Metabolic Engineering in Yeast Demonstrates That S-Adenosylmethionine Controls Flux through the Methylenetetrahydrofolate Reductase Reaction in Vivo*

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One-carbon flux into methionine and S-adenosylmethionine (AdoMet) is thought to be controlled at the methylenetetrahydrofolate reductase (MTHFR) step. Mammalian MTHFRs, the yeast MTHFR encoded by the MET13 gene is NADPH-dependent and is inhibited by AdoMet in vitro. This contrasts with plant MTHFRs, which are NADH-dependent and AdoMet-insensitive. To manipulate flux through the MTHFR reaction in yeast, the chromosomal copy of MET13 was replaced by an Arabidopsis MTHFR cDNA (AtMTHFR-1) or by a chimeric sequence (Chimera-1) comprising the yeast N-terminal domain and the AtMTHFR-1 C-terminal domain. Chimera-1 used both NADH and NADPH and was insensitive to AdoMet, supporting the view that the C-terminal domain is responsible for AdoMet inhibition. Engineered yeast expressing Chimera-1 accumulated 140-fold more AdoMet and 7-fold more methionine than did the wild-type and grew normally. Yeast expressing AtMTHFR-1 accumulated 8-fold more AdoMet. This is the first in vivo evidence that the AdoMet sensitivity and pyridine nucleotide preference of MTHFR control methylenoegenesis. 14C labeling data indicated that glycine cleavage becomes a more prominent source of one-carbon units when Chimera-1 is expressed. Possibly related to this shift in one-carbon fluxes, total folate levels are doubled in yeast cells expressing Chimera-1.

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Methylenetetrahydrofolate reductase (MTHFR)1 catalyzes the reduction of 5,10-methylenetetrahydrofolate (CH2-THF) to 5-methyltetrahydrofolate (CH3-THF), which serves as a methyl donor for the synthesis of methionine. Methionine gives rise to S-adenosylmethionine (AdoMet), which is used for numerous methylation reactions. Because the MTHFR reaction commits the methyl group carried by THF to methyl group biosynthesis, the regulation of MTHFR is crucial for one-carbon (C1) metabolism in all organisms (1, 2). The MTHFR reaction in mammalian liver is NADPH-dependent and is consequently physiologically irreversible due to the high cytosolic NADPH/NADP ratio and the large standard free energy change for the reduction of CH2-THF (3–5). This reaction therefore has the potential to deplete the cystolic CH2-THF pool (1, 2). Mammalian MTHFRs have been shown to be inhibited by AdoMet (6, 7), and this sensitivity is considered to be a key regulatory feature that prevents CH2-THF depletion (1, 2, 6). However, there is no direct evidence for the existence of this regulation in vivo.

Plant MTHFRs are NADH-dependent and are thought to be cytosolic. Due to the high cytosolic NAD/NADH ratio (8), the MTHFR reaction in planta is likely to be reversible, obviating a need for regulation by AdoMet (9). Consistent with this, plant MTHFRs are AdoMet-insensitive (9). Both Saccharomyces cerevisiae and Schizosaccharomyces pombe have two divergent copies of the MTHFR gene. The MTHFR genes of S. cerevisiae (MET12 and MET13) have been cloned and shown to encode functional enzymes (10), but neither their pyridine nucleotide requirement nor their AdoMet sensitivity has been investigated. Strains in which MET13 is disrupted require methionine for growth. However, met12 disruptants have no methionine requirement, and overexpressing Met12p does not eliminate the methionine requirement of met13 disruptants. These data indicate that the Met13p isozyme provides most of the MTHFR activity in yeast cells (10).

All known eukaryotic MTHFRs comprise an N-terminal domain that contains the catalytic site and a C-terminal domain that is implicated in the regulation of enzymatic activity (11–13). The C-terminal domain of mammalian MTHFRs contains the AdoMet-binding site and is therefore considered responsible for the enzyme’s sensitivity to this effector (11–14). The function of the C-terminal domain of plant MTHFRs, which are insensitive to AdoMet, is unknown (9). The NADH-dependent,
AdoMet-insensitive MTHFR of *Escherichia coli* (MetF) lacks the C-terminal domain (15–17).

