

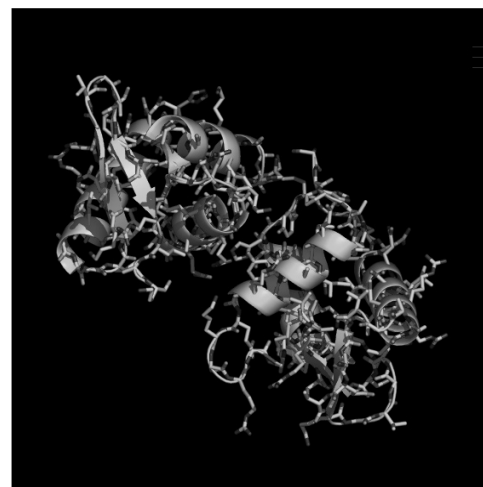
Solving the H1 Enigma: The Use of *Physarum polycephalum* as a Model System

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The discovery of DNA as the molecular code of life has shaped the biological sciences for decades. However, with this knowledge, a question must be asked: how can the nearly two meters of DNA contained within the human nucleus fit into a compartment only 0.6×10^{-6} meters in diameter? Obviously, DNA must somehow be compacted, a task made difficult by the highly negatively charged phosphate backbone as well as the sheer length of each DNA molecule. The cell solves this problem by the formation of a highly condensed state of DNA termed chromatin. The primary unit of chromatin is the nucleosome, which consists of 146 base pairs of DNA wrapped in 1.7 super-helical turns around an octamer of histone proteins H2A, H2B, H3 and H4.¹

Each histone protein consists of a characteristic histone fold domain as well as relatively unstructured amino and carboxyl terminal tails. The histone fold domains serve as the site of protein-protein interactions and allow the eight sub-particles to combine into a disk-shaped unit.¹ The highly basic nature of the histone proteins allows formation of electrostatic bonds with the negatively charged DNA. Because the formation of these bonds is independent of the DNA sequence, the entire length of DNA is able to associate homogeneously within arrays of nucleosomes. This primary level of condensation gives rise to a 10 nm fiber or “beads on a string” conformation in which short lengths of linker DNA separate each nucleosome.^{2,3} Obviously, several orders of further folding are required to achieve the compaction of DNA required by the cell. However, the mechanism of this compaction is rather poorly understood.

The association of DNA into nucleosomes and higher orders of folding vastly restricts the ability of enzymes to interact with the DNA. This means that in order for DNA replication or transcription to occur, the higher order structures must be resolved and the nucleosomes remodeled. It should also be noted that individual amino acids of the core histone tails are distinctively modified.⁴ These modifications specifically alter the properties of DNA-nucleosome binding. For example, methylation of the lysine 4 residue of H3 has been correlated with active genes while H3 methylation at lysine 9 promotes gene silencing and heterochromatin formation.⁵ Thus, specific modification of histone tails has interesting applications in gene control, as these modifications can determine the transcriptional fate at specific loci.

Functional Role of the Linker Histone H1

In addition to the core nucleosome, the linker histone (H1) is also a major part of chromatin. Association of H1 with the nucleosome protects an additional twenty base pairs of linker DNA.⁶ Composed of a short amino terminal domain, a globular domain, and a longer carboxyl terminus, the H1 protein is also highly basic in nature, but does not share the histone fold domain present in the core histones. H1 bends DNA entering and exiting the nucleosome and has long been believed to stabilize condensed chromatin.⁶ However, due to the difficulty of crystallizing H1 in association with core histones and DNA, several different models have been proposed to explain the interaction of H1 in chromatin. Traditionally, *in vitro* studies have implicated the linker histone in affecting chromatin structure several ways, including the stabilization of DNA entering the nucleosome core, restriction of nucleosome mobility and spacing and stabilization of higher order structures.⁷⁻⁹ These characteristics would place H1 in a position to globally restrict gene transcription. However, it has additionally been shown that these outcomes can be partially or completely mimicked by increased salt concentrations. Thus, a question remains: what role is H1 performing inside the cell?

The best way to begin answering this question is to perform *in vivo* experiments in which the gene encoding for H1 is removed or mutated and the effect on the organism is observed. Because the overwhelming majority of biological mechanisms are conserved from yeast through humans, it is useful to perform these studies on lower organisms as this allows simplification of the experiments and data interpretation.

