confidence level, also earlier noted by (*18*)]. Lake-level rise was significantly higher in 8 of the 11 years of low SST used by Nobre and Shukla (*5*), and was lower in 7 of 10 years of high SST. We conclude that our reconstructions of rising or overflowing levels of Lake Titicaca can be used to deduce the timing of wet conditions on the Altiplano and in Amazonia. Also, wet conditions in both regions are promoted by below-normal SST in the northern equatorial Atlantic.

Do modern controls on precipitation variability apply to the past? Melice and Roucou (*18*) showed that a 12- to 14-year periodicity of the $\delta^{18}O_{ice}$ signal persisted throughout the past 500 years of the Quelccaya record (*20*) and was correlated with SST variability in the tropical North Atlantic. Their findings imply that for the past 500 years there has been a persistent, quasi-periodic fluctuation of tropical Atlantic SST that influenced precipitation variability on the Altiplano and in Amazonia.

On longer time scales, millennial-scale cold events observed in North Atlantic SST records (*26*) during the last glacial stage were manifested as wet events in tropical South America (*14*). Anomalously low equatorial North Atlantic SSTs during the LGM (*27*), the Younger Dryas (*26*), or during Holocene cold events (*28, 29*) such as the Little Ice Age, were as much as 20 times the magnitude of the largest modern interannual SST anomalies (around 0.5°C), so the potential SST forcing of atmospheric variability during such events was far stronger than during the past century of direct measurement. We assert that this large variability of equatorial North Atlantic SST exerted a strong control on the variability of precipitation on the Altiplano and in Amazonia, on millennial and orbital time scales.

DeMenocal and co-workers (*29*) established that Holocene-age cold events of the high-latitude North Atlantic Ocean (*28*) were in phase with SST decreases, enhanced upwelling, and increased accumulation of Saharan dust, in the eastern equatorial North Atlantic. These events appear to correspond to periods of rising or overflowing level of Lake Titicaca (Fig. 3A), allowing for the possible need for a reservoir correction of about 250 years for our middle Holocene radiocarbon dates.

With few exceptions (*30, 31*), researchers have previously concluded that Amazonia was arid during the LGM, so our finding of a wetter-than-modern Altiplano and Amazonia during the LGM warrants explanation. There are three physical explanations for LGM wetness in Amazonia. First, wet season insolation was at a maximum in the southern tropics at 20,000 cal yr B.P. (Fig. 3F) (*14, 32*); thus, the South American summer "monsoon" (*33*) was maximized [and early Holocene insolation and precipitation were minimal, exactly out of phase with the northern tropics (*29, 34*)]. Second, during the LGM, zonal [cold in the east (*26*), warm in the west (*35*)] and meridional [cold in the north, warm in the south (*27*)] SST gradients in the equatorial Atlantic were favorable for enhanced SST forcing of the northeast trades and atmospheric advection of water vapor into Amazonia (*14*). Third, lower equatorial Atlantic SST favored increased gradients between land and sea-surface temperature during the austral summer, also enhancing water vapor transport into Amazonia.

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Substitution of the Thioredoxin System for Glutathione Reductase in *Drosophila melanogaster*

Stefan M. Kanzok,[1,2] Anke Fechner,[1,2] Holger Bauer,[1] Julia K. Ulschmid,[2] Hans-Michael Müller,[3] José Botella-Munoz,[4] Stephan Schneuwly,[4] R. Heiner Schirmer,[1] Katja Becker[1,2]*

The disulfide reducing enzymes glutathione reductase and thioredoxin reductase are highly conserved among bacteria, fungi, worms, and mammals. These proteins maintain intracellular redox homeostasis to protect the organism from oxidative damage. Here we demonstrate the absence of glutathione reductase in *Drosophila melanogaster*, identify a new type of thioredoxin reductase, and provide evidence that a thioredoxin system supports GSSG reduction. Our data suggest that antioxidant defense in *Drosophila*, and probably in related insects, differs fundamentally from that in other organisms.

