Title of Research Proposal

The Dynamic Nucleosome: Multiple Aspects of Positioning the +1 Nucleosome

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Summary of Research Proposal

Genome wide nucleosome positioning maps revealed the presence of global nucleosome patterns, where nucleosomes seem to be uniformly spaced at the 5' and 3' ends of genes. Yet, nucleosomes are highly dynamic, as they transiently dissociate histones, wrap and unwrap themselves in a process called nucleosome breathing or move up and down on the DNA. The +1 nucleosome is the first nucleosome downstream from the nucleosome free region. Many believe that understanding the factors that position the +1 nucleosome is the key to understanding transcriptional control by nucleosome positioning.

The proposal is an attempt to identify the factors that regulate the positioning of the +1 nucleosome. We present two main projects:

1. Identify the combinatorial effect of ATP dependent chromatin remodelers in the positioning of the +1 nucleosome
2. Delineate the mechanisms that link the stability of the +1 nucleosome to its positioning.

ATP dependent chromatin remodelers are known to treat the +1 nucleosome differently from the other histones [1]. The first project will identify the role of individual ATP dependent chromatin remodelers in the precise positioning of the +1 nucleosome. To this end, we propose a ChIP-exo-MS/MS method that would allow us to combine mass spectrometry based proteomics, while following a single nucleosome position over time. The second project will allow integration of these results into a model that seeks to identify factors that link the stability of the +1 nucleosome and its positioning.

Layperson's Summary

A cell's DNA is responsible for the functioning and characteristic of each cell in our body. Each cell contains more or less the same DNA sequence, yet our body consists of hundreds of different types of cells. The molecules and proteins that interact with the DNA are responsible for the vast differences seen between cell types in our body. Each DNA strand is about 2 m long, yet it is neatly packed into a 10μm nucleus of a cell. This packaging, apart from storing the cells regulatory information, is a way of deciding which regulatory mechanisms are allowed to impinge on the DNA. This is done by unwinding certain parts of the chromatin anytime as needed, making that section of DNA more accessible to the regulatory mechanisms. Thus, the factors that package the DNA make important contributions to a cells behavior.

When taking a closer look at the nucleus, the genetic material can be seen packed within a structure called chromatin. Chromatin is composed of repeating units of nucleosomes comparable to a bead on string structure, where each bead is a nucleosome. Each nucleosome is made up of a protein core, which is wrapped around twice by a strand of DNA. At first glance, nucleosomes can be seen to be arranged in a bead on string structure. Yet, different patterns can be seen in its arrangement. The role of nucleosomes over a strand of DNA is more than just a space occupier. The nucleosome can hide certain DNA sequences as it twists the DNA around it, and by changing the position, the hidden sequences can be revealed.

At the basic level of a gene, nucleosomes are arranged in a unique pattern. A nucleosome free area can be seen before the start of a genes coding region. This region is flanked on either side by two well-positioned nucleosomes, named the -1 and +1 nucleosome. The +1 nucleosome is positioned closer to the start of the gene. The neighboring nucleosomes, +2, +3 and +4 also show a degree of positioning. However, the spacing between the nucleosomes becomes more unequal as we move on towards the centers of the gene.

The proper positioning of the +1 nucleosome maybe the key to positioning the rest, as it can act as a barrier to modulate the positioning of its neighbors. The proposal describes the study of a collection of factors that determine the precise positioning of the +1 nucleosome. The proposal entails two projects over four years, based on Saccharomyces cerevisiae model organism combined with a novel assay and recent nucleosome mapping techniques.

Description of the Proposed Research

General Background to Research Topic

The dynamic nature of nucleosomes

Chromatin is the end product of repeating nucleosomes that stack on one another to form the complex arrangements of fibres with the aid of linker histones [2]. A 147 bp strand of DNA that wraps 1.65 turns in a left-handed superhelix, around an ocatmeric histone complex containing two copies of histone H2A, H2B, H3 and H4, forms the nucleosome [3–6]. Nucleosomes are highly dynamic and their behavior entails transient dissociations of histones, nucleosome breathing, i.e, the dynamic wrapping and unwrapping of DNA and nucleosome mobilizations where the nucleosome changes position on the DNA sequence.

Factors that affect nucleosome occupancy over genomic DNA

How a given nucleosome occupies a stretch of genomic DNA has been of interest as early as the late 1900s [7] when it was first recognized to be important for understanding the structure and function of the chromosome. Genomic maps have revealed the presence of global nucleosome patterns, where nucleosomes are uniformly spaced at the 5' and 3' ends of genes, typical nucleosome arrangements (from highly positioned nucleosomes, to the un-positioned and highly dynamic nucleosomes), as well as typical cell specific patterns [2,8].