Studies performed in yeast involving H1 knockouts showed that the absence of H1 did not significantly perturb chromatin structure or viability, meaning the organism was able to successfully complete mitosis and transcribe the required genes.¹⁰ This result was replicated in *A. immersus* and *Tetrahymena*.^{11,12} Furthermore, in lower eukaryotes studied thus far few phenotypic changes have been observed. It is interesting to note, however, that H1 has been recently implicated in aging and genome stability as yeast and *A. immersus* show shorter life spans when H1 is not present.^{11,13} The findings in lower organisms further complicate our ideas about the role of H1 in the cell. If the protein is not essential in lower organisms, why would it be conserved through humans? It is extremely

unlikely that a protein serving no function would withstand evolutionary pressures. However, because both the yeast and *Tetrahymena* H1 protein structures deviate from the common tripartite structure (yeast having two globular domains and *Tetrahymena* lacking a globular domain) the data from these organisms must be carefully analyzed.¹⁴

Another clue into the mysteries of H1 comes from null mutations in mice. Mice, like the majority of higher eukaryotes, contain several variants of H1. When researchers created null mutations of one subtype, no phenotypic difference was seen.¹⁵ However, these animals retained normal H1-to-core histone ratios thus suggesting that other H1 subtypes were compensating for the missing protein. To test this theory, researchers generated compound null mutation mice in which three H1 subtypes were absent. These mice were not viable and showed a wide range of phenotypic abnormalities which resulted in embryonic death, thus proving H1 is indeed essential in mice.¹⁵ However, these results give little indication of the actual function or mechanism of the linker histone.

Because knockout studies of H1 have shown little effect on global transcription, perhaps H1 provides a more specific function within the cell. Gorovsky examined the effect on individual genes in *Tetrahymena* and found that basal transcription of some repressed genes including (*ngoA*) was increased while the transcription of activated genes (*Cyp1*) was decreased.¹⁶ This surprising result indicates that the *in vivo* effects of H1 are indeed gene specific and may involve either up or down regulation of the transcribed gene.

Further evidence for a specific, tightly regulated function for linker histones has been shown in mice. The linker histone subtype H1b has been shown to bind in conjunction with the protein *Msx1* at the regulatory element of *MyoD*.¹⁷ The *MyoD* gene encodes a protein that controls the differentiation of skeletal muscle. When both H1b and *Msx1* bind, the *MyoD* locus is repressed, therefore the *MyoD* gene is not transcribed and muscle differentiation is inhibited in cell culture.^{17,18}

The role of H1 has also been extended to include the control of DNA repair by homologous recombination. Inhibition of the yeast H1 homologue *Hho1p* shows no significant phenotypic alteration when the protein was knocked out from yeast cells. However, the mutant strain was able to survive high levels of the DNA damaging agent methyl-methane sulfonate (MMS).¹³ Through a combination of knockout studies, it was shown that disruption of *Hho1p* increases survival in the presence of MMS only when the genes responsible for homologous recombination were available.¹³ Therefore, the presence of the linker histone *Hho1p* inhibits DNA repair by homologous recombination, which results in a reduced life span of the organism.¹³ While it is possible that the regulation of homologous repair by H1 is due to DNA condensing properties, this explanation seems unlikely as knockouts of *Hho1p* do not exhibit a noticeable change in chromatin condensation.¹³ Rather, it is possible that the presence of a linker histone covers binding sites for homologous repair machinery. If these results are conserved through humans, H1 regulation may have important consequences in cancer biology as mutation of H1 could promote tumorigenesis through enhancing rates of homologous repair.

Linker histones have also been implicated in the inhibition of ATP-dependent chromatin remodeling activities. Phosphorylation of linker histones *in vitro* inhibited the

activity of several ATP dependent chromatin remodeling complexes including γ SWI/SNF, hSWI/SNF, xMi-2, and cACF.¹⁹ This activity was shown to be independent of H1's ability to condense chromatin as the same inhibition levels were observed following modification of core histone tails. This modification prevents the DNA from folding into higher order chromatin structures. Furthermore, it was shown that the inhibitory action of linker histones on ATP dependent remodeling complexes could be countered by introducing a kinase to phosphorylate the linker histone.¹⁹ This suggests a tightly regulated pathway must be involved in the modification of linker histones at specific gene loci.