Aerobic life is based on the metabolization of molecular oxygen. The cytotoxic byproducts of this process are collectively known as reactive oxygen species (ROS). The oxidative challenge exerted by ROS is dealt with by cellular antioxidant defense systems that include low-molecular-weight compounds as well as antioxidant enzymes such as the flavoenzymes glutathione reductase (GR) and thioredoxin reductase (TrxR) (*1–4*). By catalyzing the reaction GSSG + NADPH + H^+ → 2 GSH + $NADP^+$, GR maintains high concentrations of the reducing tripeptide glutathione (GSH) and low levels of its oxidized form, glutathione disulfide (GSSG) ($NADP^+$, nicotinamide adenine dinucleotide phosphate; NADPH, reduced form of $NADP^+$). Reduced glutathione is the most abundant intracellular nonprotein thiol

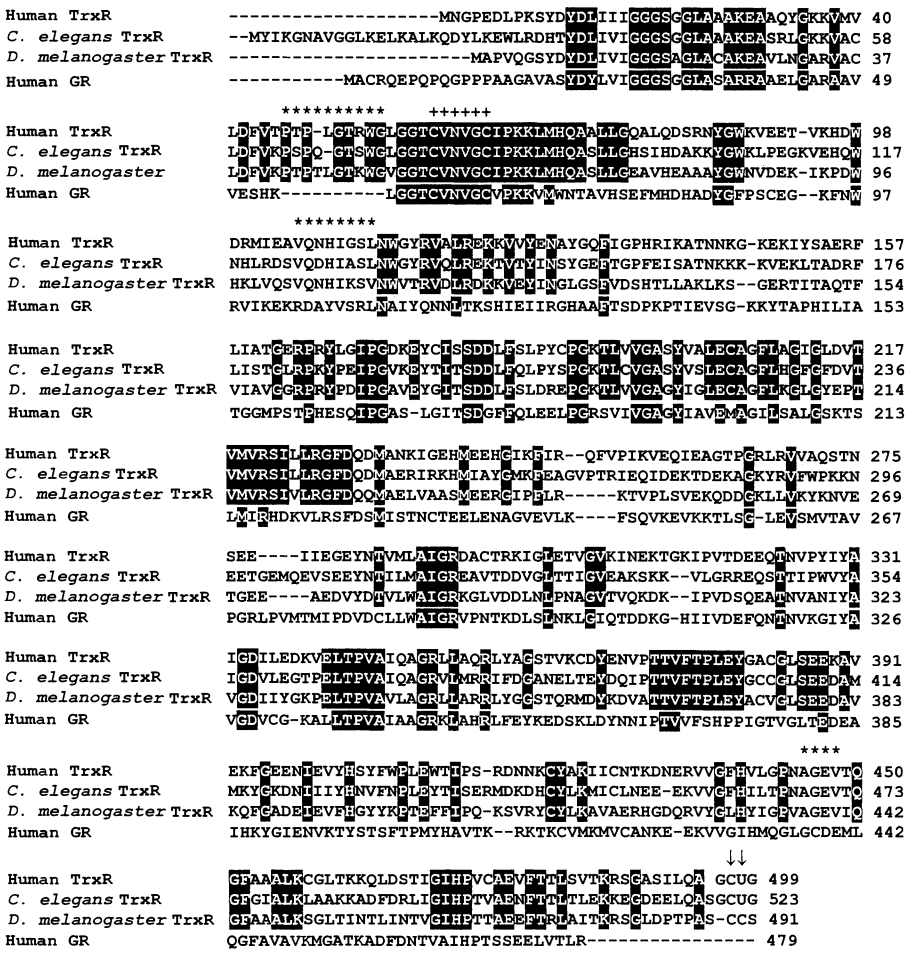

Fig. 1. Alignment of *D. melanogaster* TrxR-1 (this paper, GenBank accession number AF301144) with TrxRs from humans (GenBank accession number AAB35418) and *C. elegans* (GenBank accession number AAD41826) and with human GR (GenBank accession number P00390) (*29*). Sequence identities are shown in black boxes. Asterisk-marked chain segments distinguish high-M_r TrxRs from other members of the disulfide reductase family. Plus signs indicate amino acids of the catalytic site sequence. Vertical arrows in the COOH-terminal region mark the site of the mutations C489S and C490S.

and is an effective antioxidant. TrxR catalyzes the transfer of reducing equivalents from NADPH to thioredoxin (Trx) [NADPH + H^+ + $TrxS_2$ → $NADP^+$ + $Trx(SH)_2$], which in turn reduces a number of proteins such as Trx peroxidase and ribonucleotide reductase (*1–3*). High-molecular-weight (M_r) TrxRs (55 kD per subunit) are closely related to GR both structurally and mechanistically (*3–5*). In contrast to GR, TrxRs exhibit a rather broad substrate spectrum effected by an additional redox center, which is located on the flexible COOH-terminal extension of the protein. GSSG is, however, not reduced by TrxRs (*1–3*, *6*).