In this study, we first showed that *S. cerevisiae* Met13p is NADP-dependent and inhibited by AdoMet, like mammalian MTHFRs. We then constructed a chimeric yeast-plant MTHFR (Chimera-1) and characterized its pyridine nucleotide dependence and AdoMet sensitivity. Finally, we used metabolically engineered yeast strains expressing AdoMet-insensitive MTHFRs (AmTHFR-1 or Chimera-1) to establish the importance of the AdoMet feedback loop in vivo.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—[1-14C]Formaldehyde (53 mCi/mmol) was purchased from PerkinElmer Life Sciences PerkinElmer Life Sciences, and [6(2R,6S)-methyl-1-13C]-THF (56 mCi/mmol) was obtained from Amersham Biosciences, Inc., specific radioactivities were adjusted to the desired values with unlabeled compound. THF and [6(2R,6S)-13C]-THF were obtained from Schircks Laboratories (Jona, Switzerland). [13C]Formic acid (99% 13C) was from Cambridge Isotope Laboratories (Andover, MA). Glucose-6-phosphate dehydrogenase (recombinant *Leuconostoc mesenteroides*) enzyme and all other biochemicals were from Sigma. Glass beads were acid-beaded.

*Construction of Overexpression in Yeast—CHIMERA-1 was constructed using gene splicing by overlap extension (18). DNA fragments were amplified by high-fidelity PCR using recombinant *Pfu* DNA polymerase (Stratagene). The Met13p N-terminal domain (residues 1–333) was amplified from the MET13-pVT101-U plasmid using primers 5′-CATAGGATACGCAAAATAGGACG-3′ (forward) and 5′-GGTTTGCAAGAGATGGCTGACTCC-5′ (reverse). The AmTHFR-1 C-terminal domain (residues 327–592) was amplified from the AmTHFR1-pVT103-U plasmid using primers 5′-CCTATCTTCTGGGCAACCGTCCAAAGAGC-3′ (forward) and 5′-TGCACTGCGATCAAGAAAGAGAAGACAGAGAAGC-3′ (reverse). The two amplified fragments were mixed in a 1:1 ratio and joined by PCR using MET13 forward and AmMTHFR-1 reverse primers to generate the CHIMERA-1 DNA. The CHIMERA-1 DNA was inserted into yeast expression vector pVT103-U. The resulting construct was introduced by electroporation into *E. coli* DH10B cells. After verification by sequencing, the construct was introduced as described previously (10) into yeast strain RRY1 (Δmet12::URA3 met13::LEU2) and RRY5 (Δmet13::LEU2 met12::LEU2) for enzyme studies and into strain SCY1 (Δmet13::LEU2 met12::LEU2) for complementation.

**Yeast Strains, Plasmids, and Growth Conditions**—Synthetic minimal medium (YMD medium) contained 0.7% yeast nitrogen base without amino acids (DIFCO Bacto), 2% glucose, and the following supplements when indicated: i-serine (375 mg/liter), i-leucine (30 mg/liter), i-histidine (20 mg/liter), i-tryptophan (20 mg/liter), uracil (20 mg/liter), glycine (20 mg/liter), formate (250 mg/liter), and i-methionine (20 mg/liter). Cultures were grown at 30 °C in a rotary shaker at 250 rpm. Pre-sporulation medium contained 0.8% Bacto-yeast extract, 0.3% Bacto-peptone, 1% glucose, and 2% (w/v) sodium acetate. Synthetic minimal medium contained 1% potassium acetate and 2% Bacto-agar. The *S. cerevisiae* strains used were DAY4 (ser1 ura3–52 trp1 leu2 his4 lac1), RRY1 (Δmet12::URA3 met13::LEU2) (9), the CHIMERA-1 plasmid was used as template to construct strain RRY5 (ser1 ura3–52 trp1 leu2 his4 Δmet12 Δmet13::URA3) for enzyme studies and into strain SCY1 (Δmet12::LEU2 met13::LEU2) for complementation.

**Preparation of Extracts for NMR Analysis**—Yeast cells were grown in 500-ml cultures supplemented with [13C]formate (250 mg/liter) and unlabeled glycine, harvested at an *A*<sub>600</sub> of 3–5, and washed with water. Extracts were prepared by resuspending the cell pellet from the whole culture in 500 µl of water and lysing with glass beads by continuous vortexing at 4 °C for 20–30 min. Lysates were clarified by centrifugation, and proteins were precipitated with ethanol as described above. The deproteinized samples were submitted to Biosynthesis Inc. (Lewisville, TX) for amino acid analysis using a Beckman 7300 analyzer.