The results presented above provide a clearer indication of the role of H1 in regulating specific gene transcription, yet one is left asking what actually allows these functions to occur. H1 binds indiscriminately to DNA, yet this does not explain the specific, regulated functions observed in these studies. Perhaps, similar to the core histones, the linker histone is somehow modified in order to bring about these specific regulatory functions. It seems the linker histone story is much more complicated than simply stabilizing chromatin complexes.

Mechanism of H1

Early studies of the role of H1 clearly implicated the carboxyl Terminal Domain of H1 as necessary for the association of chromatin into higher order structures.²⁰ This effect has been attributed to the high levels of basic amino acids present in the CTD which would be capable of neutralizing the negative charge of DNA. However, Jeffery Hansen has shown by partial deletion of the CTD that the function of H1 is not due solely to charge neutralization across the entire CTD.²¹ He has further shown that while the initial binding of H1 to chromatin has some electrostatic component, specific sub-domains of the CTD are responsible for stabilization and self-association of H1. These sub-domains can be topologically rearranged within the CTD without affect on the overall function of H1.²¹ This finding is interesting in light of the usual requirement of proteins to be precisely ordered to function properly within the cell.

Through studies with *Tetrahymena*, Gorovsky has elucidated another portion of the H1 story. He has shown that phosphorylation of the linker histone regulates gene expression. However, unlike the specific post-translational modifications of the core histones which give rise to explicit alterations in chromatin structure and gene expression, phosphorylation of H1 seems to function through a less specific mechanism.¹⁶ *Tetrahymena* H1 contains a 20 amino acid stretch in the amino terminal region which has five phosphorylation sites. Gorovsky mutated these sites so as to mimic either a constitutively phosphorylated or unphosphorylated state of H1. The constitutively phosphorylated form produced similar results as seen in the knockout experiments of H1 with an increase in *ngoA* levels and decrease in *Cyp1* levels. The unphosphorylated form showed increased expression of *Cyp1*.¹⁶ Gorovsky further found that the alteration of transcription levels mediated by H1 was due to the creation of a charge patch by phosphorylation and not simply due to an alteration of the hydrophobicity or recognition of the phosphate.²² These studies are relevant to the function of H1 because as the cell undergoes a transition through various physiological conditions, the phosphorylation

of H1 may increase or decrease, thus allowing a mechanism for control.²³ In the unphosphorylated state, H1 is able to strongly bind linker DNA and thus compete with other DNA binding proteins for access to regulatory sequences, thus, in some cases, activating transcription. Conversely, in the phosphorylated state, a negative charge patch is introduced which weakens H1 interaction with linker DNA and either allows regulatory proteins to bind the DNA or even allow the nucleosome to shift and thus expose a regulatory sequence which was previously inaccessible to binding proteins.²² Additionally, Gorovsky found the location of the negatively charged region could be altered with no impact on gene transcription.

The results of Gorovsky and Hansen regarding the mobility of functional domains within the H1 protein make sense in light of the large divergence of sequence across species. Whereas core histone sequences are well conserved and modifications are specific, it seems that in H1, the precise sequence is unimportant in respect to the amino acid composition of specific domains. While the findings discussed above provide a great deal of insight into the function and mechanism of H1 inside the cell, a vast number of questions are unanswered. For example, one would like confirmation of the charge patch mechanism in an organism exhibiting a tripartite H1 structure. The topology of H1 association with the nucleosome to confer stability to chromatin is still poorly understood. Additionally, multiple other roles for H1 are likely in light of the H1b studies in mice, ATP-dependent chromatin remodeling complex interactions, and homologous repair inhibition. Overall, the study of H1 has implications not only in chromatin structure and gene expression, but also in cancer, aging, and development.