A putative *Drosophila melanogaster* GR gene has been cloned (*7*, *8*), but the gene product was not characterized. A sequence similarity search in the Berkeley *Drosophila* Genome Project database (BDGP; www.flybase.org) (*9*) using the published disulfide reductase sequence as a query (*7*, *10*) revealed an open reading frame of 1473 base pairs (bp). This gene (*dmtrxr-1*) contains three introns and occurs in two possible splice variants that differ only in their NH_2-terminal amino acids (*11*). A multiple sequence alignment with GRs and TrxRs from other species (Fig. 1) indicated characteristic TrxR motifs, including the catalytic site, the FAD- and NADPH-binding domains, and the additional COOH-terminally located redox center. Because *dmtrxr-1* is present as an expressed sequence tag (EST) in the BDGP database, the gene is transcribed in vivo. The gene published by Candas *et al.* (*7*) cannot be identified in the BDGP database. The protein deduced from this gene shares only 80% sequence identity with DmTrxR-1 and lacks characteristic GR motifs.

A Trx-like gene, deadhead (*dhd*; GenBank accession number P47938), and the corresponding gene product have been partially characterized in *D. melanogaster* (*12*, *13*). We expressed this gene (*14*) and obtained a functional Trx of 107 amino acids (12.4 kD). The active site motif (Trp-Cys-Gly-Pro-Cys) is conserved, and three-dimensional modeling [in the SwissProt database (www.expasy.ch/sprot/sprot-top.html)] revealed a classical Trx fold. Therefore, we propose "DmTrx-1" as a synonym for Deadhead.

The *dmtrxr-1* gene was isolated from a *Drosophila* cDNA library, expressed in *Escherichia coli*, and purified to homogeneity (*14*). DmTrxR-1 consists of 491 amino acids and is a homodimeric flavoprotein with Michaelis constant (K_M) values for NADPH, DmTrx-1, and 5,5′-dithio-bis(2-nitrobenzoate) (DTNB) of 6.5 μM, 7.0 μM, and 310 μM, respectively. The specific activity of DmTrxR-1 with NADPH and DmTrx-1 was found to be 24.3 U/mg [turnover number (k_{cat}) = 22.0 s^{-1}; k_{cat}/K_M = 3.1 × 10^6 M^{-1} s^{-1}]. The system followed Michaelis-Menten kinetics, and the K_M values compare well with the data for high M_r TrxRs from other species (*1–3*, *15*, *16*). This observation is supported by the fact that DmTrxR-1 (GenBank accession number AAG25639) shares rather high protein sequence identities with other disulfide reductases; for example, 56% with hTrxR (GenBank accession number AAB35418), 49% with TrxR from *Caenorhabditis elegans* (GenBank accession number AAD41826), and 33% with human GR (GenBank accession number P00390). In addition, the catalytic cycle of DmTrxR-1 resembles the mechanism of a typical flavin-containing disulfide reductase (*4*, *5*) as visualized by absorbance spectroscopy (Fig. 2).

Mammalian TrxRs are characterized by a COOH-terminal Gly-Cys-Sec-GlyOH sequence, which is involved in Trx reduction (*1–4*, *17*, *18*). In contrast, *Drosophila* TrxR has a cysteine in place of selenocysteine. In order to test whether the terminal Ser-Cys^{489}-Cys^{490}-Ser sequence of DmTrxR-1 is capable of and essential for Trx reduction, we generated the protein mutants Cys^{489} → Ser^{489} (C489S) and C490S, as well as the double mutant C489S/C490S (*19*). All mutations resulted in undetectable Trx reduction activity (specific activity < 0.01 U/mg, K_M > 2 mM). The absorbance spectra of the mutants were indistinguishable from that of the wild type, both before and after the addition of NADPH (see Fig. 2). This indicates that the reductive half reaction is not disturbed, the

[1]Center of Biochemistry, Im Neuenheimer Feld 328, Heidelberg University, D-69120 Heidelberg, Germany. [2]Interdisciplinary Research Center, Heinrich-Buff-Ring 26-32, Giessen University, D-35392 Giessen, Germany. [3]European Molecular Biology Laboratory, D-69117 Heidelberg, Germany. [4]Institute of Zoology, Regensburg University, D-93040 Regensburg, Germany.

