [$C\varepsilon D_3$]methionine. Proteins extracted from yeast cells under these conditions were analyzed for deuterated aDMA (Fig. 5d). As expected, WT and *hsl7-* yeast strains produce the most deuterated aDMA, yet it is also present in *rmt1-* and *rmt1-/hsl7-*strains.

ηMMA and δMMA are produced in rmt1-/hsl7-yeast

To detect the presence of ηMMA and δMMA in yeast lacking enzymes that produce ηMMA, mutant *rmt1-/hsl7-*yeast were grown in minimal media with [$C\varepsilon D_3$] or natural abundance methionine, and the extracted proteins were analyzed for natural abundance or deuterated ηMMA and δMMA. Figure 6a is a MS^3 produced from the primary product ion 172.2 *m/z* derived from natural abundance and deuterated

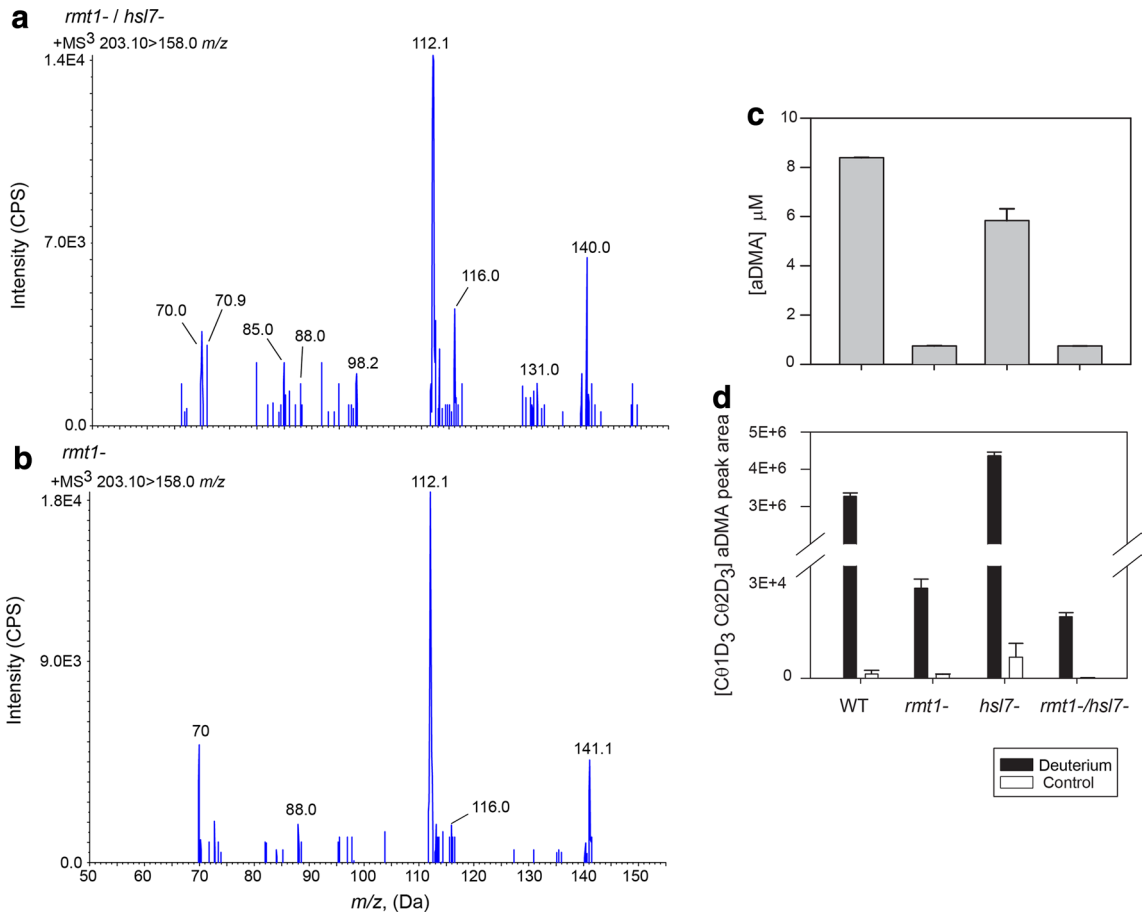

Fig. 5 The presence of aDMA in *rmt1-/hsl7-* and *rmt1-* yeast strains. MS^3 spectra for the precursor ion 203.1 *m/z* and the primary product ion 158.0 *m/z* for proteins derived from *rmt1-/hsl7-* (**a**) and *rmt1-* (**b**) yeast strains. Both spectra have a diagnostic peak at 112 *m/z* indicative of aDMA. The quantification of aDMA, from WT, *rmt1-*, *hsl7-*, and *rmt1-/hsl7-* yeast grown under log-phase conditions in YEPD is displayed in **c**. The incorporation of deuterated methyl groups into aDMA is displayed in **d**. Shown are the peak areas for deuterated aDMA derived from proteins from yeast strains grown in minimal media with natural abundance or [CεD$_3$]methionine. Concentrations depicted are mean and standard deviation for two samples, and are normalized to account for differences in total protein

ηMMA and δMMA. Figure 6b displays spectra from the primary product ion 144.0 *m/z* and its deuterated equivalent. All spectra contain the characteristic peak patterns consistent with ηMMA and δMMA that have already been demonstrated in WT yeast strains (Lakowski et al. 2013). Along with the additional conformation provided by isotopic incorporation, these data demonstrate the presence of ηMMA and δMMA within *rmt1-/hsl7-* mutant yeast.