***Physarum polycephalum* as a Model Organism for the Study of Chromatin**

The organism *Physarum polycephalum* has several specific biological properties which make it an ideal organism for the study of chromatin structure and function. During the macroplasmoidal stage of *Physarum*, the several million nuclei contained within a single cell are perfectly synchronous throughout the cell cycle.²⁴ This property not only allows the elucidation of chromatin events at specific cell cycle stages, but also vastly enhances the ability to detect these changes because one is able to observe a huge population of nuclei. Additionally, exogenous proteins applied topically to the cell are absorbed and transported to the appropriate cellular compartment.^{25,26} The utility of this property in chromatin study has been observed through the incorporation of introduced histones into the chromatin of the living cell.²⁷ This ability circumvents traditional requirements of genetic manipulation and microinjection; furthermore, it allows the introduction of biochemically relevant levels of modified proteins into the cell.

Previous Work on H1 in *Physarum*

Before much of the advanced laboratory methods for studying chromatin structure and function were available, *Physarum* provided an invaluable resource for researchers involved in this field. Because of its natural synchrony, and the large quantities of histones available from one cell, it was possible to perform studies on *Physarum* which at the time would have been impossible in other model organisms.

As early as 1973, Bradbury proposed that phosphorylation of H1 is temporally correlated with chromosome condensation and therefore with mitosis and cell division.²⁸ This result was obtained through measurement of the incorporation of radioactive phosphate at specific cell cycle stages. Bradbury saw a sharp increase in radioactively labeled H1 two hours prior to mitosis. Furthermore, the level of H1 phosphorylation was observed to drop off 20 minutes before mitosis was observed through phase contrast microscopy.²⁸

Bradbury further extended these findings to hypothesize that phosphorylation of H1 was the initiation step of mitosis. He shows the phosphorylation of H1 is directed by an increase in the levels of phosphorylating activity, which corresponds with an increase in phosphorylated H1. This result shows that H1 phosphorylation is a consequence of a regulated enzyme activity and not merely due to an increase in substrate concentration.²⁹ If this phosphorylation activity did cause chromosome condensation, H1 could be implicated in the control of cell division which would in turn have broad implications in cancer biology.

To confirm the hypothesis that phosphorylation of H1 controls mitosis, in 1976 Bradbury used a heterogeneous chromatin extract of Ehrlich ascites containing Growth Associated Histone Kinase (HKG). In vitro, the extract was shown to phosphorylate calf thymus H1.³⁰ When the extract was added to *Physarum*, mitosis was advanced by twenty minutes. This result could indicate that it was indeed the phosphorylation of H1 which promoted mitosis. However, any experiment using an extract rather than purified protein must be considered carefully as the result of the experiment may be due to several factors present in the extract. Furthermore, the level of extract added was many times higher than the activity of HKG normally present in the cell. Considering this high level of enzyme and the relatively short time of advancement, these experiments provide little evidence that H1 phosphorylation is causatively connected to chromatin condensation.

Fischer found further confirmation that H1 phosphorylation occurs prior to mitosis in 1980. However, no dephosphorylation was seen prior to the onset of mitosis.³¹ Furthermore, through studies with affinity chromatography on a DNA-cellulose column, Fischer showed that phosphorylated H1 is less strongly bound to DNA than the unphosphorylated form.³¹ This finding seems to suggest that phosphorylated H1 is not the determining factor for the onset of mitosis. The release of H1 by phosphorylation could allow other factors to bind the DNA competitively, and thus enable modification of chromatin structure.

Early results involving H1 phosphorylation and the correlation to the onset of mitosis do not provide satisfactory evidence of a mechanism of these events, nor do they provide even a direct causal relationship. The basic nature of H1 is the major property which allows binding to DNA; thus, it makes sense that phosphorylation, and thus the introduction of negative charges to the H1 protein, would loosen interaction with DNA. How this would introduce a condensation event is unclear unless the release of H1 provides the opportunity for other condensing proteins to bind. As H1 has been shown to stabilize the intrinsic ability of the core histones to condense into higher order chromatin structures, and as no other stabilizing protein has been found to bind to chromatin, it

seems unlikely that it is, as early studies suggest, the release of H1 by phosphorylation that causes the condensation of DNA. Despite the conflicting results, early studies provide a solid basis of the utility of *Physarum* in the study of H1.