*To whom correspondence should be addressed at the Interdisciplinary Research Center, Heinrich-Buff-Ring 26-32, Giessen University, D-35392 Giessen, Germany. E-mail: becker.katja@gmx.de

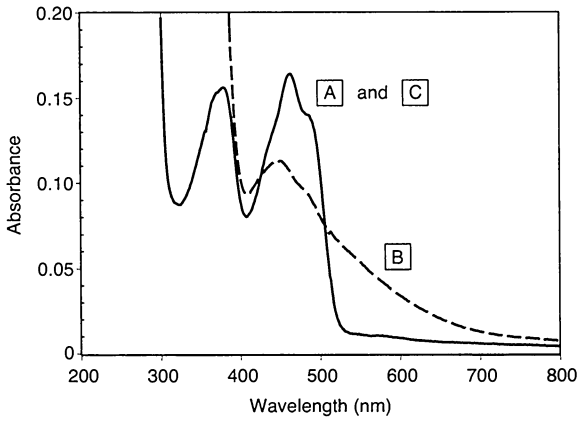

Fig. 2. Flavin absorbance spectra of DmTrxR-1 in oxidized form, after reduction with NADPH and reoxidation with Trx. Spectra were determined for 14 μM DmTrxR-1 subunit in 5 mM NaH_2PO_4 and 50 mM Na_2SO_4 (pH 7.0) without NADPH (A) or with 200 μM NADPH (B). By analogy with other disulfide reductases (*28*), the reaction of the enzyme with NADPH leads to cleavage of an active site disulfide, and one of the nascent thiolates forms a charge transfer complex with the flavin. This gives rise to the absorbance around 540 nm. Because of its COOH-terminal redox pair, the enzyme might have taken up more than two electrons (*4*, *28*). After autoxidation of NADPH, the enzymatic cycle was completed by addition of 25 μM PfTrx-1. The resulting spectrum (C) was identical with the original spectrum (A).

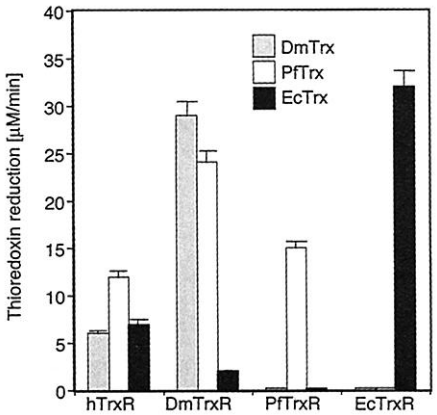

Fig. 3. Reduction of Trxs from different species by TrxR from humans (hTrxR), *D. melanogaster* (DmTrxR), *P. falciparum* (PfTrxR), and *E. coli* (EcTrxR) at 25°C. Each assay mixture contained 1 ml of 100 mM potassium phosphate (pH 7.4), 100 μM NADPH, 10 nM TrxR, and 10 μM Trx. Reduction rates below 1 μM/min represent background activities.

dissociation constant for NADPH being below 4 μM for all mutants. The spectrum of oxidized protein could not, however, be restored by DmTrx-1. Thus, the COOH-terminal Cys^{489}-Cys^{490} pair of DmTrxR-1 is required for reduction of Trx. These data speak against the proposition that selenocysteine in the COOH-terminal Cys-X sequence of high M_r TrxRs is a chemical necessity (*18*).

In order to study the trans-species reactivities of TrxRs and their substrates, we examined the Trx systems from *Drosophila*, the malaria parasite *Plasmodium falciparum*, and *E. coli*. DmTrxR-1 efficiently reduced its cognate Trx as well as Trx from *P. falciparum*, the specific activities being at least twice as high as for human TrxR (hTrxR) (Fig. 3).[4] *E. coli* Trx was, however, a much less efficient substrate. Deadhead, a typical Trx, was reduced by hTrxR but not by the enzymes from *P. falciparum* and *E. coli*. These data are consistent with the fact that the Trx reducing sites differ greatly among TrxRs from higher eukaryotes, protozoal parasites, and prokaryotes (*4*).