A previously developed, MRM^3 assay was used to quantify ηMMA and δMMA in yeast proteins from WT, *rmt1-*, *hsl7-*, and *rmt1-/hsl7-* strains (Lakowski et al. 2013). The normalized concentrations of ηMMA and δMMA derived from yeast grown in YEPD media under log-phase growth conditions are depicted in Fig. 6c. The concentration of ηMMA in WT yeast is threefold greater than in *hsl7-* and sixfold greater than in *rmt1-* and *rmt1-/hsl7-* strains. This result is consistent with the reduction in enzymes capable of making ηMMA. The concentration of δMMA is reduced in mutant compared with WT strains, which is unexpected as all of these strains contain the *RMT2* gene.

Discussion

For the first time, we demonstrate definitively the presence of sDMA on yeast proteins. Hsl7p is capable of producing ηMMA and sDMA on calf thymus histones in vitro, but sDMA was not detected on yeast proteins (in vivo) (Miranda et al. 2006; Sayegh and Clarke 2008). Difficulties in detection of sDMA, and lower concentrations of sDMA on yeast proteins, in part, explain its absence in previous studies. However, these studies were conducted with yeast grown in the log phase rather than stationary phase or heat-shock conditions where sDMA concentrations are increased, which further explains why sDMA has remained undetected in yeast until now. We demonstrate that sDMA