A nucleosome free region is usually seen upstream from the transcription start site. The first nucleosome downstream from the nucleosome free region, the +1 nucleosome, is highly positioned. The rest of the nucleosomes, the +2, +3, +4, etc, are less precisely positioned and show a decrease in positioning over the body of the gene. Often, a well positioned -1 nucleosome is found immediately upstream of the nucleosome free region (figure 1). As nucleosomes are highly dynamic [9] the positioning of these nucleosomes is an end result of combinations of factors. These include; influences of the underlying DNA sequence and non nucleosomal factors.

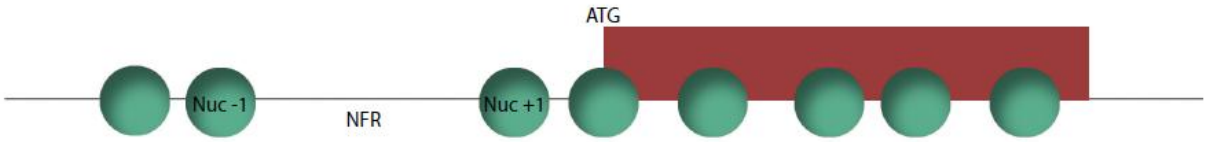


Figure 1: Schematic diagram of a typical gene promoter. The gene (brown box), its promoter (black line) and associated nucleosomes (green) are shown. The first nucleosome upstream from the transcription start site (ATG) is the +1 nucleosome (Nuc +1) and the 1 nucleosome found immediately upstream of the nucleosome free region is the -1 nucleosome (Nuc -1).

The nucleosome positioning code

There is considerable debate on the relative importance of DNA sequence on nucleosome localization [10]. Studies on reconstituted nucleosomes show high DNA sequence preferences [2]. This influence is different from an interaction between the amino acids of a DNA binding factor and a few bases of DNA. Nucleosome formation is based more on the ability of a certain stretch of DNA sequence to wind around a histone core [4]. It is generally accepted that there could be a nucleosome positioning code where the pattern of nucleosome positioning is determined primarily by the genomic DNA sequence. Thus the pattern of nucleosome positioning can be predicted using the DNA sequence [11,12].

Certain dinucleotides, AT, TA, GC modulate the DNA structure such that it wraps easily around a nucleosome [7]. These dinucleotides appear at distances of multiples that are 10.4 (10.4xn) bases, which corresponds to the nucleosome DNA structural period [12]. This 10.4xn base periodicity is thought to facilitate DNA bending in that direction as it also corresponds to the minimum energy of DNA folding in the nucleosome [12].

Unlike the dinucleotides, homopolymeric sequences of poly (dA:dT) and poly (dG:dC) are inhibitory for nucleosome binding as these stretches are stiffer and do not easily wrap around nucleosomes [7,13,14]. These poly (dA:dT) stretches are major determinants of nucleosomal organization as promoter nucleosome free regions correspond to these sites [10,15]. Additionally, the strong correlation between the positioning of poly(dA:dT) tracts and +1 nucleosomes suggests a contribution to the positioning of the +1 nucleosome [16,17]. The Poly(dA:dT) tracts could be thought of as barriers for the establishment of the +1 nucleosome [17], which is thought to be the key to positioning neighboring nucleosomes. This idea is related to the statistical

positioning model [18] and the barrier nucleosome model [19], which states that nucleosomes on a strand of DNA would appear to be well-positioned because of the constraints or barriers that ensure the precise positioning of the +1 nucleosome and thereby restrict the positions of neighboring nucleosomes.

Role of non-nucleosomal factors on nucleosome positioning

Even though nucleosome occupancy is strongly determined by the DNA sequence, it has now become clear that some aspects of positioning are not determined by sequence alone [4]. Supporting this idea, studies have shown that the spacing of nucleosomes differ in different cell types and organisms even when the same DNA sequence is used [2,10,18]. These differences cannot, by definition, be due to intrinsic DNA sequence, but rather protein factors from the host organism. Thus, a dynamic interplay between the underlying DNA sequence and non nucleosomal factors such as cooperation between ATP dependent chromatin remodelers, DNA-binding proteins, the transcription machinery and the passage of the RNA polymerase II are thought to result in stable positioning of nucleosomes [10,20]. Additionally, incorporation of histone variants and histone covalent modifications that change nucleosomal characteristics are also thought to influence nucleosome mobility and positioning [21].