Comparative inhibition analyses were conducted on DmTrxR, hTrxR, and human GR (hGR), using the most frequently used disulfide reductase inhibitors (*16*, *20*, *21*). DmTrxR is less accessible to modification by the cytostatic agent carmustine than are the other two enzymes, and it was not inhibited by the phenylisoalloxazine derivatives tested nor by methylene blue. The organic gold compound auranofin, which is known to inhibit hTrxR in almost stoichiometric quantities, is but a weak inhibitor of DmTrxR-1 (*6*). The herbicide paraquat is a turncoat inhibitor of hGR (*21*). Under our assay conditions, 10 μM paraquat inhibited DmTrxR, hTrxR, and hGR to a similar degree of ~30%; at 1 mM paraquat, no enzyme activity was detected. These findings should be considered when interpreting data from studies that use high concentrations of paraquat for generating ROS in order to investigate the transcriptional control of antioxidant enzymes (*7*, *8*).

A previous study on other organisms has shown that various Trx systems, including the systems of *P. falciparum*, *E. coli*, and *D. melanogaster*, can reduce GSSG (*22*). When 100 μM GSSG was tested as a substrate of 10 nM DmTrxR-1, the activity was below the detection limit. However, after adding 10 μM Deadhead, NADPH was completely consumed by the enzyme, the initial activity being 4.3 mU/ml. In additional experiments, the rate of the reaction was found to depend on the concentrations of DmTrxR, Deadhead, and GSSG. Taken together, these results indicate the following reaction sequence for GSSG reduction by the Trx system

$$NADPH + TrxS_2 + H^+ \rightarrow NADP^+ + Trx(SH)_2 \quad (1)$$

$$Trx(SH)_2 + GSSG \xrightarrow{k_2} TrxS_2 + 2GSH \quad (2)$$

Reaction 1 follows Michaelis-Menten kinetics, whereas reaction 2 represents a chemical reaction characterized by the rate constant k_2, which was determined to be 170 M^{-1} s^{-1} (= 0.01 μM^{-1} min^{-1}) (*23*). This value would allow high GSSG fluxes on the order of 10 to 100 μM/min in *D. melanogaster* cells in the absence of GR. Indeed, extensive searches of the *D. melanogaster* genome (*10*) exclude the existence of a gene encoding a typical GR. The lack of GR in *Drosophila* was confirmed experimentally by studies on cultured *Drosophila* Schneider cells and on adult Oregon R flies (*24*). Concentrated extracts showed only traces of GSSG reduction. We therefore isolated putative NADPH-binding enzymes from *Drosophila* Schneider cells or adult flies by affinity chromatography (*24*). The column eluates showed no activity when tested under GR assay conditions with 0.1 to 1 mM GSSG. However, addition of 10 μM DmTrx-1 led to a high and constant rate of GSSG reduction. Thus, a typical NAD(P)H-dependent GR is missing in *Drosophila*, and the observed glutathione reduction is largely supported by the Trx system. This interpretation is corroborated by the fact that the sum of reactions 1 and 2 formally corresponds to the GR-catalyzed reaction. In addition, in extracts of the malaria vector *Anopheles gambiae* a significant GSSG reduction could only be achieved by addition of Trx (*25*). This finding supports the hypothesis that other dipteric insects lack GR as well. The spurious identification of GR activity in insect tissues [reviewed in (*8*)] is explainable by the presence of TrxR and Trx in the extracts or by the fact that DTNB was used as a seemingly specific disulfide substrate for GR.

Because of their tracheal respiratory system, insects are particularly exposed to ROS and depend on high concentrations of intracellular GSH. As demonstrated by Sohal *et al.* (*26*), the [2 GSH]/[GSSG] ratio can serve as an in vivo indicator for oxidative stress response in *Drosophila*. According to our data, the Trx system is capable of maintaining sufficiently high GSH concentrations.

The demonstrated absence of a GR in the fruit fly and the identification of a Trx system as a major player in glutathione metabolism may induce new experimental approaches to biological processes that are based on the molecular mechanism of redox homeostasis, such as adaptation to oxidative stress, aging of aerobically living organisms, and protection from parasites (*27*, *28*).

