Cover Page



Universiteit Leiden



The handle <u>http://hdl.handle.net/1887/35931</u> holds various files of this Leiden University dissertation

Author: Deventer, Sjoerd van Title: Tracking the big ones : novel dynamics of organelles and macromolecular complexes during cell division and aging Issue Date: 2015-10-21

Chapter 2:

Recombination-Induced Tag Exchange to track old and new proteins

Kitty F. Verzijlbergen¹, Victoria Menendez-Benito², Tibor van Welsem¹, **Sjoerd J. van Deventer**², Derek L. Lindstrom³, Huib Ovaa², Jacques Neefjes², Daniel E. Gottschling³, Fred van Leeuwen¹

Proceedings of the National Acadamy of Sciences USA, Jan 2010, 107 (1): 64-8

¹ Division of Gene Regulation and
² Division of Cell Biology 2,
Netherlands Cancer Institute, Amsterdam, The Netherlands
³ Division of Basic Sciences
Fred Hutchinson Cancer Research Center, Seatle, USA

Abstract

The dynamic behavior of proteins is critical for cellular homeostasis. However, analyzing dynamics of proteins and protein complexes *in vivo* has been difficult. Here we describe Recombination-Induced Tag Exchange (RITE), a novel genetic method that induces a permanent epitope-tag switch in the coding sequence after a hormone-induced activation of Cre recombinase. The time-controlled tag switch provides a unique ability to detect and separate old and new proteins in time and space, which opens up new opportunities to investigate the dynamic behavior of proteins. We validated the technology by determining exchange of endogenous histones in chromatin by biochemical methods and by visualizing and quantifying replacement of old by new proteasomes in single cells by microscopy. RITE is widely applicable and allows probing spatio-temporal changes in protein properties by multiple methods.

Introduction

Proteins are dynamic molecules. Their abundance is controlled by synthesis and degradation and they can be subject to post-translational processing, modification and demodification. In addition, most proteins are very mobile and undergo interactions with multiple other protein partners (1-4). However, little is known about the dynamics of proteins within macromolecular complexes in vivo (2, 4). Studying time-dependent changes in physical properties of proteins or protein turnover requires methods to distinguish resident (old) proteins from new proteins. Current methods that do so are usually based on fluorescent reporters or differential chemical labeling. For example, fluorescence recovery after photo bleaching relies on exchange of the old bleached protein by non-bleached proteins (1, 3, 4). Alternative methods involve time-dependent changes in fluorescence, non-specific pulse-chase labeling of proteins with labeled amino acids, or labeling with chemical dyes that specifically bind to short tags (5-7). Although suitable for detection of proteins by microscopy or mass spectrometry, a limitation of these methods is that they do not provide a handle for biochemical analysis of old and new proteins and their complexes. To solve this problem and to eliminate the requirement for chemical labels or UV light we developed Recombination-Induced Tag Exchange (RITE), a novel method in which a genetic epitope tag is switched by transient induction of a site-specific recombinase. As a consequence, old and newly synthesized proteins are differentially tagged, which enables monitoring of protein dynamics by multiple techniques, as illustrated here. In contrast to inducible expression strategies (8-12), differential tagging by a time-controlled site-specific protease (13), or the labeling methods described above, RITE allows parallel detection and purification of old and new proteins under physiological conditions and over long periods of time.

We used RITE to probe the stability of chromatin. Photo-bleaching experiments using histones tagged with fluorescent reporters suggest that chromatin is a static complex (14). However, recent work suggests that chromatin is more dynamic than previously anticipated (15). For example, ectopically induced histones can be incorporated into chromatin of non-dividing yeast cells and gene activation of certain promoters is accompanied by transient loss of histones (8-12, 16). In metazoans, the histone H3 variant H3.3 can be assembled into chromatin by a replication-independent transcription-

coupled process (17-19). We took advantage of RITE to determine whether endogenously expressed canonical histones undergo replication-independent exchange. RITE can also be used to visualize proteins by microscopy. To demonstrate this we applied RITE to the proteasome, a highly conserved and essential macromolecular complex critical for degradation of proteins by proteolysis (20). Using fluorescent RITE we could visualize the replacement of old by new proteasomes in the nucleus and cytoplasm of dividing cells.

Results

Recombination-Induced Tag Exchange (RITE) outline

RITE can be applied by integration of a RITE cassette downstream of any gene of interest, resulting in a C-terminal tag situated between two LoxP sites with an orphan tag downstream. Upon a transient time-controlled activation of the site-specific Crerecombinase, recombination between the tandem LoxP sites results in exchange of the 'old' tag by an orphan 'new' tag in the coding sequence leading to an epitope-tag switch (Fig. 1). After switching, all newly synthesized mRNAs will encode for proteins containing the new epitope tag. The LoxP recombination sites are part of the coding sequence, which eliminates the need for introns and allows the tag cassette to be introduced directly at the 3' end of any gene of interest to generate a switchable tag. As a consequence, the differentially tagged proteins are encoded by a single gene and under control of the endogenous promoter. Recombination can be induced using a constitutively expressed Cre recombinase fused to the human estrogen binding domain (EBD). This fusion protein is sequestered by heat shock proteins and inactive (21). The nuclear activity of Cre-EBD can be rapidly activated by the addition of β -estradiol, which releases the fusion protein from heat shock proteins (21). A major advantage of RITE is that the genetic switch is permanent.



Fig. 1. Outline of Recombination-Induced Tag Exchange (RITE). RITE cassettes contain two epitope tags (old and new) the first of which is in between two LoxP sites. Integration of a RITE cassette downstream of an open reading frame (ORF) results in a protein tagged with an 'old' tag (blue). The 'old' tag is preceded by an invariant flexible spacer (S) and a short peptide encoded by the LoxP sequence (LoxP), and is followed by a transcriptional terminator (stop) and a selectable marker (select). Upon induction of Cre recombinase, site-specific recombination between the tandem LoxP sites in the genome results in loss of the 'old' tag and fusion of the ORF to the 'new' tag. After the switch, newly synthesized proteins will contain the 'new' tag (yellow), whereas existing proteins will contain the 'old' tag. Old and new proteins are expressed from the same gene by the native promoter.

Therefore, after the switch both old and new proteins can be followed in the original cells and their descendants under any condition of interest. We applied this strategy in haploid yeast cells and integrated RITE cassettes by homologous recombination at endogenous gene loci.

