Supporting Online Material for

Genome-Wide Kinetics of Nucleosome Turnover Determined by Metabolic Labeling of Histones

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Materials and Methods

Cell culture and azidohomoalanine treatment of cells

Drosophila S2 cells were grown to late-log phase in Schneider's insect medium supplemented with 5% fetal bovine serum in standard 150 cm² cell culture flasks at 25°C. The medium was then replaced with Shields and Sang minimal media containing all amino acids except methionine (Met), and cells were incubated for 30 minutes to reduce cellular Met pools. The medium was then replaced with Shields and Sang medium containing all amino acids except Met and including 4 mM azidohomoalanine (Aha). Aha was either custom synthesized by Toronto Research Chemicals or purchased from Anaspec (catalog # 63669). Cells were further cultured for various time periods as indicated in the text and subsequently washed with 1X PBS (11.9 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl, pH = 7.4) prior to processing. For pulse-chase experiments, Shields and Sang medium with 4 mM Met was added to the cells after Aha treatment, and cells were incubated for an additional 1.5 hours. One flask of cells was used for each CATCH-IT experiment, and ~10⁷ cells remained in each flask after the media changes.

For experiments using total protein extracts, cells were treated as above and protein was extracted by lysing cells in 1X PBS containing 1% SDS, followed by sonication to reduce viscosity. This extract was heated to 95°C for 5 minutes, centrifuged at 16,000 x g for 5 minutes to remove insoluble material, and the supernatant was used for the cycloaddition reaction after a 1:10 dilution in 1X PBS. Cycloheximide treatment was performed by including 50µg/mL cycloheximide in the media along with 4 mM Aha. This cycloheximide concentration did not affect cell growth or viability.
**Biotin coupling and chromatin extraction**

After Aha treatment, cells were collected and washed once in 1X PBS and then lysed by resuspension in ice-cold TM2 buffer (2 mM MgCl₂, 10 mM Tris, 1% NP-40, pH = 7) with vortexting 5 times for 5 seconds. Nuclei were collected at 100 x g for 5 minutes at 4° and washed twice in TM2 buffer without NP-40. Nuclei were then resuspended in 200 µL of HB 125 buffer (0.125 M Sucrose, 15 mM Tris, 15 mM NaCl, 40 mM KCl, 0.5 mM Spermidine, 0.15 mM Spermine, pH = 7.5) and cycloaddition reagents were added as follows: biotin alkyne, prepared as previously described (I), was added to a final concentration of 0.5 mM and a freshly prepared mixture of CuSO₄ and ascorbic acid (50 mM and 250 mM, respectively) was added to give final concentrations of 1 mM CuSO₄ and 5 mM ascorbic acid. Nuclei were placed on a rocking platform at 4° for 30 minutes and then pelleted at 100 x g for 5 minutes at 4°. Nuclei were again resuspended in 200 µL of HB125 buffer and the cycloaddition reaction was repeated as described above using fresh reagents. Performing two rounds of cycloaddition was found to be required for coupling of biotin to all available Aha molecules. After the final cycloaddition, nuclei were resuspended in 250 µL of HB125 with 1 mM EDTA and 2 mM CaCl₂, and warmed to 37° in a water bath. Ten units of micrococcal nuclease (MNase) were then added and cells were kept at 37° for 3.5 minutes with occasional gentle mixing; this digestion yielded mostly mononucleosomes. MNase reactions were stopped by adding EDTA to 2 mM and pelleting nuclei by centrifugation at 100 x g for 5 minutes at 4°. About 80-90% mononucleosomes were obtained. Nuclei were then resuspended in 300 µL of CSB 350 buffer (1X PBS with 350 mM NaCl, 2 mM EDTA, 0.1% Triton X-100) and rocked at 4° for 2 to 16 hours to extract soluble chromatin. This procedure extracts ~60% of total chromatin (data not shown). The soluble chromatin fraction was then centrifuged at 13,000 x g for 5 minutes to remove all insoluble
material. Ten microliters of this soluble chromatin from each sample was saved as “input” DNA for comparison to “pulldown” DNA on microarrays.