ATP dependent chromatin remodelers establish desired spacing between nucleosomes

The role of ATP dependent nucleosome remodeling complexes were highlighted by Zhang et al.,(2011)[16]. The addition of whole cell extracts and ATP resulted in *in-vitro* reconstitution of nucleosome positions and occupancy levels around the 5′ ends of nearly all 4,785 tested yeast genes whereas incubating reconstituted nucleosomes in the absence of ATP had no such effect. ATP dependent nucleosome remodeling complexes are a class of enzymes known to use the energy of ATP hydrolysis to move, destabilize, restructure or eject nucleosomes. A deletion analysis of the ATP dependent remodelers, ISW1, ISW2 and CHD1 shows that regular positioning of the majority of nucleosomes is lost in the absence of these enzymes [17]. Thus, ATP dependent remodeling enzymes are good candidates for directing the positioning of the majority of nucleosomes within the Saccharomyces cerevisiae genome *in vivo*. ATP dependent remodelers act on multiple nucleosomes directing their movement away from default sequences,

to either locations dictated by the complex or to locations that establish desired spacing between nucleosomes [22–25].

A role for DNA binding proteins

A strong correlation exists between certain DNA binding proteins (Abf1, Reb1, Rap1, Mbp1 and Cbp1) and the positioning of nucleosomes [1,11]. This has lead to the suggestion that these proteins may act as barrier elements to restrict the movement of nucleosomes. These observation strengthen the barrier model for nucleosome positioning [18,19], where the movement of the -1 and +1 nucleosomes is forcibly prevented by other factors.

Role of the transcription machinery

Finally, many believe that some aspect of the transcription machinery may have a role in the positioning of the +1 nucleosome and thereby affect spacing of downstream nucleosomes. The mechanistic connection between the +1 nucleosome and transcriptional start sites and the directional positioning of nucleosomes have linked transcription initiation and related complexes to the positioning of the +1 nucleosome [10]. In a study that introduced genomic sequences from evolutionary divergent yeast species in to *Saccharomyces cerevisiae*, the spacing relationship between the +1 nucleosome and transcription start site shifted to resemble a more *S. cerevisiae* like pattern. This lead to the conclusion that the pre-initiation complex maybe involved in the fine-tuning the position of the +1 nucleosome [10].

The position of the +1 nucleosome has also been observed to vary considerably depending on the transcriptional activity of the gene [1]. In repressed genes, the transcription start site is located closer to the midpoint of the +1 nucleosome. Active genes tend to position the +1 nucleosome further away from the transcription start site. This has led to the suggestion that the transcription initiation machinery may have a role in the positioning of the +1 nucleosome.

Additionally studies have shown that the passage of RNA polymerase II the polymerase shifts nucleosomes in a direction away from polymerase passage [26]. This has lead to the suggestion that the RNA polymerase passage may have a role in nucleosome positioning [27].

Role of nucleosome stability on nucleosome positioning

Histones are deposited primarily in the S-phase in a DNA replication dependent manner in yeast. After they are deposited, they can either be moved, replaced or evicted [28]. In many species, transcription leads to the replacement of histone H3 by H3.3 histone variant [28]. In *Saccharomyces cerevisiae*, whose only H3 variant is H3.3 [28], passage of RNA polymerase II generally results in the eviction of nucleosomes [29,30] which are then reassembled behind the polymerase [31]. In some cases, polymerase passage may not evict the entire histone octamere, but a single H2A-H2B dimer, while rest of the hexasomal complex is retained [32,33]. Thus, a measurement of histone H3 turnover is a measure of nucleosome stability. Studies have found that in Saccharomyces cerevisiae nucleosomes at promoters are replaced more rapidly than at coding regions [28]. Promoter nucleosomes also show precise spacing patterns, signifying a connection between stability of nucleosomes and positioning.

The composition of the nucleosomes is another factor that determines nucleosome stability. In *Saccharomyces cerevisiae* H2A.Z histone variant is enriched at the two positioned nucleosomes flanking the nucleosome free region [21,34,35]. The incorporation of H2A.Z in to Saccharomyces cerevisiae chromatin is mediated by the SWR1 chromatin remodeling complex (also known as SWR-C) [36] and is replication-independent [37,38]. SWR1 removes H2A-H2B dimers and replaces them with Htz1-H2B dimers [39]. Studies by Dion et al., (2007) suggest that nucleosomes enriched with the variant H2A.Z have high turnover rates [28]. Similar results were also presented by Zhang et al., (2005) and Watanabe et al, (2013), where H2A.Z bearing nucleosomes, possibly in combination with histone H3 acetylated at lysine 56 (H3K56Ac) , were seen to be unstable and susceptible to loss [39,40]. It is thought that replacement of Histone H2A, by the H2A.Z functions to erase histone marks, and thereby preventing the spread of chromatin states [40]. Li et al., (2005) demonstrates that once positioned to a certain location, H2A.Z containing nucleosomes are not easily influenced by chromatin remodelers and are less influenced by nucleosome mobilization driven by these complexes[21] .