Application of RITE to Histone H3

First RITE was applied to histone H3 to investigate the stability of histones within chromatin. One of the two histone H3 genes was tagged with a RITE cassette containing two small epitope tags, HA and T7 (H3-HA \rightarrow T7) (Fig. 2A). The second histone H3 gene was deleted. As a consequence, in this strain all histone H3 proteins were tagged (Fig. 2A). Yeast cells expressing the tagged histones are viable (Fig. 2B). Since histone H3 is essential, this demonstrates that the tagged H3 proteins are functional. After addition of the hormone β -estradiol, which has no detectable effect on growth or transcription (22), most of the cells had undergone recombination within two hours (Fig. 2C). To confirm that the genetic switch at the DNA level yields differentially tagged proteins, switched starved cells (see below) were released in fresh media and harvested at several time points after re-entry into the cell cycle. Immunoblot analysis demonstrated replacement of old histone H3-HA protein by new H3-T7 in dividing cells (Fig. 2D). The replacement of one tagged protein by the other is in contrast to previously used 'inducible-expression' strategies, which involve



Fig. 2. Application of RITE to endogenous histone H3. (A) One of the two genes encoding histone H3 in yeast (HHT2) was tagged with a RITE cassette (H3-RITE) containing short epitope tags: HA (old) and T7 (new). The other gene encoding histone H3 (HHT1) was deleted. A Hygromycin resistance gene (Hygro) was used to select against illegitimate recombinants. The tag switch was under control of a constitutively expressed hormone-dependent Cre recombinase (Cre-EBD78). (B) Growth of wild-type and H3 RITE-tagged (before [HA] and after [T7] the switch) yeast cells spotted in a ten-fold dilution series. (C) The efficiency of recombination in the cell population was determined by Southern blot analysis of genomic DNA digested with HindIII (H) before (Pre) and after (Post) addition of the hormone β -estradiol. An invariant fragment was used as a control (Ctrl). (D) Detection of old (HA) and new (T7) histone H3 by quantitative immunoblot analysis of whole cell lysates of equal numbers of starved switched cells released into fresh media. The number of population doublings was calculated by staining the cells with NHS-TER (see supplementary methods). (E) The percentage of old H3-HA plotted against the number of population doublings. The measured HA/T7 ratios of the blot in panel D were converted into H3-HA percentages by using standard curves of samples with known percentages of H3-HA and H3-T7 (see supplementary methods).

ectopic expression of a tagged (new) version of a protein by an inducible promoter in the presence of an endogenous copy. Because of ongoing synthesis of the endogenous gene copy, endogenous histones represent old as well as new proteins. As a consequence, the induced and endogenous proteins quickly reach a new steady state. Tagging a single endogenous gene with a RITE cassette eliminates this problem and allows simultaneous tracking of old and new proteins over many cell divisions.

Immunodetection of protein turnover in replicating and non-replicating cells

Quantification of the immunoblot shown in Fig. 2D showed that replacement of old H3-HA by new H3-T7 occurred at a rate faster than expected when only dilution due to replication is taken into account, suggesting histone turnover by replication-independent mechanisms (Fig. 2E). The fact that RITE introduces a permanent genetic switch after a transient signal allowed direct comparison of histone exchange in different cell cycle stages. To minimize new histone mRNA and protein expression during the recombination process the tag switch was performed in nutrient-starved cells, here referred to as G0 (Fig. 3A). Switched H3-HA \rightarrow T7 cells were released into fresh medium containing nocodazole to arrest the cells after passage through one S-phase in G2/M (Fig. 3A and Fig. S2). During S-phase, like the DNA, the amount of histones gets duplicated and incorporated into the chromatin. As expected, cells at the estimated start of the G2/M cell cycle block (t=3 h) showed an approximately equal abundance of old H3-HA and new H3-T7 (Fig. 3B-C). To investigate replication-independent histone exchange, the switched H3-HA T7 cells were released into fresh media containing α -factor to arrest the cells in G1, to prevent passage through S-phase (Fig. 3A). New H3-T7 was detected at the start of the cell block (t=2 h) and increased further during the next three hours (t=5 h). Moreover, the abundance



Fig. 3. Global histone exchange determined by immunodetection. (A) Yeast strains were grown to saturation (here referred to as G0) in complete medium and recombination was induced overnight (switch) by addition of hormone (Fig. S1). Cells were released in fresh media and arrested in G1 (alpha factor) or G2/M (nocodazole). Samples were taken at the estimated start of the arrest (2 h G1 and 3 h G2/M) and three hours later. (B) Quantitative immunoblot analysis of old and new histone H3 in whole-cell lysates using antibodies against HA (old, blue), T7 (new, yellow) or an antibody raised against the spacer-LoxP sequence (LoxP) recognizing 'old' and 'new' proteins simultaneously. (C) Relative H3-T7/H3-HA ratios (New/Old) were calculated based on the ratio of the top band (H3-HA) and bottom band (H3-T7) of the LoxP blot (absolute values) and the ratio of HA and T7 signals (arbitrary units).

Chapter Two



Fig. 4. Replication-independent transcription-coupled histone turnover quantified by affinity purification. (A) Analysis of chromatin-bound histones by ChIP of HA (old) and T7 (new) histone H3 quantified by real-time quantitative PCR (gPCR). Histone exchange (ratio of new/old) was determined for promoters of the indicated genes and an intergenic region on chromosome V (No-ORF). The genes are ranked by estimated transcription frequency in log-phase, from low (white) to high (black) frequency. The result shown is the average of two individual experiments (± SEM). The box is a zoom-in of the G0 time points. (B) Relative mRNA expression levels were determined by reverse-transcriptase qPCR (RTgPCR). An S-phase sample of the same H3-RITE strain was used as a reference sample. A wild-type strain without a RITE tag showed very similar expression profiles (Fig. S4). (C) Histone turnover H3-T7→HA cells without in any arrest was determined by induction of Cre-recombinase in log-phase cells (OD660=0.25). The percentage of cells that had undergone recombination (Rec) is indicated for each time-point (determined by a colony plating assay). Histone replacement at promoters was determined by ChIP (HA/T7).

of new histone H3-T7 after five hours in G1 was similar to that of cells arrested in G2/M, which had undergone one round of genome duplication and therefore contain at least 50% new H3-T7 and 50% old H3-HA (Fig. 3B-C). Thus, yeast cells that had been arrested in G1 for the duration of around three cell doubling times had replaced approximately half of the old H3-HA protein by new H3-T7 in the absence of DNA replication.

Affinity purification of old and new histones in chromatin

Since soluble histones represent a minor fraction of the total histone pool (23), these results suggested that the G1-arrested cells had incorporated new histone H3-T7 into chromatin. To address this question we took advantage of the possibility of using the epitope tags for affinity purification of chromatin fragments containing old and new histones. Following chromatin immunoprecipitation (ChIP) the ratio of new H3-T7 over old H3-HA was determined by real-time quantitative PCR (qPCR) for promoter regions of a set of genes with different transcriptional properties and for an intergenic region (Fig.

