A Novel Approach for Studying Histone H1 Function *in Vivo*

Giorgia Siriaco, Renate Deuring, Gina D. Mawla,¹ and John W. Tamkun²

Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, California 95064

ABSTRACT In this report, we investigate the mechanisms that regulate *Drosophila* histone H1 expression and its association with chromatin *in vivo*. We show that histone H1 is subject to negative autoregulation and exploit this result to examine the effects of mutations of the main phosphorylation site of histone H1.

KEYWORDS *Drosophila*; histone H1; chromatin; gene expression

HISTONE H1 is a key determinant of higher-order chromatin structure. This conserved linker histone contains a winged helix domain that binds the nucleosome near the site of DNA entry and exit; the regions flanking this domain interact with core histones and linker DNA to package nucleosome arrays into 30-nm fibers *in vitro* (Harshman *et al.* 2013). Consistent with its biochemical properties, the loss of histone H1 function leads to chromosome decondensation, defects in gene expression, genomic instability, and lethality (Godde and Ura 2008; Happel and Doenecke 2009; Lu *et al.* 2009; Siriaco *et al.* 2009; Vujatovic *et al.* 2012; Lu *et al.* 2013). These findings have stimulated great interest in the mechanisms that regulate the expression, assembly, and function of histone H1.

Several factors make *Drosophila* an excellent model organism for studying histone H1 function *in vivo*. Unlike most higher eukaryotes, which contain multiple, functionally redundant histone H1 variants (Godde and Ura 2008; Happel and Doenecke 2009), *Drosophila* has only one somatic histone H1 variant, the product of the *His1* gene. In addition, a subset of *Drosophila* tissues contains gigantic polytene chromosomes that arise from repeated rounds of DNA replication in the absence of cell division, making it much easier to directly score chromosome defects resulting from the loss of histone H1 function.

In this study, we used a *Drosophila* strain expressing histone H1 tagged with green fluorescent protein (H1-GFP) to investigate the regulation of histone H1 expression and its association with chromatin. A GAL4-inducible transgene encoding histone H1 with GFP fused to its C terminus (*UAS-H1-GFP*) (Fasulo *et al.* 2012) was expressed in the salivary glands of larvae bearing insertions of *UAS-H1-GFP* and an *eyGAL4* driver (Hazelett *et al.* 1998). Live imaging revealed that H1-GFP is associated with polytene chromosomes of *eyGAL4 UAS-H1-GFP* larvae; no unbound H1-GFP was detected in the nucleoplasm (Figure 1A). The staining of polytene chromosome squashes with DAPI revealed that the banding patterns of H1-GFP and DNA are highly coincident (Figure 1, B and C), as previously observed for the endogenous histone H1 protein (Corona *et al.* 2007).

The expression of histone H1 must be tightly regulated, as even modest changes in the level of histone H1 can have dramatic effects on nucleosome repeat length, global nucleosome density, and chromatin compaction (Blank and Becker 1995; Woodcock *et al.* 2006; Routh *et al.* 2008; Siriaco *et al.* 2009). In budding yeast, core histones are subject to negative autoregulation (Gunjan *et al.* 2006; Eriksson *et al.* 2012). Autoregulation also maintains constant levels of core histones in *Drosophila* (McKay *et al.* 2015). These observations prompted us to examine whether similar mechanisms are used to regulate histone H1 levels in *Drosophila*. As expected, the activation of the *UAS-H1-GFP* transgene in the larval salivary gland using the strong *eyGAL4* driver caused a large (~10-fold) increase in the total level of histone H1 RNA (*i.e.*, endogenous plus GFP-tagged H1; Figure 2A). This increase was accompanied by a compensatory 10-fold decrease in the expression of the endogenous histone H1 protein, as determined

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¹Present address: Department of Biology, Massachusetts Institute of Technology, 31 Ames Street, 68-333, Cambridge, MA 02139.