**NMR Analysis**—NMR spectra were obtained on a Varian Unity-Inova Spectrometer with a 1H frequency of 500 MHz and a 13C frequency of 125 MHz. [13C] data were collected with an acquisition time of 1.3 s with a 3-s delay and 90° pulse angle. Two-level composite pulse was used for proton decoupling with a power level of 48 dB during acquisition and 40 dB during delay. A total of 2000–20000 scans of 64,000 data points were acquired over a sweep width of 25 kHz. Data processing included exponential line broadening of 2 Hz. [6(2R,6S)-13C]AdoMet was quantified by comparing NMR peak heights with a standard sample of known concentration.

**Folate Determination**—Yeast strains were grown, harvested, and washed as described for AdoMet and methionine determination. Pelleted cells were suspended in twice their wet weight of extraction buffer (50 mM Hepes, 50 mM 2-(cyclohexylamino)ethanesulfonic acid, 0.2 mM EDTA, 0.2 mM sodium ascorbate, pH 7.9). Glass beads were added at 1.5 times the wet weight of the pellet. The samples were heated at 100 °C for 10 min and lysed by vortexing for 4 min. After centrifugation (20,000 × *g*, 30 min), supernatants were decanted, their volumes were measured, and they were stored at −70 °C until analysis. Folate species were separated by HPLC and quantified using the *Lactobacillus casei* microbiological assay (21, 22).

**RESULTS**

**NADP Dependence and AdoMet Sensitivity of Yeast MTHFR Met13p**—Recombinant Met13p was overexpressed in RRY3, a met12 met13 double mutant that totally lacks MTHFR activity and is a methionine auxotroph (10). The pyridine nucleotide preference and AdoMet sensitivity of recombinant Met13p were tested by measuring enzyme activity radio metrically in the forward (reductive) direction in 100 mM potassium phosphate buffer, pH 7.2 (9). Met13p was found to be strictly NADP-dependent and to be inhibited by AdoMet.
Role of MTHFR Sensitivity to S-Adenosylmethionine

Fig. 1. Pyridine nucleotide preferences and S-adenosylmethionine sensitivities of recombinant MTHFRs. Desalted crude extracts of RRY3 (Δmet12 Δmet13) yeast cells expressing Met13p (A), Chimera-1 (B), or AtMTHFR-1 (C) were assayed for NADPH-CH₂-THF oxidoreductase activity using NADH or NADPH (200 μM) as reductant, plus or minus 1 mM AdoMet; CH₂-THF concentration was 0.5 mM. Extracts were preincubated for 15 min at 24 °C with buffer or AdoMet before the assays. Data are means ± S.E. (n ≥ 3).

(Fig. 1A). Activity with NADH as reductant was below the detection limit of the assay (<2% of activity with NADPH).

Construction of a Chimeric Yeast-Plant MTHFR—A chimeric MTHFR (Chimera-1) was constructed by fusing the N-terminal (catalytic) domain of Met13p to the C-terminal domain of a plant MTHFR (AtMTHFR-1 from Arabidopsis thaliana; Ref. 9). The domains were spliced together by overlap extension PCR (18) within a short, conserved amino acid sequence (VRPIFW) that is immediately downstream of a region identified as the interdomain bridge in mammalian MTHFRs (12). Fig. 2A shows the domain structure of the chimeric enzyme and its parents schematically; Fig. 2B shows the sequence of the Chimera-1 polypeptide.

Complementation of a Yeast Δmet13 Mutant by CHIMERA-1—The CHIMERA-1 DNA was cloned into the yeast expression vector pVT103-U and introduced into strain SCY1 (MET12 Δmet13). A MET12" strain was used to avoid any possible growth effects of a met12 mutation. The transformation yielded methionine-independent colonies whose growth was comparable with that of the wild-type strain DAY4, or SCY1 transformed with MET13-pVT101-U (Fig. 3). No complementation was observed with the vector alone, and retransformation of SCY1 with rescued plasmid containing CHIMERA-1 restored methionine prototrophy, confirming that the complementation is due to the plasmid-encoded chimeric enzyme (data not shown). Arabidopsis MTHFR (AtMTHFR-1), expressed from pVT103-U, was previously shown to complement the methionine requirement of a yeast met13 mutation (9).