4A). Histone exchange in chromatin was already detectable in switched G0 cells prior to release. After supplementation of fresh medium containing α -factor, exchange increased in the transition to the G1 arrest, and increased further during the arrest (2 and 5 h G1). Strikingly, five hours after release into the G1 block, the replacement of old H3-HA by new H3-T7 was quantitatively similar at different loci to that of cells that had just duplicated their genome and histone content (3 h G2/M). This confirms that cells arrested in G1 had undergone rapid replication-independent exchange of chromatin-bound histones (Fig. 4A). However, histone exchange was not restricted to the G1 phase. Cells arrested in G2/M (from 3 h until 6 h) and even cells arrested by nutrient depletion (G0 pre until G0 post) accumulated new H3-T7 during the arrest, albeit slower (Fig. 3B and 4A). Identical results were obtained with a strain in which the old and new tags were swapped (H3-T7 \rightarrow HA; Fig. S3), showing that the characteristics of new histone deposition were not determined by the specific epitope tags. We conclude that replication-independent histone exchange is a common feature of arrested cells but the rate of exchange can vary between cell cycle phases.

RITE allowed a direct and quantitative comparison between G1 and G2/M cells, which demonstrated that cells arrested in G1 replaced half of the old histones by new histones within five hours by replication-independent mechanisms. Analysis of mRNA expression levels during the different phases of the cell cycle showed that the rate of histone exchange was coupled to the level of transcription at each time point or to previous transcription events (Fig. 4B, Fig. S4). Analysis of the inducible GAL1 promoter showed that induction of transcription caused an increase in histone exchange (Fig. S4), suggesting that transcription leads to histone exchange. In addition, transcription-coupled histone exchange also occurred in coding regions, at rates similar to the rates found at promoters (Fig. S5). Transcription-coupled histone exchange might be a specific property of arrested cells that cannot replace histories by replication-dependent mechanisms. To investigate this possibility, histone exchange in chromatin was determined in log-phase cells that had been grown for many generations without a growth arrest (Fig. 4C). In these cycling cells new histone H3-HA was also incorporated more efficiently in highly transcribed genes (Fig. 4C), suggesting that transcription-coupled histone exchange occurred on top of replication-dependent histone deposition. In addition, monitoring of old and new histones during successive cell divisions showed that transcription-coupled histone deposition was maintained during at least three cell divisions (Fig. S6). Thus, biochemical purification of old and new histones revealed that chromatin is a very dynamic macromolecular complex in dividing as well as non-dividing cells and that transcription is a key determinant of chromatin instability.

Fluorescent RITE to monitor proteasome replacement in time and space

The methods discussed above probe protein dynamics in pools of cells. To visualize the behavior of old and new proteins in single cells, a fluorescent RITE cassette was constructed that switches from a green fluorescent protein (GFP) tag to a monomeric red fluorescent protein (mRFP) tag (Fig. 5A). To illustrate the use of fluorescent RITE, a constituent protein of another macromolecular complex, the proteasome, was tagged. Specifically, we constructed a yeast strain where the only endogenous PRE3 gene, encoding a catalytic β -subunit of the proteasome, was tagged with the GFP \rightarrow mRFP RITE cassette. This strain has a normal growth rate, indicating that the RITE-tagged subunit is functional, since deletion or mutation of PRE3 is lethal. We note that yeast cells expressing

Chapter Two



Fig. 5. Spatio-temporal analysis of old and new proteasomes by microscopy. (A) Schematic representation of fluorescent RITE. (B) PRE3-GFP \rightarrow mRFP cells were grown to saturation (G0) and recombination was induced overnight (switch). Subsequently, cells were released in fresh media (release 1) and samples were taken at the indicated time points. Nine hours after the first release, cells were again supplemented with fresh media (release 2). Time points 3, 6, 9, and 24 h correspond to approximately 0.3, 2, 3, and 8 cell divisions, respectively. (C) Representative confocal microscopy images of PRE3-GFP \rightarrow mRFP grown as indicated in panel B and of control strains (PRE3-GFP and PRE3-mRFP). Hoechst was used as a nuclear counterstaining (blue). Scale bar represents 4 μ m. (D) The GFP and mRFP fluorescent intensities of micrographs from panel C were quantified and the value shown for each time-point is an average of the mean fluorescence intensity in the nuclei, cytoplasm and total surface of 400 cells (± SD). Dashed lines indicate GFP and mRFP signals in control cells expressing GFP or mRFP only (the bottom dashed lines indicate background levels)

H3-GFP→mRFP were inviable, indicating that not every protein can be safely tagged with the larger GFP→mRFP RITE cassette. To visualize the replacement of old by new proteasomes by microscopy, recombination was induced in G0 (Fig. 5B), during which very little proteasome synthesis occurs. Since the proteasome is a stable complex, many old proteasomes (Pre3-GFP) remain that are slowly replaced by new proteasomes (Pre3-

mRFP) (Fig. 5C). When the cells were released in fresh media the old proteasomes were more swiftly replaced by new proteasomes due to dilution during cell division (Fig. 5C). In yeast and mammalian cells the proteasome is present in both the nucleus and the cytosol (24). Quantification of GFP and mRFP signals showed that in the switched yeast cells, the appearance of new proteasome and loss of old proteasome followed similar kinetics in the two compartments (Fig. 5D). Thus fluorescent RITE enables visualization of replacement of old by new proteins in living cells in time and space during cell cycle arrests and during successive cell divisions.

Discussion

Here we show that RITE is a versatile method to study different parameters of protein dynamics such as protein turnover and exchange of subunits in macromolecular complexes. In contrast to other methods such as pulse-chase labeling, inducible expression, methods based on differential fluorescence, or TimeStamp (3, 5, 6, 13, 25), RITE provides the unique possibility to simultaneously monitor old and new proteins and to do so by multiple techniques. RITE has important additional advantages over existing technologies. It does not require addition of UV light, chemicals or labels, circumventing the need for expensive ultra sensitive mass spectrometry technologies. Furthermore, since no heterologous inducible promoters are required to differentially express old and new proteins, tagged genes are regulated by their endogenous promoter and the switch can occur without perturbation under any condition of interest. Protein replacement of the stable proteasomes and histones could be assessed over long time periods in dividing and non-dividing cells, indicating that RITE is suitable to study the dynamics of long-lived proteins, which are typically difficult to study with more traditional methods. RITE should also be applicable to shorter lived proteins, however. Although it takes about two hours until the majority of the cells has switched, switched cells can already be detected as early as fifteen minutes after activation of Cre. RITE may be less suitable for studies of very short-lived proteins.