²Corresponding author: 350 Sinsheimer Labs, Department of Molecular, Cell and Developmental Biology, 1156 High St., University of California, Santa Cruz, CA 95064.
E-mail: tamkun@ucsc.edu

Figure 1 Histone H1 tagged with GFP is functionally equivalent to the endogenous histone H1 protein. (A) Confocal analysis reveals that H1-GFP is primarily associated with chromatin and localizes in a normal banding pattern in live *eyGAL4 UAS-H1-GFP* salivary gland polytene chromosomes. Chromosomes were imaged as previously described (Fasulo *et al.* 2012). Scale bar, 10 μm . (B) H1-GFP is normally distributed (as evidenced by GFP fluorescence) in fixed *eyGAL4 UAS-H1-GFP* salivary gland polytene chromosomes and colocalizes with DAPI. Arrowhead marks chromocenter. Chromosomes were prepared as previously described (Corona *et al.* 2007). Scale bars, 10 μm . (C) Comparison of the pixel intensity and pattern of the boxed chromosome arm in B reveals that H1-GFP and DAPI are highly overlapping. Chromosome boundaries were identified and the sum pixel intensity within chromosomes was calculated using Volocity (Improvision) software.

by Western blotting using an antibody that recognizes both the endogenous and GFP-tagged H1 proteins (Figure 2, B and C). As a result, the total level of histone H1 protein did not increase significantly and H1-GFP became the predominant form of histone H1 in this tissue (Figure 2, B and C). The degree of endogenous H1 repression was sensitive to the dosage of the *UAS-H1-GFP* transgene, with the strongest repression observed in *eyGAL4 UAS-H1-GFP* homozygotes (data not shown). Similar results were obtained using the ubiquitously expressed *daGAL4* driver (Gerber *et al.* 2004; see below). Flies ubiquitously expressing the *UAS-H1-GFP* transgene were viable and showed no developmental delay (data not shown). These findings indicate that negative autoregulation maintains relatively constant levels of histone H1 *in vivo*.

The suppression of endogenous histone H1 levels via the expression of H1-GFP did not cause obvious changes in either the structure or banding pattern of salivary gland polytene chromosomes (Figure 1, A and B). By contrast, even the partial knockdown of histone H1 levels by RNAi blocks development and causes dramatic defects in polytene chromosome structure (Lu *et al.* 2009; Siriaco *et al.* 2009). These findings indicate that histone H1-GFP is functionally equivalent to the endogenous histone H1 protein and can be used to study histone H1 function *in vivo*.

The association of histone H1 with chromatin is highly dynamic, with the bulk of H1 in the genome undergoing exchange in less than a minute (Lever *et al.* 2000; Contreras *et al.* 2003; Siriaco *et al.* 2009). Recent studies implicating histone H1 exchange in the regulation of cellular pluripotency and differentiation have heightened interest in the molecular mechanisms underlying this process (Meshorer *et al.* 2006; Alvarez-Saavedra *et al.* 2014; Christophorou *et al.* 2014). In both *Tetrahymena* and mammals, the phosphorylation of histone H1 weakens its affinity for chromatin (Roth and Allis 1992) but its effect on histone H1 assembly and chromosome compaction remains poorly understood.

Drosophila is a good model organism for studying the role of this modification since it has a single zygotic H1 isoform that contains only one major phosphorylation site: histone H1 serine 10 (H1S10) (Villar-Garea and Imhof 2008; Bonet-Costa *et al.* 2012). Standard genetic approaches cannot be used to study histone H1 modification in *Drosophila*, however, due to the presence of ~ 100 identical, functionally redundant copies of the gene encoding histone H1 (*His1*) interspersed with genes encoding the core histones H2A, H2B, H3, and H4 in the histone gene cluster (HIS-C) (McKay *et al.* 2015). Previous studies of histone H1 function in most higher eukaryotes, including *Drosophila*, have therefore been limited to the analysis of phenotypes resulting from the

protein in *eyGAL4 UAS-H1-GFP* salivary gland nuclei shows a twofold increase in H1-GFP. For quantification, Western blots were scanned and the sum pixel intensity for each band was calculated using Volocity (Improvision) software.

knockdown of histone H1 levels by RNAi (Lu *et al.* 2009; Siriaco *et al.* 2009). Our discovery that H1-GFP represses the expression of the endogenous H1 protein allowed us to circumvent this obstacle and characterize phenotypes associated with mutations affecting specific histone H1 residues.

Transgenic strains bearing GAL4-inducible transgenes encoding GFP-tagged histone H1 proteins with amino acid substitutions that mimic ($H1^{S10E}$) or block ($H1^{S10A}$) S10 phosphorylation were generated by *P*-element-mediated transformation. The *UAS-H1^{S10A}-GFP* and *UAS-H1^{S10E}-GFP*

transgenes were then expressed in the larval salivary gland using the *daGAL4* driver to study the effect of the S10E and S10A mutations on histone H1 function *in vivo*. As observed for H1-GFP (Figure 2B and Figure 3A), the expression of either $H1^{S10A}$ -GFP or $H1^{S10E}$ -GFP dramatically reduced the level of endogenous histone H1 such that the mutant form of histone H1 was the predominant linker histone in the larval salivary gland (Figure 3A).