**Streptavidin pulldown and removal of H2A/H2B and non-histone chromatin proteins**

Use of a stringent washing procedure to remove H2A/H2B is important to obtain valid nucleosome kinetic measurements, and has the additional advantage of removing essentially all non-histone chromatin proteins (2). One reason is that the turnover of H2A/H2B dimers is a process distinct from that of H3/H4 (3, 4). Therefore, performing the CATCH-IT procedure on nucleosomes containing H2A/H2B dimers would result in profiles and rate measurements that reflect a mixture of signals from H2A/H2B and H3/H4 turnover that cannot be deconvolved, and thus would not be useful. Also, most newly incorporated H2A and H2B are recycled from pre-existing nucleosomes, whereas nearly all newly incorporated H3, H3.3 and H4 are synthesized de novo, both during replication (5) and outside of replication (3, 6). Therefore, metabolic labeling of H2A + H2B would grossly underestimate the actual turnover rate of H2A/H2B dimers. Moreover, since H2A/H2B flank the central H3/H4 tetramer, and the DNA wraps around the entire particle, turnover as measured by H3/H4 necessarily requires eviction of H2A/H2B. Indeed, H2A/H2B dimers turn over much faster than H3/H4 tetramers (7), and so would not alter our conclusions concerning the highly dynamic nature of nucleosomes at epigenetic regulatory elements. Also, the large majority of histone modifications of interest in the chromatin field are on H3 and H4, and we anticipate future applications of CATCH-IT to determine the dynamics of these many important modifications.

To the 300 µL of soluble chromatin for each sample, 25 µL of M280 Streptavidin Dynabeads (Invitrogen catalog # 112.05D) was added and this mixture was rocked at 4° for 1.5
hours. Dynabeads were collected on a Dynal magnet rack, supernatant was removed, and beads were resuspended in 1 mL of Urea wash buffer (4M urea, 0.3 M NaCl, 1 mM EDTA, 20 mM Tris, pH 8) with rocking at 4° for 5 minutes to remove H2A/H2B dimers and other DNA-binding proteins (2).

**Preparation of DNA for microarrays**

Beads were decanted as above and resuspended in 1 mL of CSB 350 with rocking at 4° for 5 minutes. Beads were finally decanted and resuspended in 100 µL CSB 350 with 1% SDS and treated with RNase A at 37° for 10 minutes, then with Proteinease K at 70° for 10 minutes. Input DNA was given the same treatments, and both were purified using the Qiagen MinElute DNA purification kit. DNA samples were then amplified using the Sigma WGA2 whole genome amplification kit. Amplified input and pulldown DNAs were then labeled with Cy3 and Cy5 dyes, respectively, using random priming with Cy dye-labeled random hexamers according to the NimbleGen ChIP-chip protocol.

For comparison to CATCH-IT purified chromatin, we also purified biotinylated H3.3-containing nucleosomes from an S2 cell line expressing biotinylated H3.3 as described previously (8). The procedures for chromatin extraction, streptavidin purification, and DNA labeling were essentially the same as those used for the CATCH-IT chromatin purification, with the exceptions that streptavidin-bound H3.3 nucleosomes were not washed with the urea/NaCl buffer and whole genome amplification was not performed.

**Microarray hybridization and data analysis**
Profiling was performed on single custom designed 2.1 million feature isothermal microarrays (GEO GPL6888) purchased from NimbleGen, Inc. and hybridized and scanned by the Fred Hutchinson Center Genomics Facility using NimbleGen protocols. Data are available from GEO (GSE19788). Data were processed and log₂-ratios converted to standard deviates as previously described (9). Chromatin log₂-ratios were aligned with binding sites from ChIP-chip data obtained from GEO and/or original publications for GAF (GSM409071) (10), Zeste (11) EZ and PSC (12), and for ORC2 GSE18942 (13).

**Estimation of mean lifetimes from CATCH-IT data**

H3/H4 is incorporated behind the replication fork, and this will bring in Aha, but this process itself is would not involve replacement, in that there are no resident histones on newly synthesized DNA to be replaced, and the old histones are found to segregate conservatively to daughter strands (14). Only when H3/H4 is replaced by H3.3/H4 in a replication-independent process that follows is the process of turnover completed. Another round of turnover will replace H3.3/H4 with H3.3/H4 and this difference will not be detected, i.e., saturation is reached (see Fig. S5D-E). We therefore estimate kinetics based on modeling cycles of incorporation followed by replacement, reporting the mean lifetime of histones as representing a single full cycle of turnover. Note that this method will be insensitive to nucleosomes undergoing replication because the average turnover signal from a population of cells undergoing replication should be homogenous across the genome in unsynchronized cells. Therefore the presence of such a population in the culture should, at worst, only reduce the dynamic range of the microarray measurements. Also, this reduction is expected to be small, because it takes only a second or two to replicate through a nucleosome, ~1/50,000th of the cell cycle, so that each nucleosome in the
whole cell population undergoing replication will be diluted by ~50,000 nucleosomes that are not.