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10. Analysis of the DNA sequence was performed by BLAST searches of the BDGP database. In addition, a second putative Trx gene (*dmtrx-2*, GenBank accession number AF236866) was identified. The 106–amino acid protein shows 37% sequence identity with DmTrx-1 and 49% with human Trx (JH0568).
11. The *dmtrxr-1* gene was identified after plasmid rescue using an Eco RI digestion of total genomic DNA of a *Drosophila* line carrying a transposon insertion in the X chromosome. The transposon insertion is located downstream of base 93 of a putative *Drosophila* disulfide reductase sequence. This *dmtrxr-1* sequence was found to be identical with the BDGP clot 1274. DNA sequencing was performed with the Pharmacia ALF express system. Based on the genomic sequence derived from the BDGP, the gene structure of *dmtrxr-1* allows two alternative transcripts that differ in the 5′ untranslated region and the first 15 bp. This corresponds to a difference in the NH_2-terminal amino acids: MAPVQ (BDGP EST GM02264; GenBank accession number AF301144) versus MSTKG (BDGP EST GM14215; GenBank accession number AF301145) (*29*). The present work exclusively contains data on the MAPVQ variant (Fig. 1). Furthermore, a putative DmTrxR-2 (GenBank accession number AAF64152) has been identified in the BDGP database, exhibiting 76% protein sequence identity with DmTrxR-1 and 50% with hTrxR. The deduced protein contains an NH_2-terminal peptide of 27 amino acids that is likely to represent a mitochondrial targeting sequence. Multiple TrxRs have been described in other organisms, including humans, mice, and *C. elegans* (*1–3*).
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14. The *dmtrxr* primers were designed according to the BDGP *trxr* sequence (BDGP clot 1274). For *dmtrxr-1*, the forward primer introduced a Sac I restriction site (5′-GCGCGAGCTCGCGCCCGTGCAAGGATCC-3′), and the reverse primer introduced a Hind III restriction site (5′-GCGCAAGCTTTCGACACGTCCCAGGCG-3′). The *dmtrx* primer design was based on the *dhd* sequence by Salz *et al.* (*12*). For *Dmtrx-1*, the forward primer (5′-GCGCGGATCCGCATCCGTACGCACCATGAA-3′) and the reverse primer (5′-GCGCAAGCTTTACGCCTTCACCAGCTTGG-3′) introduced the restriction sites Bam HI and Hind III, respectively. The primers were used in a hot start polymerase chain reaction (PCR) using a *D. melanogaster* head cDNA library as a template (at 95°C for 30 s, 55°C for 1 min, and 72°C for 30 s, for 30 cycles). The resulting amplified PCR fragments were cloned into the *pQE-30* (Qiagen) expression vector, which placed a hexahistidyl tag at the NH_2-terminus of the proteins. The plasmid constructs were introduced into *E. coli* XL1-Blue cells (Stratagene). Gene expression was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C in an overnight culture. The recombinant proteins were purified over a Ni-NTA agarose column. As judged from silver-stained SDS–polyacrylamide gel electrophoresis, the resulting proteins were >98% pure. The yield was 20 mg of DmTrxR-1 and 10 mg of DmTrx-1 per 1 liter of cell culture.
15. DTNB reduction assays for TrxR activity were spectrophotometrically conducted at 25°C in 100 mM potassium phosphate of pH 7.4, containing 200 μM NADPH and 3 mM DTNB as a disulfide substrate (*6*). Trx reduction assays with the Trxs from other species as substrates were performed in the same buffer but in the presence of 100 μM NADPH (millimolar absorbance coefficient at 340 nm = 6.22 mM^{-1} cm^{-1}). GR activity was also assayed at 340 nm and 25°C, but in 47 mM potassium phosphate, 200 mM potassium chloride, and 1 mM EDTA (pH 6.9) in the presence of 100 μM NADPH and 1 mM GSSG.
16. hTrxR was isolated from placenta and hGR was recombinantly produced in *E. coli* (*6*). *E. coli* TrxR, *E. coli* Trx, and human Trx were kind gifts of C. H. Williams Jr. (University of Michigan). *P. falciparum* TrxR (PfTrx) was recombinantly produced as described (*22*). The expression vector *pQE30* was purchased from Qiagen. All chemicals were of the highest available purity.
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19. The DmTrxR-1 mutants C489S and C490S and the double mutant C489S/C490S were generated by site-directed mutagenesis using a plasmid DNA template. In all three cases, the same forward primer introducing a Sac I restriction site was used (5′-GCGCGAGCTCGCGCCCGTGCAAGGATCC-3′). A Hind III restriction site was introduced by the following mutagenesis reverse primers: for C489S, 5′-GCGCAAGCTTTAGCTGCAG**G**AGCTGGCCG-3′; for C490S, 5′-GCGCAAGCTTTAGCTG**G**AGCAGCTGGCCG-3′; for C489S/C490S, 5′-GCGCAAGCTTTAGCTG**G**AG**G**AGCTGGCCG-3′. Mutated bases are in bold; restriction sites are underlined. Gene expression and protein purification were conducted as described for the wild-type enzyme.
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23. Rate constants were determined using an enzymatic $TrxS_2$ reducing system and subsequently transforming it to a GSSG reducing system (*22*). In a cuvette containing 100 μM NADPH, 10 nM DmTrxR-1 subunit, and 10 μM $TrxS_2$, Trx reduction was measured. After the reaction had come to an end, GSSG was added. The system resumed NADPH consumption at a rate that now represents a Trx flux leading to GSSG reduction. On the basis of the employed concentrations, k_2, the rate constant for the reaction between reduced Trx and GSSG, was determined to be 170 M^{-1} s^{-1}, which is equal to 0.01 μM^{-1} min^{-1}. This value was confirmed by varying the total Trx concentration between 5 and 20 μM and the DmTrxR-1 subunit concentration between 1 nM and 10 nM.
24. *D. melanogaster* Schneider cells were grown in Schneider's medium (Gibco BRL). A cell pellet of 750 μl was resuspended in 3 ml of 100 mM potassium phosphate (pH 7.4) and lysed by freezing and thawing three times. The centrifuged lysate was assayed for enzyme activities and then applied to a 0.5-ml 2′,5′–adenosine diphosphate (ADP) Sepharose column. After the column was washed with 10 volumes of the phosphate buffer, disulfide reductases were eluted with 2 mM NADPH. To test the enzyme activity of whole flies, a fly homogenate was obtained by chilling 300 adult OREGON-R flies (~200 mg) and subjecting them to sonication in 5 ml of 100 mM potassium phosphate buffer (pH 7.4). Enzyme activity was determined in the extract or in eluate fractions of the 2′-5′-ADP Sepharose column.
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29. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