Pyridine Nucleotide Preference and AdoMet Sensitivity of Chimera-1—Recombinant Chimera-1 overexpressed in yeast strain RRY3 was tested for pyridine nucleotide preference and AdoMet sensitivity as described above for Met13p. Chimera-1 was found to use both NADH and NADPH (Fig. 1B), which was unexpected because its yeast and plant parents use either one or the other (Fig. 1, A and C). However, as anticipated, Chimera-1 was totally insensitive to AdoMet (Fig. 1B), like the plant enzyme that contributes its C-terminal region (Fig. 1C).

Kinetic Properties of Met13p, AtMTHFR-1, and Chimera-1—Velocity versus [S] plots for both Met13p and AtMTHFR-1 showed substrate inhibition by CH₂-THF that was especially marked in the case of Met13p (Fig. 4). The Kₘ and Kᵥ values for these two enzymes for CH₂-THF were accordingly calculated as described by Cleland (23). Chimera-1 showed no inhibition by CH₂-THF within the range tested (Fig. 4). Its Kᵥ values for CH₂-THF were therefore estimated from Hanes plots; these values were similar whether NADH or NADPH was the reductant and fell midway between those of the parent enzymes (Table I). Because the activities of Met13p and AtMTHFR-1 were inhibited by CH₂-THF, apparent Kₘ values of these enzymes for NADPH and NADH were compared with those of Chimera-1 using a nonsaturating CH₂-THF concentration of 50 μM (Table I). The apparent Kᵥ values of Chimera-1 for both NADP and NADH were closer to that of AtMTHFR than to that of Met13p.

Construction of Yeast Strains with a Chromosomal Copy of CHIMERA-1 or AtMTHFR-1—To study how AdoMet sensitivity and pyridine nucleotide specificity impact the function of MTHFR in vivo, we engineered strains in which the coding sequence of AtMTHFR-1 or Chimera-1 replaced that of the chromosomal MET13 gene. The introduced sequences were thus present as single chromosomal copies under the control of the native promoter, ensuring an expression level comparable to that of MET13. Replacement of the MET13 open reading frame with Chimera-1 and AtMTHFR-1 gave strains SCY4 and SCY6, respectively. The growth rates of these strains in YMD medium supplemented with serine, histidine, leucine, tryptophan, and uracil were similar to that of the wild-type (doubling times, 2.5 ± 0.2 h). Thus, both the chimeric enzyme and the plant enzyme complemented the methionine requirement when expressed from the chromosomal MET13 promoter.

Levels of AdoMet and Methionine in DAY4, SCY4, and SCY6 Yeast Strains—AdoMet and free methionine were determined by HPLC. In the experiment shown in Fig. 5, strain SCY6 expressing the AdoMet-insensitive, NADH-dependent AtMTHFR-1 accumulated 8-fold more AdoMet than the DAY4 strain expressing the native Met13p enzyme. Strain SCY4 expressing the AdoMet-insensitive, NADH- or NADPH-dependent enzyme Chimera-1 accumulated even more AdoMet (140-fold more AdoMet than DAY4) (Fig. 5A). Along with the greatly elevated AdoMet level, strain SCY4 accumulated 7-fold more methionine than DAY4 (Fig. 5B). AdoMet hyperaccumulation was seen in SCY4 cultures harvested at a range of cell densities; its magnitude varied from 75-fold to 254-fold above wild-type in four independent experiments (data not shown).

Origin of the C₁ Units Used for AdoMet Synthesis—The yeast strains used in this study are all ser1 strains that are blocked at phosphoserine aminotransferase and thus require serine for growth; glycine (or glycine plus formate) can substitute for serine. Use of ser1 strains allows controlled introduction of C₁ units via the cytosolic C₁-THF synthase by providing formate or via the mitochondrial glycine cleavage system by providing glycine (24). To identify the source(s) of C₁ units, we used production of AdoMet, we determined the methyl-¹³C enrichment of AdoMet in cultures supplemented with [¹³C]formate and unlabeled glycine. The methyl-¹³C enrichment under these conditions measures the relative contributions to methyl group synthesis of C₁ units originating from cytosol and mitochondria (25, 26). The methyl-¹³C enrichment values were 0.92 for the
wild-type expressing Met13p and 0.54 for a strain expressing Chimera-1. These data show that with wild-type Met13p, 90% of AdoMet comes from [13C]formate, whereas only 54% comes from [13C]formate when Chimera-1 replaces Met13p. Thus, AdoMet hyperaccumulation is associated with a far greater contribution from mitochondrial glycine cleavage to the C1 unit pool.