We aim to model the situation in which all histones in the genome initially have only methionine (at time $t = 0$), and during a brief Aha treatment, incorporate histones H3 (or H3.3) and H4 with Aha in place of methionine. We assume that at time $(t)$ Aha becomes incorporated into the histones occupying a particular site in the genome in direct proportion to the number of histone molecules at that site that have Met, and this number decreases as a result of turnover until saturation is reached. This relationship implies a negative exponential distribution of the fractional occupancy of Aha at each site represented by an array probe (Fig. S7). If $N$ is the fractional occupancy of Aha at the site, then $1-N$ is the fraction of Met, and $dN/dt = -\lambda(1-N)$, where $\lambda$ is the turnover rate. Solving for $N$ at time $t$, $N(t) = N_\infty [1-exp(-\lambda t)]$, where $N_\infty$ is the fractional occupancy of Aha at saturation, and $\lambda = -\ln[1-N(t)/N(\infty)]/t$. The mean lifetime of nucleosomes at this site is $1/\lambda$.

We estimated $\lambda$ at each array probe as follows:

1) We used 1,874,619 of the ~2.1 million probes from chromosomes 2L, 2R, 3L, 3R, 4 and X, excluding heterochromatic chromosomes.

2) We computed the raw ratios, $R(t) = \text{Aha incorporation}/\text{Total chromatin}$, for each probe from Cy5/Cy3 pixel measurements. For robustness, we used all three time points (20', 40', 60') in scaling.

3) We removed the saturated probes and probes with $R(t) < 75$th percentile of the random sequence probes on the same arrays to avoid low signal. This left 1,567,850 remaining probes for Experiment 1 and 1,788,127 for Experiment 2.
4) Because the computation of lambda requires that \( R(t) < R(\infty) \), we rescaled \( R(t) \) to lie between zero and one by selecting minimum and maximum values representing the initial and saturation states. Different strategies for determining minimum and maximum values yielded similar results, so we used the simplest, which selected the minimum and maximum values as the 0.1 and 99.9 percentiles respectively of \( R(t) \). This method dampens the contribution of extreme values of \( R(t) \), and the choice of such extreme limits compresses the dynamic range and provides very conservative values to avoid possible underestimation of mean lifetimes. We then rescaled the raw ratios as \( N(t) = \frac{R(t) - \text{min}(t)}{\text{max}(t) - \text{min}(t)} \), setting \( N(t) = 0 \) if \( < 0 \) and \( N(t) = 1 \) if \( > 1 \).

5) We computed \( \lambda \) as \(-\ln(1-N(t))/t\) for \( t = 20' \) at each probe.

6) We averaged probe \( \lambda \) values at genomic locations of interest, such as at TSS + 100 bp for genes, and estimated mean lifetimes at these locations as the reciprocal of the averaged \( \lambda \)s.

We restricted our estimation of mean lifetimes to the initial \( t=20' \) Aha labeling interval to avoid making assumptions about whether or not Aha is incorporated uniformly with time, and any delay in incorporation will lead to an overestimate of mean lifetime (underestimate of turnover rate).

References

**Figure S1: Metabolic labeling of total protein and histones in S2 cells.**

(A) Schematic diagram of the CATCH-IT method. Upper panels show the structure of Met and Aha, and lower panel shows the reaction scheme. (B) Streptavidin western blot of cycloaddition reaction products. Reactions were performed on total protein extracts from Met- or Aha-treated S2 cells, with or without Cu(I) or biotin-alkyne as indicated above the blot. Asterisks indicate the positions of endogenous biotinylated proteins. (C) Streptavidin western blot of cycloaddition reaction products from total protein extracts of cells treated with Aha in the presence or absence of the translation inhibitor cycloheximide. (D) Streptavidin western blot of nuclear protein extracts from cells treated with Aha from 1.5 or 3 hours and coupled to biotin through cycloaddition reactions. Positions of histones are indicated to the right of the blot.