Neither the S10A or S10E mutation caused obvious defects in the binding of histone H1 to chromatin *in vivo*. As observed

Figure 3 Mutations in the major phosphorylation site do not alter histone H1 function *in vivo* (A) Western analysis of salivary gland protein extracts from *UAS-H1-GFP*; *daGAL4*, *UAS-H1^{S10A}-GFP*; *daGAL4* and *daGAL4 UAS-H1^{S10E}-GFP* third-instar larvae. Endogenous H1 protein levels are reduced compared to wild-type control, suggesting that H1-GFP, $H1^{S10A}$ -GFP, and $H1^{S10E}$ -GFP are the main forms of histone H1 in salivary gland nuclei. Both endogenous H1 and H1-GFP were detected using antibodies against H1 (Active Motif, 39575). (B) Fixed salivary gland polytene chromosomes from *UAS-H1-GFP*; *daGAL4*, *UAS-H1^{S10A}-GFP*; *daGAL4* and *daGAL4 UAS-H1^{S10E}-GFP* third instar larvae. H1-GFP, $H1^{S10A}$ -GFP, and $H1^{S10E}$ -GFP are normally distributed on polytene chromosomes and colocalize with DAPI. Scale bars, 10 μ m. (C) Confocal images of *UAS-H1-GFP*; *daGAL4*, *UAS-H1^{S10A}-GFP*; *daGAL4* and *daGAL4 UAS-H1^{S10E}-GFP* third-instar salivary gland nuclei. Confocal images were obtained as described in Fasulo *et al.* (2012). H1-GFP, $H1^{S10A}$ -GFP, and $H1^{S10E}$ -GFP localize normally to chromosomes. Bottom: magnification of a single section through the salivary gland nuclei shown in the top. Images were taken at comparable gains. Scale bars, 10 μ m. (D) Exchange rate of $H1^{S10A}$ -GFP is reduced compared to $H1^{S10E}$ -GFP and H1-GFP.

Table 1 Quantitation of histone H1 exchange using FRAP assays

Genotype	$t_{1/2}$ (sec)	Mobile fraction (%)
<i>UAS-H1-GFP; daGAL4</i>	20.63 ± 6.9	79 ± 15
<i>UAS-H1^{S10A}-GFP; daGAL4</i>	31.27 ± 13.5	72 ± 16
<i>daGAL4 UAS-H1^{S10E}-GFP</i>	20.03 ± 10.3	72 ± 11

The mobile fraction and residence half-times of wild-type and mutant histone H1 tagged with GFP were determined by FRAP. Each value represents the average of at least 10 experiments (average $n = 15$). For FRAP experiments, 10 single imaging scans were acquired followed by 60 bleach pulses of 267 ms within a square region of interest (ROI) measuring $2 \times 2 \mu\text{m}$. Every second, 160 images were then collected. For imaging, the laser power was attenuated to 8% of the bleach intensity. Two further ROIs, measuring $2 \times 2 \mu\text{m}$, were used to measure total fluorescence and background. Recovery curves were generated and analyzed using easyFRAP software (Rapsomaniki *et al.* 2012).

for the wild-type histone H1 protein, the distribution of both H1^{S10A}-GFP and H1^{S10E}-GFP on fixed polytene chromosome squashes was coincident with DAPI staining (Figure 3B). Live analyses also revealed that the H1^{S10A}-GFP and H1^{S10E}-GFP proteins associated with chromatin in a normal banded pattern; the majority of both mutant proteins were bound to chromatin and neither H1^{S10A}-GFP nor H1^{S10E}-GFP was observed in the nucleoplasm (Figure 3C). The expression of either H1^{S10A}-GFP or H1^{S10E}-GFP caused a slight (~25%) reduction in both the size and DNA content of salivary gland polytene chromosomes, suggesting they may have a subtle effect on endoreplication (Figure 3B and data not shown). However, the banding pattern, morphology and overall structure of polytene chromosomes of larvae expressing H1^{S10A}-GFP or H1^{S10E}-GFP in place of the endogenous H1 protein were surprisingly normal (Figure 3, B and C). We therefore conclude that H1S10 mutations have little or no effect on chromatin compaction *in vivo*.