Intracellular Folate Levels in Yeast Strains DAY4, SCY4, and SCY6—The pools of 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, 5-CH3-THF, and THF were determined by HPLC combined with microbiological assay (21, 22). The most abundant folate in all strains was 5-CH3-THF, constituting ≥68% of the total folate pool. The other folates (10-formyltetrahydrofolate, 5-formyltetrahydrofolate, and THF) each represented <15% of the total in all strains (Table II). The most striking difference between the strains was that SCY4 expressing Chimera-1 had more than twice the total folate content of the wild-type strain DAY4 (156 versus 66 nmol g−1 wet weight), although the relative amounts of the individual folates were essentially unchanged. Another difference was that strain SCY6 expressing AtMTHFR-1 showed only a slight increase in total folate relative to the wild-type DAY4, but its 10-formyltetrahydrofolate and THF pools were doubled.

FIG. 2. The primary structure of Chimera-1 and its parent enzymes. A, schematic representations of Chimera-1 and its parents, Met13p (black) and AtMTHFR-1 (white). The pyridine nucleotide specificity of each enzyme is indicated. Chimera-1 comprises the N-terminal domain of Met13p and the C-terminal domain of AtMTHFR-1. The gray bar marks the hydrophilic bridge region between the domains, and the white triangle shows the position of the AdoMet-binding site identified by photoaffinity labeling of mammalian MTHFR (11, 13). B, amino acid sequence of Chimera-1. The sequence from Met13p is in black, and the sequence from AtMTHFR-1 is in gray.

FIG. 3. Functional complementation of yeast MTHFR mutants by Chimera-1. Similar numbers of cells of DAY4 (wild-type) (1), SCY1 (MET12 Δmet13) (2), or SCY1 transformed with pVT101-U containing MET13 (3) or pVT103-U containing CHIMERA-1 (4) were plated on synthetic minimal medium plus or minus methionine. Strain SCY4, whose chromosomal copy of MET13 is replaced by CHIMERA-1, is shown (5).

FIG. 4. Dependence of recombinant MTHFR activity on CH2-THF concentration. Desalted extracts of RRY3 (Δmet12 Δmet13) yeast cells expressing Met13p, AtMTHFR-1, or Chimera-1 were assayed for NAD(P)H-CH2-THF oxidoreductase activity using an NADH or NADPH concentration of 100 μM and CH2-THF concentrations of 10–1500 μM. Enzyme activities are expressed as a percentage of the maximum activity obtained for each. Maximum activities (in nmol min−1 mg−1 protein) were as follows: Met13p, 12.1; AtMTHFR-1, 27.9; and Chimera-1, 92.2. Only the values obtained with NADPH as reductant are shown for Chimera-1; values with NADH were very similar. All data shown are means of duplicate observations; S.E. bars were smaller than the data points.
Characterization of yeast Met13p shows that its properties differ from those of other MTHFRs. Met13p is almost completely reduced NADPH/NADP ratio (1.2). The enzymes are about 30 residues shorter than the human enzyme. The assay is carried out in the presence of phosphate, for this procedure. The relative low cytosolic NADH/NAD ratio may limit the extent of AdoMet accumulation due to the lack of feedback inhibition by AdoMet. The relative low cytosolic NADH/NAD ratio may limit the extent of AdoMet accumulation due to the lack of feedback inhibition by AdoMet.
reaction freely reversible (9). Reliable measurements of cysteolic NAD(P)/H/NAD(P) ratios in yeast are lacking. However, studies with yeast cysteolic CH2-THF dehydrogenase show that the NADP-dependent reaction is reversible in vivo, whereas the NAD-dependent reaction runs only in the oxidative direction (29). This indirect evidence suggests that the yeast cysteolic NADP pool is far more reduced than the NAD pool. An AdoMet-insensitive MTHFR that is driven by the aggregate cysteolic NAD(P)/H/NAD(P) ratio might therefore be expected to direct more C1 units to CH2-THF, and thence to AdoMet, than one driven solely by the NADH/NAD ratio.