**Figure S2: Removal of H2A/H2B dimers from biotinylated H3.3 nucleosomes captured on streptavidin beads.**

H2A and H3 western blots on streptavidin bead-bound (B) and wash (W) fractions of biotinylated H3.3 nucleosomes. Biotinylated nucleosomes were bound to streptavidin beads and washed with a solution containing 4 M urea and 0.3 M NaCl for the indicated times.
Gene ends analysis of CATCH-IT profiles from (A) a 3-hr Aha treatment, and (B) a 3-hr Met treatment in parallel. All 9820 genes from FlyBase r5.13 with annotated 5′ and 3′ ends were grouped by gene expression quintiles (top 20% to bottom 20% based on GEO# GSM333845), aligned by gene ends, and the log$_2$-ratios of pulldown DNA/input DNA averaged across genes. Although a distinct pattern is seen for the Met control, the fact that it closely follows the G+C content of the genome (C) suggests that it reflects an artifact arising from an imbalance between the two DNA samples used in co-hybridization to arrays. Because only background levels of DNA were obtained from the Met pulldown, a likely possibility is that the imbalance arises from bias in whole genome amplification.

**Figure S3: CATCH-IT patterns require Aha.**
Figure S4: Reproducibility of CATCH-IT patterns.
Gene ends analysis of CATCH-IT profiles from two independent experiments (A and B) using 3-hr Aha treatments. All 9820 genes from FlyBase r5.13 with annotated 5' and 3' ends were grouped by gene expression quintiles (top 20% to bottom 20% based on GEO# GSM333845), aligned by gene ends, and the log₂-ratios of pulldown DNA/input DNA averaged across genes. R = 0.97 (comparing A and B in left panels) and 0.92 (right panels) for all probes in all genes represented in the figure.
Figure S5: CATCH-IT kinetics over genic regions.
Gene ends analysis of a CATCH-IT experiment from a 3 hour Aha pulse followed by a 1.5 hour Met chase. All 9820 genes from FlyBase r5.13 with annotated 5′ and 3′ ends were grouped by gene expression quintiles (top 20% to bottom 20% based on GEO#GSM333845), aligned by gene ends, and the log$_2$-ratios of pulldown DNA/input DNA averaged across genes. Scatter plots show the correlation between the pulse and the difference between the pulse and chase, using only genic probes from different gene expression quintiles, showing little difference between the lowest quintiles (unexpressed genes, panel A) with offsets increasing with increasing expression levels (panels B-D).
Figure S6: Profiles of CATCH-IT short time-course experiments.
Gene ends analysis of CATCH-IT profiles from (A) 20’, (B) 40’ and (C) 60’ Aha treatments, and from calculated differences between the time points (D-E). All 9820 genes from FlyBase r5.13 with annotated 5’ and 3’ ends were grouped by gene expression quintiles (top 20% to bottom 20% based on GEO# GSM333845), aligned by gene ends, and the log₂-ratios of pulldown DNA/input DNA averaged across genes. Averages from two independent CATCH-IT experiments are shown. Saturation is approached over the course of the experiment, such that profiles for 40’ and 60’ are nearly superimposable.
**Figure S7: Kinetics of nucleosome turnover at Zeste sites.**
A) Average CATCH-IT time course signals over Zeste and EZ+PSC binding sites. B) Same as in (A) but for a pulse-chase experiment with a 3-hr Aha pulse and 1.5-hr Met chase. C) Average biotinylated H3.3 signals over Zeste and EZ+PSC binding sites. D) Average signals from chromatin salt fractions over Zeste and EZ+PSC binding sites.

**Figure S9. Kinetic model for turnover.**
Estimated turnover time curves for two values of \( \lambda \) are shown. The exponential decay constant, \( \lambda \), is estimated from the data, and examples of turnover processes at two hypothetical sites turning over at different rates are represented by the curves.
Figure S9. Kinetics of nucleosome turnover at sites of GAF binding. Quintile plots of various chromatin signals ranked by peak score for GAF binding sites.
Table S1. Mean lifetime estimates based on 20' time-course CATCH-IT profiles

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