We also investigated the potential effect of H1S10 phosphorylation on histone H1 exchange in salivary gland nuclei using FRAP assays. The H1^{S10A} mutation increased the residence half-time ($t_{1/2} = 31$ sec) but not the mobile fraction of histone H1 (Figure 3D and Table 1). By contrast, the H1^{S10E} mutation did not alter histone H1 exchange ($t_{1/2} = 21$ sec vs. 20 sec for H1-GFP; Figure 3D and Table 1). These data suggest that even large changes in the rate of histone H1 exchange do not cause obvious changes in chromosome structure *in vivo*. Thus, mutations that block or mimic the

phosphorylation of H1S10—the main site of histone H1 phosphorylation—have minimal effect on histone H1 function in this tissue. Consistent with these findings, flies expressing high levels of either mutant protein under the control of the strong, ubiquitously expressed *daGAL4* driver were healthy, fertile, and phenotypically normal (data not shown). It remains possible that the phosphorylation of other sites of the histone H1 protein modulate its activity *in vivo*.

Although the mechanism by which histone H1 regulates its own expression is unknown, we suspect that it occurs at the level of protein stability. In our studies, we rarely observed free H1-GFP in the nucleoplasm, even using the strongest available GAL4 drivers, suggesting that the unbound histone H1 pool is unstable and rapidly degraded. Consistent with this possibility, we have been unable to express mutant forms of the histone H1 protein lacking the winged-helix domain required for nucleosome binding (data not shown). Histone H1 stability could also be dependent on a limiting interaction partner, as has been observed for the centromere-associated histone H3 variant CENP-A, which is unstable unless it is bound to the CENP-A assembly factor Cal1 (Schittenhelm *et al.* 2010; Chen *et al.* 2014).

Our serendipitous finding that histone H1 is autoregulated allowed us to establish a system for investigating mutations in the main phosphorylation site of histone H1. Until recently, it was impossible to conduct studies of *Drosophila* histone genes due to the presence of multiple, functionally redundant copies of the genes encoding histone H1 and all four core histones in the HIS-C gene cluster. Although a strategy for overcoming this obstacle has recently been developed and used to analyze phenotypes associated with *Drosophila* core histone mutations (Gunesdogan *et al.* 2010; Hodl and Basler 2012; Pengelly *et al.* 2013; McKay *et al.* 2015), it is relatively labor intensive and its utility for studying histone H1 function has not been established. By expressing transgenes encoding mutant H1 proteins under the control of a strong, GAL4-inducible promoter, we were able to test their ability to functionally replace the endogenous histone H1 protein. In theory, this approach could be used to study mutations in other residues of histone H1 or other proteins subject to negative autoregulation.

Table 2 List of oligonucleotides used for the construction of transgenes and the quantification of RNA levels by RT-PCR

Primer	Sequence
RP49-F	5' CACCAGGAAGTCTTGAATCCCGG 3'
RP49-R	5' AGATCGTGAAGAAGCGCACCAAG 3'
His1-F	5' CAGCAAATGGTGGACGCTTC 3'
His1-R	5' GCGAATATGTACCAATACTGC 3'
H1-F	5' TATCTCGAGTGCTTTTGGCAGCCGTAGTCTCGC 3'
H1S10A-R	5' ACCGAATTCGCATGTCTGATTCTGCAGTTCGAACGTCCGCTGCCCCAGTG 3'
H1S10E-R	5' CCGAATTCGCATGTCTGATTCTGCAGTTCGAACGTCCGCTGAGCCAGTGG 3'

The primer names and corresponding sequences are shown. To generate GAL4-regulated transgenes encoding C-terminal GFP-tagged histone H1 S10A and S10E substitutions, fragments were amplified from *Drosophila* genomic DNA using the H1-F, H1S10A-R, and H1S10E-R primers, subcloned into the *EcoRI* and *XhoI* sites of pENTR1A, and transferred to the *P*-element transformation vector pTWG (Brand and Perrimon 1993; Rørth *et al.* 1998). Transformants were generated by *P*-element-mediated transformation using the Df(1)w67c2 strain (Spradling 1986). Homozygous viable transformants used in this study include UAS-H1^{S10A}-GFP (line 1A4B) and UAS-H1^{S10E}-GFP (line 2G1), which are located on the second and third chromosomes, respectively.

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