The finding that expressing an NADH-dependent, AdoMet-insensitive plant MTHFR in yeast causes methionine and AdoMet overaccumulation raises the question of what prevents this from occurring in plants. Plants have the S-methylmethionine cycle, an auxiliary feature exclusive to plant C1 metabolism (30, 31). In this apparently futile cycle, AdoMet donates a methyl group to methionine, giving S-methylmethionine. S-Methylmethionine then donates a methyl group to homocysteine, yielding two molecules of methionine. The net effect of the cycle is thus to convert AdoMet back to methionine. In vivo radiolabeling data and metabolic modeling have recently indicated that the S-methylmethionine cycle serves to stop accumulation of unutilized AdoMet and that it may be the main mechanism whereby plants achieve short-term control of AdoMet levels (31). Because S. cerevisiae does not have this cycle, AdoMet might be expected to accumulate in yeast expressing the plant enzyme, as in fact occurs. The 140-fold hyperaccumulation of AdoMet has no apparent ill-effect on yeast growth, at least under our culture conditions. A likely reason is that most of the excess AdoMet is sequestered in the vacuole and hence excluded from the metabolically active pool, as occurs when AdoMet is overproduced in yeast cultured with a high level of methionine in the medium (32). It should be noted that although AdoMet hyperaccumulation was a robust phenomenon in strain SCY4 grown as we describe in medium containing formate and glycine, it did not occur when these supplements were replaced by serine. The basis for this effect is now being investigated.

In wild-type yeast, the dominant methylated products are phosphatidicholine head groups in membranes (~12 μmol methyl units g−1 wet weight) and protein-bound methionine (~14 μmol g−1 wet weight, assuming 100 mg protein g−1 wet weight and a methionine content of 2 mol %) (33) (Proteome Analysis at EBI, 2001, www.ebi.ac.uk/proteome). The total level of methyl end products is therefore at least 26 μmol g−1 wet weight. In relation to this, the AdoMet pool in the wild-type strain DAY4 (31 nmol g−1 wet weight) is very small (<0.2%). However, in the AdoMet-hyperaccumulating strain, the AdoMet pool expands to ~4 μmol g−1 wet weight, which adds ~15% to the methyl budget. To investigate the source of the extra methyl groups, we compared the 13C-enrichment of the AdoMet methyl group in these two strains supplied with [13C]formate and unlabeled glycine. In the wild-type, DAY4, formate was the principal source of the AdoMet methyl group, consistent with the previous data on the origin of choline methyl groups in this strain (29). Surprisingly, even though the production of methyl groups in the AdoMet-hyperaccumulating strain changed by only ~15%, the formate contribution to the AdoMet methyl group dropped dramatically from >90% to 54%, with the remainder presumably coming from glycine cleavage in the mitochondria. If we make the reasonable assumption that AdoMet methyl 13C enrichment faithfully reflects that of methionine, this finding shows that AdoMet hyperaccumulation is associated with a profound change in the relative importance of cysteolic and mitochondrial C1 fluxes.

Consistent with the above inference, and equally surprising, strain SCY4 expressing Chimera-1 showed a doubling of total cellular folate content compared with the wild-type. This folate pool expansion could be a response to the shift in C1 fluxes occasioned by unregulated AdoMet synthesis. Whatever the mechanism, the data imply that cross-talk between C1 metabolism and folate synthesis or degradation is an important factor in control of intracellular folate levels.

REFERENCES
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Additions and Corrections

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Page 4059, Fig. 2B: The amino acids 51–250 should read as indicated in the sequence shown below. DNA sequencing indicated these minor errors in the published sequence.

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51 IDITWAGGGLSRLSLSTDLVATAGSVLGETCHGLCTNTMPSMIDDLE
101 NAYHSCQHILALGQPDRDAGSANVFVEEGGQYAKDILKYIKSKYGZHFA
151 IGVAGYECHELFERGDKLDELYLKQGDAQDPFIITQNYDYDGNFINW
201 CSQVRAGGEMTVPIDTYPWTTYAAFLRRAGQGQISIPQHFSRSRLDPKRD
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Fig. 2B

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Clustering of CD40 ligand is required to form a functional contact with CD40.

Heike Grassmé, Jürgen Bock, Jutta Kun, and Erich Gulbins

Page 30292, legend to Fig. 2A: “Cy3-labeled” should be “Cy2-labeled.” The second sentence under A should be: “Surface ceramide was detected by FACS analysis of cells incubated with Cy2-labeled goat anti-ASM.” This does not affect the results of the paper.

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