The differential tagging of histone H3 showed that endogenously expressed canonical histones undergo turnover within chromatin in a transcription-dependent manner. Our results are in agreement with previous histone H3 turnover studies using time-controlled induced expression of a tagged ectopic histone copy in yeast (8-12, 16, 26). The direct comparison to replication-dependent assembly of new histones indicates that replication-independent histone exchange occurs at a high rate. This was unexpected when one considers the regulated expression of histones. We note that while H3 mRNA indeed peaks in S-phase when chromatin is duplicated, its expression is lower but still substantial outside of S-phase (Fig. 4B). This supports the idea that canonical histones are synthesized outside of S-phase for replication-independent histone exchange. Especially in starved cells, H3 mRNA is relatively abundant (Fig. 4B). The high rate of histone exchange suggests that post-translational modifications in chromatin are continuously being erased in dividing and non-dividing cells. Thus, replication-independent histone exchange might provide cycling and non-cycling cells with a means to replace old histones that have acquired damage or that need to be epigenetically re-set.

Using fluorescent RITE, replacement of old by new proteasomes in time and space was determined by microscopy. The amount of old proteasomes decreased at a very similar rate in the cytosolic and nuclear compartments, suggesting an even segregation during cell division and/or a fast re-equilibration between proteasomes in both compartments.

Likewise, the appearance of new proteasome in both compartments followed similar kinetics, indicating that the translocation of new proteasome subunits into the nucleus is a relatively fast phenomenon (Fig. 5D).

RITE is a widely applicable tool to dissect novel mechanisms and functions of protein dynamics. For example, RITE-tagged genes of interest and the Cre recombinase can be efficiently introduced into the collection of yeast deletion strains by one round of genetic crossing, which allows genome-wide genetic screens for identification of factors involved in protein dynamics. RITE can also be applied to investigate whether new and aging proteins have different properties such as age-related post-translational modifications or whether they show differential segregation between mother and daughter cells. Finally, although we have validated RITE in budding yeast, with minor modifications RITE technology may be adapted for use in higher eukaryotes. The RITE cassettes are universally applicable and conditional versions of Cre recombinase have already been developed for many cell systems or even whole organisms (27).

Materials and Methods

Yeast strains and growth conditions

Yeast strains and growth conditions are described in Table S1 and the supplementary methods. RITE cassettes contain an invariant short peptide spacer sequence (GGSGGS) that was found to be required for viability of strains carrying tagged histones. The spacer and ITSYNVCYTKLS peptide encoded by the LoxP DNA sequence are present in front of the epitope tags both before and after the switch. RITE cassettes were PCR amplified and targeted to the 3' end of the endogenous genes by homologous recombination to tag the C-terminus and ensure regulation by the endogenous promoter. The hormonedependent Cre-EBD (Cre-EBD78) was described previously (22). A constitutively expressed copy was stably integrated in the yeast genome. For RITE experiments, yeast cells were arown overnight in YPD in the presence of Hygromycin B (200 µg/ml, Invitrogen). The cells were then diluted 1:10 into fresh YPD and incubated for 30-36 hours. Recombination was induced by the addition of 1μ M β -estradiol (E-8875 Sigma-Aldrich). Subsequently, cells were diluted 1:25 in fresh YPD media to release the cells back into the cell cycle. Cells enter G1 arrest upon addition of 0.5 ng/µl of α -factor and G2/M arrest upon addition of 15 µg/ml Nocodazole (Sigma-Aldrich). Detailed protocols for ChIP, RT-PCR, immunoblot, Southernblot, FACS, and microscopy are described in the supplementary methods and Table S2.

Acknowledgements

We thank C. Logie for helpful suggestions, F. van Diepen, A. Pfauth and L. Oomen for technical assistance, and G. Filion for statistical help. We thank members of the van Leeuwen lab and M. Fornerod for suggestions and critical reading of the manuscript. FvL was supported by the EU 6th framework program (NOE 'The Epigenome' LSHG-CT-2004-503433) and by a VIDI fellowship from The Netherlands Organization for Scientific Research (NWO-ALW). DLL was supported by postdoctoral fellowship #PF-04-041-01-GMC from the American Cancer Society. VMB was supported by a long-term EMBO fellowship.

References

- 1. Gorski SA, Dundr M, Misteli T (2006) The road much traveled: trafficking in the cell nucleus. Curr. Opin. Cell Biol. 18:284-290.
- Russel D *et al.* (2009) The structural dynamics of macromolecular processes. Curr. Opin. Cell Biol. 21:97-108.
- Reits EA, Neefjes JJ (2001) From fixed to FRAP: measuring protein mobility and activity in living cells. Nat. Cell Biol. 3:E145-E147.
- D'Angelo MA, Hetzer MW (2008) Structure, dynamics and function of nuclear pore complexes. Trends Cell Biol. 18:456-466.
- Adams SR, Tsien RY (2008) Preparation of the membrane-permeant biarsenicals FIAsH-EDT2 and ReAsH-EDT2 for fluorescent labeling of tetracysteine-tagged proteins. Nat. Protoc. 3:1527-1534.
- Mann M (2006) Functional and quantitative proteomics using SILAC. Nat. Rev. Mol. Cell Biol. 7:952-958.
- Subach FV *et al.* (2009) Monomeric fluorescent timers that change color from blue to red report on cellular trafficking. Nat. Chem. Biol. 5:118-126.
- Schermer UJ, Korber P, Horz W (2005) Histones Are Incorporated in trans during Reassembly of the Yeast PHO5 Promoter. Mol Cell 19:279-285.
- Linger J, Tyler JK (2006) Global replicationindependent histone H4 exchange in budding yeast. Eukaryot Cell 5:1780-1787.
- 10. Dion MF *et al.* (2007) Dynamics of replicationindependent histone turnover in budding yeast. Science 315:1405-1408.
- 11. Rufiange A *et al.* (2007) Genome-wide replication-independent histone h3 exchange occurs predominantly at promoters and implicates h3 k56 acetylation and asf1. Mol Cell 27:393-405.
- Jamai A, Imoberdorf RM, Strubin M (2007) Continuous histone H2B and transcriptiondependent histone H3 exchange in yeast cells outside of replication. Mol Cell 25:345-355.
- Lin MZ, Glenn JS, Tsien RY (2008) A drugcontrollable tag for visualizing newly synthesized proteins in cells and whole animals. Proc. Natl. Acad. Sci. U S A 105:7744-7749.
- Kimura H, Cook PR (2001) Kinetics of core histones in living human cells: little exchange of H3 and H4 and some rapid exchange of H2B. J. Cell Biol. 153:1341-1353.
- 15. Henikoff S (2008) Nucleosome destabilization in the epigenetic regulation of gene expression. Nat. Rev. Genet. 9:15-26.
- 16. Kim HJ *et al.* (2007) Histone chaperones regulate histone exchange during transcription. EMBO J.
- 17. Mito Y, Henikoff JG, Henikoff S (2005) Genomescale profiling of histone H3.3 replacement

patterns. Nat Genet 37:1090-1097.