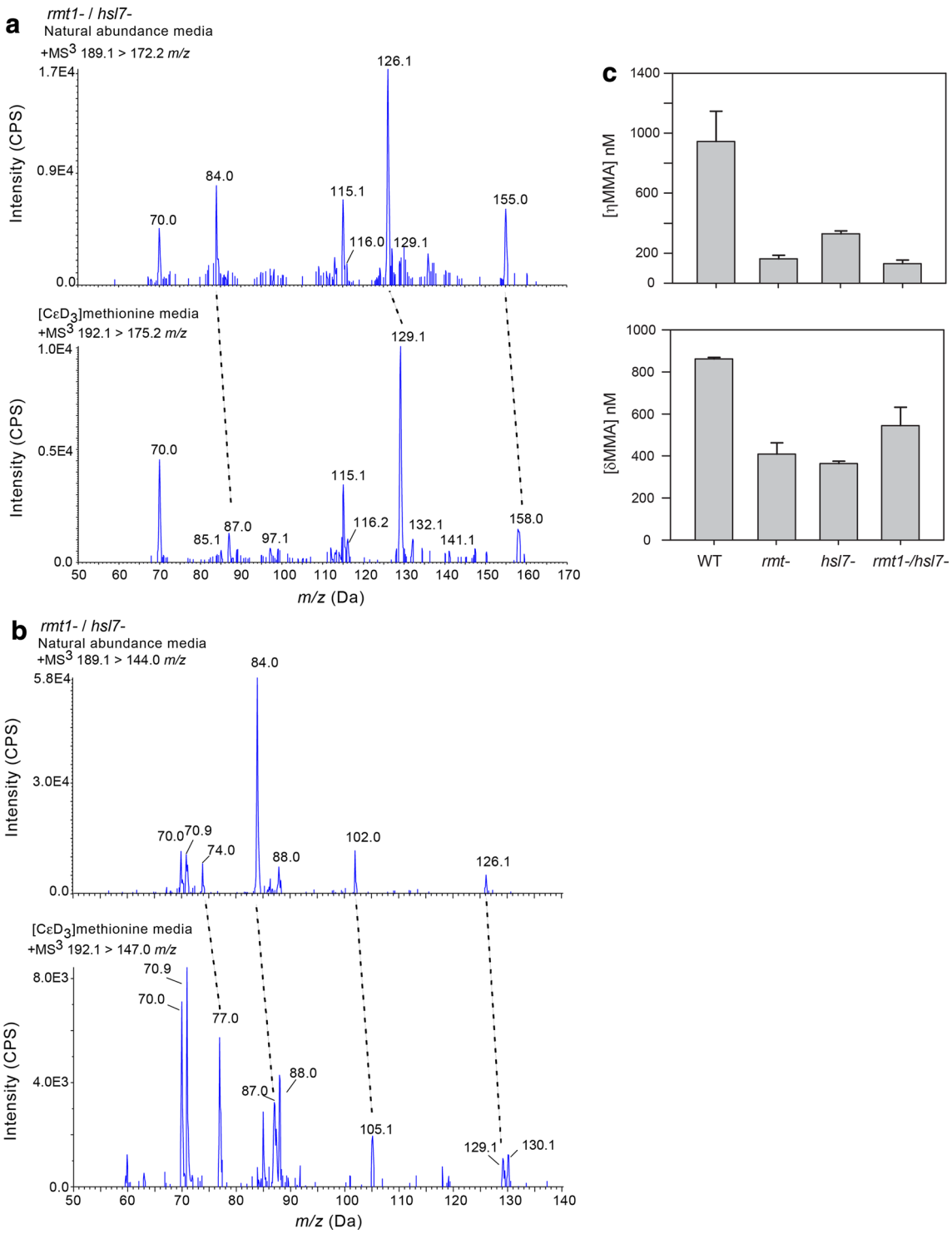

Fig. 6 Natural abundance and deuterated ηMMA and δMMA derived from *rmt1-/hsl7-* yeast protein. Displayed are secondary product ions produced from the primary product ions 172.2 *m/z* (**a**) and 144.0 *m/z* (**b**) from both ηMMA and δMMA derived from *rmt1-/hsl7-* yeast grown in natural abundance (*top*) and [$C\varepsilon D_3$]methionine minimal media. Peaks in the *bottom* spectra that have a single deuterated methyl group are 3 Da heavier than those that have natural abundance methyl groups. The quantification of ηMMA and δMMA is shown from WT, *rmt1-*, *hsl7-*, and *rmt1-/hsl7-* yeast grown under log-phase conditions in YEPD (**c**). The concentrations depicted are mean and standard deviation of two samples, and are normalized to account for differences in total protein

increased by as much as sevenfold and aDMA decreased by 11-fold during heat shock and stationary phase as compared to log-phase growth on yeast proteins. Additionally, the concentrations of ηMMA, and δMMA decreased by half during heat shock and stationary phase compared to log-phase growth of wild-type yeast. Our results show that under stressful conditions, sDMA increases, and aDMA, δMMA and ηMMA decrease on yeast proteins. As both heat shock and stationary-phase conditions lock yeast in the G1 phase of the cell cycle (Herman 2002; Verghese et al. 2012), the observed aDMA and sDMA concentrations may also reflect global changes in protein methylation patterns attributable to entry into the G1 phase.

Previous studies have noted a link between the response to nutrient deprivation conditions encountered during stationary-phase growth and arginine methylation. Ras2p regulates the nitrogen starvation response (Broek et al. 1985; Parrini et al. 1996) and has been shown to associate with Rmt1p (Jackson et al. 2012). The recycling of amino acids is also a component of stationary growth (Herman 2002), and some proteins involved in this process are arginine methylated. ECM29 is part of the proteasome core particle, which recycles proteins and is monomethylated at R542 and dimethylated at R1112. The vacuolar protein sorting-associated protein 52, monomethylated on R224, is required for the recycling of proteins from endosomes (Pang et al. 2010). Recently, a direct link between the activity of Rmt1p and starvation has been established. It was found that the stability of the mRNA for B-type cyclin CLB2, involved in regulating progression to the M phase, was increased by Rmt1p methylation of heterogeneous ribonucleoproteins to promote their nuclear localization and thereby stabilize CLB2 mRNA (Messier et al. 2013). Interestingly, our results are congruent with this study since we find that aDMA concentrations on yeast proteins are 11-fold lower during stationary phase growth (Fig. 4d).

Yeast lacking *rmt1*- produce less aDMA rather than eliminate it altogether. As Rmt1p is the only RMT known to produce aDMA, this result suggests the presence of an additional RMT (other than Rmt1p) capable of forming aDMA on yeast proteins. With respect to sDMA, *hsl7*- yeast produce nearly half the sDMA of their WT counterparts (Fig. 3c, and d); therefore, sDMA production in yeast appears dependent upon Hsl7p under log-phase growth conditions. Nevertheless, sDMA production is not eliminated in *hsl7*- strains. In fact, under stationary-phase growth, the *hsl7*- strain produces more than twofold additional sDMA than WT yeast grown under the same condition (Fig. 4). We have previously observed in HeLa cells that ectopically express enzymes that only produce aDMA, increased the production of both aDMA and sDMA on proteins, suggesting changes in expression or activity of other PRMTs (Pak et al. 2011). A potential explanation is that when specific RMT enzymes are knocked out, additional narrow substrate specificity enzymes compensate by broadening substrate specificity. This phenomenon has been observed previously in PRMT1 knockout cells (Dhar et al. 2013) and comports with our in vitro data that suggest that PRMTs will methylate histones that they do not normally prefer if they are the only available substrates (Lakowski et al. 2010b). These results suggest that enzymes other than Hsl7p may be producing sDMA in yeast. This study adds to an increasing body of evidence that additional protein methyltransferases remain to be discovered in yeast (Niewmierzycka and Clarke 1999; Lipson et al. 2010; Low and Wilkins 2012; Young et al. 2012; Erce et al. 2013).

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Compliance with ethical standards

Conflict of interest The authors have declared no conflict of interest.

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