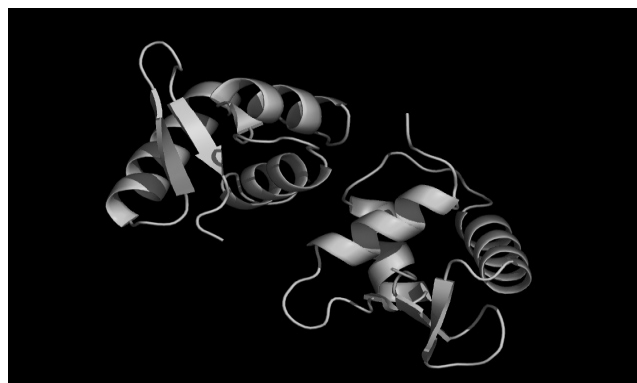
New Approaches to the Study of H1 in *Physarum*

The easy availability and more sophisticated laboratory techniques available for studies of yeast and other lower organisms shifted attention away from *Physarum* in the late 1980's. However, the properties of *Physarum* remain an untapped resource for the elucidation of chromatin structure and function at specific cell cycle stages. One of the *Physarum* characteristics which is useful in biochemical analysis is the ability to incorporate exogenous proteins placed on the surface of the macroplasmidium. Hayes and Thiriet studied the ability of *Physarum* to incorporate three different somatic linker histones into chromatin. Each linker histone was localized into the nucleus and incorporated into the chromatin.²⁷ It was also shown through salt release experiments that exogenous linker histone is less tightly bound during S phase than during G2 phase of the cell cycle. Additionally, when linker histone was incorporated during G2 phase a decrease in DNA transcription level was observed.³² This moderation of transcription was dependent on the type of linker histone, with H1 decreasing transcription by 60% while H1^o and H5 showed 30% reduction.³² These findings are interesting in light of the lack of a G1 phase in *Physarum*. Thus, the cell must prepare for the events of DNA replication which occur during S phase before mitosis. This consideration may explain the delay of mitosis and requirement for high levels of H1 phosphorylation observed previously in *Physarum*.

Studies in *Physarum* have been retarded relative to those in yeast or *Tetrahymena* due to the absence of highly characterized genetic resources. However, recently a high titer cDNA library has been created by our lab; this library contains the DNA sequences of protein coding regions of the *Physarum* genome. Furthermore, *Physarum* has recently been selected for genome sequencing. The combination of the genome sequence and the cDNA library should propel research in *Physarum* as these resources will allow the power of *Physarum* as a model organism to be further realized.

Specifically, our lab has also screened the library for the complete coding sequence of the *Physarum* H1 protein. This sequence will allow further studies of the mechanism of H1 to be addressed in vivo. As previously described, *Physarum* incorporates exogenous proteins into chromatin. With the complete coding sequence of H1, it will be possible to mutate this sequence at specific locations and determine the effect of these mutations on chromatin structure and function at specific cell cycles. *Physarum* is well suited to these studies because unlike mammalian systems, only one subtype of H1 is present. This allows a more precise analysis of the effect of H1 on chromatin as there are no compensatory effects by other H1 subtypes.

In using these methods, it should be possible to examine the effect of phosphorylation of H1 because specific residues can be modified to assume a phosphorylated or unphosphorylated state and thus determine which phosphorylations are important to transcriptional regulation. This should allow further testing of the proposed charge patch mechanism proposed in



Cartoon structure of Histone H5

Tetrahymena. Additionally, further investigation of the role of H1 in chromatin condensation, the regulatory events that promote H1 phosphorylation, and the global and local results of this phosphorylation will be possible through the study of H1 in *Physarum*.

Conclusions

Although the scientific community has made great advances in determining the function and mechanism of H1 in chromatin structure and function, much remains unexplored. As in the case of many biological systems, the field of chromatin studies was once seen as a rather unimportant aspect of gene function and regulation. However, as current studies advance, it is obvious that the role of chromatin is far more vital than simply DNA condensation. Studies of H1 provide insight into the role of DNA and chromatin binding factors, which provide the precisely regulated control of transcription. Certainly, these findings have broad reaching effects for the control of human pathology and aging. Through the development of H1 studies in *Physarum*, we are one step closer to unfolding the complex web of H1 interactions in the cell.

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