- Wirbelauer C, Bell O, Schubeler D (2005) Variant histone H3.3 is deposited at sites of nucleosomal displacement throughout transcribed genes while active histone modifications show a promoter-proximal bias. Genes Dev 19:1761-1766.
- Chow CM *et al.* (2005) Variant histone H3.3 marks promoters of transcriptionally active genes during mammalian cell division. EMBO Rep. 6:354-360.
- 20. Finley D (2009) Recognition and processing of ubiquitin-protein conjugates by the proteasome. Annu. Rev. Biochem. 78:477-513.
- 21. Logie C, Stewart AF (1995) Ligand-regulated sitespecific recombination. Proc. Natl. Acad. Sci. U S A 92:5940-5944.
- 22. Lindstrom DL, Gottschling DE (2009) The Mother Enrichment Program: A Genetic System for Facile Replicative Life Span Analysis in *Saccharomyces cerevisiae*. Genetics 183:413-422.
- 23. Gunjan A, Verreault A (2003) A Rad53 kinasedependent surveillance mechanism that regulates histone protein levels in *S. cerevisiae*. Cell 115:537-549.
- Reits EA et al. (1997) Dynamics of proteasome distribution in living cells. EMBO J. 16:6087-6094.
- 25. Yen HC *et al.* (2008) Global protein stability profiling in mammalian cells. Science 322:918-923.
- Choi ES, Shin JA, Kim HS, Jang YK (2005) Dynamic regulation of replication independent deposition of histone H3 in fission yeast. Nucleic Acids Res 33:7102-7110.
- 27. Branda CS, Dymecki SM (2004) Talking about a revolution: The impact of site-specific recombinases on genetic analyses in mice. Dev. Cell 6:7-28.

Supplemental Figures:



Fig. S2



Figure S2. Cell cycle progression and arrest monitored by flow cytometry.

FACS analysis of DNA content to monitor release of starved cells from G0 (1C) into the G1 (1C) and G2/M (2C) blocks. Asterisks indicate the analyzed time points.



Figure S3. Histone exchange is independent of the order of the RITE tags

Starved switched cells (see Fig. 3) containing a 'swapped-tag' cassette that switches from T7 to HA (H3-T7 \rightarrow HA) were released into fresh media and arrested in G1 or G2/M. ChIP of T7 and HA was quantified by qPCR for the genes indicated. The 5 h G1 arrest time-point was not analyzed because this BAR1 wild-type strain degrades of-factor and escapes from the arrest after four hours.



Recombination-Induced Tag Exchange



2

Figure S4. Histone exchange correlates with mRNA expression levels. (A) To confirm that the RITE tags did not affect gene expression, mRNA was isolated from a wild-type strain (NKI2036) and relative expression levels were calculated at each indicated time. The relative expression levels are identical to those of the RITE tagged strain (Fig. 4B). The relative mRNA expression patterns correlated very well with the observed histone exchange rates. For example, ADH2 was active under low glucose conditions in starved cells and repressed in cells released in glucose-rich media. Indeed, ADH2 belonged to the genes with high exchange in arrested cells or shortly after release and then dropped to the low exchange at later time points (Fig.s 3C and 3D). ACT1, which was induced in cells arrested by α -factor, specifically peaked late in the G1 arrest (Fig. 3C). Finally, relative exchange at HHT2 was highest early in the G2/M arrest. Although HHT2 expression was low in G2/M, cells at this time point have just exited S-phase, during which transcription of histone genes was induced (Fig. 3D). Therefore, the presence of new H3-T7 might be a mark of previous transcription events. (B) To analyze the causal effect of transcription on histone exchange, cells were starved and switched in medium containing raffinose and subsequently released into medium containing glucose or galactose, to repress or induce the GAL1 gene, respectively. Cells were arrested in G1 for 4 hrs. (C) FACS analysis of cells starved in raffinose media and released in media containing nocodazole (G2/M arrest) and either glucose or galactose. Starved cells released in media with galactose re-entered more slowly into the cell cycle and showed lower overall new histone expression at this time point. Therefore, subsequent exchange ratios were determined relative to ADH1. (D) Histone exchange (ChIP T7/HA) at GAL1, HHT2 and IMD1.Upon activation of GAL1, deposition of new H3-T7 was increased at the GAL1 promoter indicating that transcription enhanced histone exchange.

Chapter Two



Figure S5. Histone exchange occurs at promoters and coding sequences. (A-D) RITE-ChIP showed that new H3-T7 was readily incorporated in promoters by a transcription-coupled process. However, histone exchange at promoter regions, which represent less than a guarter of the yeast genome, was insufficient to explain the global deposition of new H3-T7 (~50% of the total H3 pool) observed by immunoblots (Fig. 3B). Therefore histone exchange in coding sequences was also determined. Histone exchange at promoters (PRO) was determined as in Fig. 3C and compared to exchange in coding sequences (ORF) of ACT1 and ADH1, and in addition of two long genes FMP27 and PMA1 to exclude effects of proximal promoter sequences. The location of the analyzed regions and the distance between promoter and ORF regions is indicated. (E) Relative mRNA expression levels in the H3-HA→T7 strains as shown in Fig. 3D but now including the expression of PMA1 and FMP27. Ectopically expressed histories in yeast have been shown to be predominantly incorporated in promoter regions largely irrespective of the level of transcription, whereas the lower level of exchange in ORFs correlated with transcription rates (1-7). Using RITE we found by ChIP that exchange of endogenous histones in ORFs was nearly as high as in promoters and this notion was supported by the global turnover of bulk histones that we detected by immunoblot (Fig. 3B). In addition, the rate of exchange of endogenous histones in ORFs as well as promoters correlated with transcription levels. Thus, transcription-coupled replication-independent exchange of histones occurred in promoters and coding regions.