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DNA Replication-Independent Silencing in *S. cerevisiae*

Ann L. Kirchmaier and Jasper Rine*

In *Saccharomyces cerevisiae*, the silent mating loci are repressed by their assembly into heterochromatin. The formation of this heterochromatin requires a cell cycle event that occurs between early S phase and G_2/M phase, which has been widely assumed to be DNA replication. To determine whether DNA replication through a silent mating-type locus, *HMRa*, is required for silencing to be established, we monitored heterochromatin formation at *HMRa* on a chromosome and on a nonreplicating extrachromosomal cassette as cells passed through S phase. Cells that passed through S phase established silencing at both the chromosomal *HMRa* locus and the extrachromosomal *HMRa* locus with equal efficiency. Thus, in contrast to the prevailing view, the establishment of silencing occurred in the absence of passage of the DNA replication fork through or near the *HMR* locus, but retained a cell cycle dependence.

Heritable states of gene expression are central to the development of life. Gene repression and activation play pivotal roles in the differentiation of totipotent cells into different cell types, each of which selectively and stably expresses only a subset of the genes in the genome. DNA replication can play a role in changing patterns of gene expression (*1–3*) and thus is a possible mechanism for disrupting chromatin states before their reprogramming and for the de novo establishment of those states. There are also clear examples of changes in gene expression and differentiation that occur independently of DNA replication (*4*).

For years, one of the strongest suggestions of a role for DNA replication in establishing heritable transcriptional states came from studies of yeast mating types. In *S. cerevisiae*, mating competence requires

Division of Genetics and Development, Department of Molecular and Cell Biology, University of California, 401 Barker Hall, Berkeley, CA 94720, USA.

*To whom correspondence should be addressed. E-mail: jrine@uclink4.berkeley.edu