ele





2

Figure S6. Histone exchange in successive cell divisions. Starved switched H3-T7→HA cells were released into fresh media and harvested after one, two, and three rounds of cell division. To count cell divisions we developed a convenient and stable fluorescent cross linker, NHS-TER, which labels the yeast cell wall. Upon cell division, daughter cells or buds synthesize cell wall de novo and emerge as unlabeled cells whereas the mother cells retain the label (8) (A). Thus, the fraction of unlabeled cells, which can be quantitatively determined by FACS, is a measure of the number of cell doublings. (B) The cell wall of the starved cells was labeled with NHS-TER prior to induction of Cre recombinase to guantitatively determine the number of cells in the population that had undergone cell division. Mother cells retain the old cell wall and daughter cells make cell wall de novo, as confirmed by confocal microscopy (right panel; blue is Hoechst DNA staining). The percentage of unlabeled cells identified by FACS (left panel) indicates the percentage of new-born daughter cells in the population and corresponds to the percentage of chromatin that is new. (B) Starved H3-T7→HA cells stained with NHS-TER prior to Cre-induction were released after the switch into fresh media and samples were taken at the indicated time points to capture cells after 1, 2, and 3 cell divisions. The number of cell divisions in the population was confirmed by FACS. The percentage of cells that had undergone a cell division (d) relates to the percentage of unlabeled cells (u) by 100*u=d/(100+d). (C) Histone replacement at promoters in samples described in panel B was determined by ChIP (HA/T7).

Supplementary References:

- Linger J, Tyler JK (2006) Global replicationindependent histone H4 exchange in budding yeast. Eukaryot Cell 5:1780-1787.
- 2. Dion MF, Kaplan T, Kim M, Buratowski S, Friedman N *et al.* (2007) Dynamics of replicationindependent histone turnover in budding yeast. Science 315:1405-1408.
- Rufiange A, Jacques PE, Bhat W, Robert F, Nourani A (2007) Genome-wide replication-independent histone h3 exchange occurs predominantly at promoters and implicates h3 k56 acetylation and asf1. Mol Cell 27:393-405.
- Kim HJ, Seol JH, Han JW, Youn HD, Cho EJ (2007) Histone chaperones regulate histone exchange during transcription. EMBO J.

- Jamai A, Imoberdorf RM, Strubin M (2007) Continuous histone H2B and transcriptiondependent histone H3 exchange in yeast cells outside of replication. Mol Cell 25:345-355.
- Kaplan T, Liu CL, Erkmann JA, Holik J, Grunstein M et al. (2008) Cell cycle- and chaperone-mediated regulation of H3K56ac incorporation in yeast. PLoS Genet. 4:e1000270.
- Gat-Viks I, Vingron M (2009) Evidence for gene-specific rather than transcription ratedependent histone H3 exchange in yeast coding regions. PLoS Comput. Biol. 5:e1000282.
- Park PU, McVey M, Guarente L (2002) Separation of mother and daughter cells. Methods Enzymol. 351:468-477.

Supplemental Table 1:

Strain:	Relevant genotype	Reference
BY4705	$MAT\alpha ade 2\Delta:: HisG his 3\Delta 200 leu 2\Delta 0 lys 2\Delta 0 met 15\Delta 0 trp 1\Delta 63 ura 3\Delta 0 \tag{1}$	
BY4724	MATa lys2Δ0 ura3Δ0	(1)
BY4742	$MAT\alpha his 3\Delta 1 leu 2\Delta 0 lys 2\Delta 0 ura 3\Delta 0 \tag{1}$	
NKI2036	MATa his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δhhf1-hht1::LEU2 This study	
NKI2148	MATa his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δhhf1-hht1::LEU2 HIS3::Pt- dh3_CRE_EBD78 bar1Δ::HisG hht2::HHT2-LoxP-HA-HYG-LoxP-T7	
NKI2158	MATa his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δhhf1-hht1::LEU2 CYC1te- rm::Ptdh3_CRE_EBD78_HIS3 hht2::HHT2-LoxP-T7-HYG-LoxP-HA	
NKI4004	MATa his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 ura3Δ0 Δhhf1-hht1::LEU2 This study hht2::HHT2-LoxP-HA-HYG-LoxP-T7	
NKI4009	MATa his3Δ200 leu2Δ0lys2Δ0 met15Δ0 ura3Δ0 Δhhf1-hht1::LEU2 This study hht2::HHT2-LoxP-T7	
Y7092	MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 (2)	
NKI4101	MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 pre3::PRE3-V5-loxP-HA-yEGFP-HYG-loxP-T7-mRFP	
NKI4103	NKI4101 + lyp1Δ::NATMX-GPD_CRE_EBD78 This study	
NKI4104	MATα can1Δ::STE2pr-Sp_his5 lyp1Δ his3v1 leu2Δ0 ura3v0 met15Δ0 LYS2 This study lyp1Δ::NATMX-GPD_CRE_EBD78 pre3::PRE3-V5-loxP-T7-mRFP	

1. Brachmann CB, Davies A, Cost GJ, Caputo E, Li J *et al.* (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14:115-132.

2. Tong AH, Boone C (2006) Synthetic genetic array analysis in *Saccharomyces cerevisiae*. Methods Mol. Biol. 313:171-192.

Supplemental Table 2:

Gene name:	Primer name:	Primer Sequence:
ADH1	ADH1 PRO II fwd	CCGTTGTTGTCTCACCATATCC
ADH1	ADH1 PRO II rev	GTTTCGTGTGCTTCGAGATACC
ADH1	ADH1 ORF II fwd	TAGGTTCTTTGGCTGTTCAATACG
ADH1	ADH1 ORF II rev	CGGAAACGGAAACGTTGATGACACCG
HHT2	HHT2 QFor1	GTGCCAAACGACCACAGTTG
HHT2	HHT2 QRev1	GGGCGTGCCAATAGTTTCAC
HHT2	HHT2 QFor2	AAGCCCCAAGAAAACAATTAGC
HHT2	HHT2 QRev2	AAGCCCCAAGAAAACAATTAGC
ACT1	ACT1 Qfor	СТСТТТТАТСТТССТТТТТТССТСТСТ
ACT1	ACT1 Qrev	CGTGAAAAATCTAAAAGCTGATGTAGTAG
ACT1	ACT1 QforORF	TCGTTCCAATTTACGCTGGTT
ACT1	ACT1 QrevORF	CGGCCAAATCGATTCTCAA
ADH2	ADH2 PRO II fwd	AACACCGGGCATCTCCAAC
ADH2	ADH2 PRO II rev	AAGTCGCTACTGGCACTC
ADH2	ADH2 ORF I fwd	ACACCCACGACGGTTCTTTC
ADH2	ADH2 ORF I rev	CAAGATTGGCGCGACTTCAG
IMD1	QFOR IMD1	TTTCGTGGGCTAGTACATTTTACCT
IMD1	QREV IMD1	TGATAAGAAAAGTAAGGCAAGGAATAGA
IMD1	Q IMD1 for-2	TTTGCAGGCTTCCCTGTCA
IMD1	Q IMD1 rev-2	TGATGGCACCCACCAACTTT
SIR3	SIR3 Qfor	CGAAAACGCTATTCTTTCCAAAA
SIR3	SIR3 Qrev	CCCCTGTAAGGAAGGTGATGAA
SIR3	SIR3 ORF Qfor 1	GACGGCCGAGAGAATTTTGTAT
SIR3	SIR3 ORF Qrev 1	CTTCAAGCCCACCATCATCA
PMA1	PMA1 PRO Qfor1	TGGTGGGTACCGCTTATGCT
PMA1	PMA1 PRO Qrev1	TGTTAGACGATAATGATAGGACATTTGA
PMA1	PMA1 ORF Qfor1	AAATCTTGGGTGTTATGCCATGT
PMA1	PMA1 ORF Qrev1	CCAAGTGTCTAGCTTCGCTAACAG
FMP27	FMP27 PRO Qfor1	AGGGAGACATGAAAAAGGGTCTT
FMP27	FMP27 PRO Qrev1	TCTCTGAGATGTCTAGGCCCTTTTA
FMP27	FMP27 ORF Qfor1	TGGACAGCATTGCCATAGAAGA
FMP27	FMP27 ORF Qrev1	TGTACTAATTCACTCAACATAACCATTGTT
NoORF	NoORF Qfor	GGCTGTCAGAATATGGGGCCGTAGTA
NoORF	NoORF Qrev	CACCCCGAAGCTGCTTTCACAATAC

Supplementary Methods

Yeast strains and plasmids

All S. cerevisiae strains used in this study are derived from S288C strains BY4705, BY4727, BY4741, BY4742 (1) and are listed in Table 1. Plasmids pRS400, pFvL99 and pFvL100 were used for gene replacements by KanMX4, NatMX4, and HpHMX4, respectively (2, 3). To generate pFvL99 and pFvL100, the PacI-Bsml KanMX4 insert of pRS400 was replaced by a PacI-BsmI fragment of pAG25 or pAG32 (4), respectively. The drug resistance cassettes were amplified using the standard pRS primers. RITE cassettes were constructed by restriction enzyme based cloning of PCR fragments in a modular fashion to generate the following basic construct: Notl-spacer-LoxP-Kpnl-Tag1-Spe1-stop-ADH1term-BamHI-HygroMX-Xbal-LoxP-Sall-Tag2-BsrGI-stop. The following modules were used: spacer: GGTGGATCTGGTGGATCT, LoxP: ATAACTTCGTATAATG¬TATGCTATACGAAG-TTATCA, HA: TACCCATACGATGTT¬CCTGACTATGCG, T7: ATGGCAAGCATGAC-TGGTGGACAGCAAATGGGT, HphMX: 1.8 kb fragment amplified from pFvL100 AGATTGTACTGA¬GAGTGCAC..... CGGTGTGAA¬ATACCGCACAG, ADH1 terminator: amplified from pFA6a-3HA-KanMX (5) CTTCTAA¬ATAAGCGA...... GGGATAACAGGGTAA. The encoded short peptide spacer sequence (GGSGGS) was found to be required for viability of strains carrying tagged histones. The 34bp LoxP DNA sequence is part of the coding region (resulting in the peptide sequence ITSYNVCYTKLS) and is present in front of the epitope tags both before and after the switch. RITE cassettes were PCR amplified and targeted to the 3' end of the endogenous genes by homologous recombination to tag the C-terminus and ensure regulation by the endogenous promoter. The hormone-dependent Cre-EBD was described previously (6). We used a derivative of this construct (Cre-EBD78) which is constitutively expressed and contains several mutations to make the recombinase more tightly dependent on β -estradiol (7). A TDH3 promoter fragment, Cre-EBD78, and a CYC1 terminator sequence were cloned into pRS303 (1) to generate pTW040, which was linearized with Eco47III or Mlul to integrate the construct at the HIS3 locus or CYC1 locus, respectively.

Galactose induction

To perform a galactose induction the cells were grown to saturation in YP containing 3% raffinose. Since the cells cycle a little slower in raffinose than in glucose twice the amount of cells was grown for the same amount of time as previously. In order to release the cells into the cell cycle, one half of the culture was resuspended in YPD, the other half in YP + 1% raffinose + 2% galactose. Both media contained 0.5 ng/µl of α -factor. To determine the speed of release in either media, the cells were grown identical to previously, but released into media containing 15 µg/ml Nocodazole. Cells were harvested every hour for FACS analysis of DNA content.

Polyclonal antibody production

A polyclonal antibody was obtained by immunizing rabbits using the peptide GGSGGSITSYNVC*YTKLS against the spacer and LoxP sequence (the asterisk indicates the cysteine present as a sulfhydryl necessary for conjugation). For immunization 2 mg of the peptide was covalently conjugated to Imject Mariculture Keyhole Limpet Hemocyanin (mcKLH) (Pierce) as a carrier protein. The concentration of the conjugated hapten was determined using Bradford (Bio-Rad). Per immunization 100µg in 1ml PBS was injected. Each rabbit received 3 boosts with 1 month intervals, two rabbits were immunized. Each antibody was tested for specificity using a WT, an HA and a T7 tagged strain.

Southern Blotting

For Southern blotting $5x10^8$ cells were spun and frozen at -80°C. A histone H3 (HHT2) specific probe was made by PCR amplification using the primers: HHT2_HindIII_for: GAATCTTTCTGTGACGCTTGG and HHT2_HindIII_rev GGGGAAGAACAGTTGGAAGG, resulting in a 650bp amplicon covering the region 576144 to 576794. When used on genomic DNA which was digested using the HindIII enzyme, the three bands recognized are specific for before the switch (3000bp), after the switch (931bp) or as an internal control (1538bp). Radioactive Southern blotting was performed using 50μ Ci of 32P-dCTP; incubation was done overnight at 65° C.

Quantitatve Immunoblotting

For immunoblotting $5x10^7$ cells were spun, washed once with cold 1xTE+0.2mM PMSF, pellet was frozen (3). Whole-cell extracts were obtained from approximately $5x10^7$ cells by the classical glass beads breakage method using 200µl of glass beads and SUMEB (8) complemented with PMSF (1 mM), benzamidine (5 mM), pepstatin (1 µg/ml), leupeptin (1 µg/ml) and DTT (1 µM). The resulting lysate was separated onto a 16% polyacrylamide gel and blotted onto 0.45 µm nitrocellulose membrane. Membranes were blocked with 2% Nutrilon (Nutricia) in PBS. Primary antibody incubations were performed overnight in Tris-buffered saline-Tween with 2% Nutrilon, anti-HA (12CA5), anti-T7 (Abcam, 1:1000) and a polyclonal antibody obtained against the LoxP peptide (1:2500). Secondary antibody incubations were performed for 45 minutes using LI-COR® Odyssey IRDye® 800CW (1:12.000).

Immunoblots were subsequently scanned on a LI-COR Odyssey® IR Imager (Biosciences) using the 800 channel. Signal intensities were determined using Odyssey LI-COR software version 3.0. Ratios of T7/HA were converted into %HA values by using a standard curve of samples with known amounts of H3-HA and H3-T7. These samples were generated by mixing cells expressing either only H3-HA (NKI4004) or H3-T7 (NKI4009) in various ratios of cell numbers. Blots of the standard curve and the experimental samples were processed simultaneously.

Reverse-transcription

Ranking of genes based on estimated transcription frequencies was based on genome-wide mRNA expression and stability data from Holstege *et al.* (9). Total yeast RNA was prepared from 5×10^7 cells of each of the indicated growth condition using the RNeasy kit (Qiagen) according to the manufacturer's protocol (10). RNA samples were treated with RNase free DNAse (Qiagen), and cDNA was made by using Super-Script II reverse transcriptase (Invitrogen). To obtain an S-phase sample, cells were synchronized for three hours in G1 using 0.5 ng/µl α -factor, released after two washes with YPD (containing 0.1mg/ml ProNase E if strain was bar1 Δ) and isolated every 0.5 h. By FACS analysis it was determined that 0.5 h after release the maximum amount of cells were in S-phase.

Chromatin immunoprecipitation

ChIP was performed as described previously (3, 10, 11). Approximately $1x10^{\circ}$ cells were fixed with 1% formaldehyde for 15 minutes room temperature. The formaldehyde was quenched with 125mM glycine by shaking 5 minutes at room temperature. Cells were washed once in cold TBS + 0.2 mM PMSF, pellet was frozen at -80°C. The chromatin was sheared using a bioruptor (Diagenode) for 6 minutes with 30 seconds intervals at high. The obtained fragments have an average size of 500bp, as determined on a 2% TAE gel stained with ethidium bromide and quantified using TINA software. The isolated chromatin of the equivalent of $5x10^7$ cells was immunoprecipitated overnight at 4°C using magnetic Dynabeads (Invitrogen) which were previously incubated with antibody o/n at 4°C.

Real-time PCR

ChIP DNA and cDNA was quantified in real-time PCR using the SYBR® Green PCR Master Mix (Applied Biosystems) and the ABI PRISM 7500 as described previously (2, 10). An input sample was used to make a standard curve, which was then used to calculate the IP samples, all performed in the 7500 fast system software. As a measurement for exchange, the amount DNA of the T7-IP was divided over the HA-IP. Primers used for qPCR are listed in Table S2.

Staining cells with N-hydroxysuccinimide-Tetra-Ethylrhodamine (NHS-TER)

A 20% aqueous solution of an isomeric mixture of 5(6)-carboxyrhodamine (Rhodamine WT) was obtained from Abbey Color, Philadelphia, PA. The free acid was precipitated with concentrated hydrochloric acid (two equivalents) as described (12). The precipitate was collected by centrifugation, and resuspended in 1M HCl. This procedure was repeated twice and the precipitate was frozen and freeze-dried to remove residual traces of water. The free acid was converted in an active N-hydroxysuccinimide (NHS) ester by condensation with N-hydroxysuccinimide mediated by the agent di-isopropylcarbodiimide (DIC). This is a relatively simple and very economical procedure compared to other fluorescent labeling approaches. To stain yeast cells, cultures were washed twice with PBS. Cells resuspended in PBS, NHS-TER was added (0.8 mg NHS-TER per 10⁸ cells) and incubated at room temperature for 15 minutes. Cells were washed 8 times with PBS and then resuspended in YPD medium. For each time point 10⁷ cells were fixed for FACS analysis or confocal microscopy. The samples were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature and washed with water. Cells were briefly sonicated. For confocal microscopy the pellet was resuspended in 1 ml water and cells were stained with Hoechst (1 µg/ml) as a DNA stain. The pellet was resuspended in 50 µl water and 2 µl of this solution was mounted in Vectashield mounting medium on a concanavalin-A coated cover slip. Confocal analyses were performed using a Leica TCS SP2 confocal system, equipped with Diode 405 and 561 lasers. Images were taken using a 63x 1.4 objective. Emission windows 415-540 and 571-700 and Kalman averaging were used. For FACS analysis the cells were resuspended in 500 µl water.

FACS analysis of DNA content and cell doubling

The DNA content was measured using SYTOX Green in flow cytometry as described previously (2, 13), detection was done using a 530/30 filter. For FACS analysis of DNA content 1x10⁷ cells were spun briefly at maximum speed, resuspended in 1ml of 70% ethanol, and kept at -20°C. NHS-TER stained cells were detected using a 585/42 filter of the FACS calibur (Becton-Dickinson). For each measurement 100.000 cells were counted. Analysis was performed using FCS express 2. To determine the percentage stained cells (mother) versus unlabeled cells (daughter), NHS-TER stained cells were harvested at indicated time points. Additionally, cells were also counted using a count chamber and a wide field microscope. The signal in channel FL2 was divided into two regions based on a 100% and an unlabeled control, these regions were applied to all samples. The number The percentage of

labeled cells (L) was used to calculate the number of population doublings (Dp) by: L=100*0.5^Dp.

Microscopy

 $5x10^6$ cells were pelleted, washed once with water and fixed with 4% formaldehyde for 10 minutes at room temperature. Cells were then washed with water and nuclei were stained with Hoechst 33342 (Invitrogen, UK, 1 µg/ml) for 15 minutes at room temperature. Cells were then washed and resuspended in 100 µl water. Resuspended cells were mixed with one volume Vectashield mounting solution (Vector Laboratories) and mounted onto ConA-coated cover slips. The images were made using a Leica AOBS LSCM (Leica Microsystems), using a 405nm, a 488 and a 563nm laser to visualize Hoechst, GFP, and mRFP, respectively. Images were analyzed using customized Cell Profiler (open-source cell image analysis software). For each time point, 4 different micrographs, each of them containing approximately 100 yeast cells were quantified using the pipeline described below.

Confocal microscopy pipeline

Can be found at: www.pnas.org/content/107/1/64

Recombination-Induced Tag Exchange