Positioning of the +1 nucleosome

Genome wide nucleosome arrangement patterns find that the +1 nucleosome is strongly positioned and the degree of positioning decreases with more downstream nucleosomes. As discussed a highly positioned +1 nucleosome would create the constraints needed for establishing proper spacing of downstream nucleosomes [4]. Yet there is a gap on knowledge in the events leading to the precise positioning of the +1 nucleosome.

A role for ATP dependent nucleosome remodelers have been suggested in the repositioning of the +1 nucleosome. Studies show that nucleosome remodelers handle the +1 nucleosome differently and certain remodelers, ISW1a, ISW2, RSC and SWI/SNF show preferential binding to the +1 nucleosome [1]. It is believe that the positioning and stability of the +1 nucleosome is a result of the combinatorial action of all these remodelers.

A recent study exposed the cooperative role of DNA binding factors and Chromatin remodelers in the positioning of the +1 nucleosome[1], highlighting the important role played by DNA binding factors . The study describes the role of the ATP dependent chromatin remodeler ISW2, which binds the +1 nucleosome in an orientation-specific manner and moves it towards the nucleosome free region NFR until it encounters a barrier such as Reb1.

As described before, the role of transcription machinery in nucleosome positioning mainly focuses on the positioning of the +1 nucleosome. The high correlation between transcription start sites and the +1 nucleosome have lead to the idea that the positioning of the +1 nucleosome may depends on DNA sequences linked to the process of transcriptional initiation[11]. These observations have suggested a role for influences of DNA sequence in the positioning of the +1 nucleosome. Additionally, the presence of poly Poly (dA:dT) tracts that dictate nucleosome free regions, have also been suggested to contribute to the positioning of the +1 nucleosome [16].

Another factor that affects the positioning of the +1 nucleosome is nucleosome stability. The passage of RNA polymerase II results in the eviction of nucleosomes, including the +1 nucleosome, calling its stability and precise positioning to question. Additionally, the composition of the +1 nucleosome of *Saccharomyces cerevisiae* is unique as it is enriched for the H2A.Z histone variant [1,41]. As H2A.Z containing nucleosomes are relatively unstable[40] and have

higher turnover rates [28], a delicate association between histone replacement and eviction should play a role in the positioning of the +1 nucleosome.

What determines nucleosome positions? Are these nucleosome positions mainly dictated by the ATP dependent chromatin remodelers? Is the continuous and dynamic association of a remodeler required to maintain the nucleosome over unfavorable sequences? Alternatively do events leading to transcription initiation have a prominent role in its positioning? Nucleosomes dynamic, moving rapidly between intrinsically favorable and unfavorable locations, while at the same time, being highly unstable, especially in active genes. How does nucleosome stability and factors that modulate its movement effect the positioning of one of nature's most positioned nucleosomes?

Main Goals

We aim to identify the factors that regulate the positioning of the +1 nucleosome. In order to achieve this, we will,

Research project 1:

Identify the combinatorial effect of ATP dependent chromatin remodelers in the positioning of the +1 nucleosome. We will look in to the following aspects in greater detail.

i. Identify the Multiple remodelers acting on the +1 nucleosome
ii. What is the role of the individual remodelers?

Research project 2:

Discover the mechanisms that link the positioning of the +1 nucleosome and its stability. In detail,

i. What is the link between the stability of the +1 nucleosome and its positioning?
ii. How do other factors such as (a) the presence of chromatin remodelers, (b) general dNA binding factors (Reb1), (c) RNA Polymerase II passage and (d) H2A.Z is incorporation, effect the stability and positioning of the +1 nucleosome

The main goal of the proposal is to understand the factors involved in positioning of the +1 nucleosome. The proposal contains 2 main projects. The first project will identify the role of individual ATP dependent chromatin remodelers in the precise positioning of the +1 nucleosome. The second project will allow integration of these results into a model that seeks to identify the mechanisms that link the stability of the +1 nucleosome and its positioning.

Research Plan and Approach

Experimental setup: Research project 1

1.1 Identify the Multiple remodelers acting on the +1 nucleosome

Hypothesis:

1. ATP dependent chromatin remodelers show specificity towards the nucleosomes they target.
2. The +1 nucleosome is targeted differently from the rest of the nucleosomes.
3. The targeting of the +1 nucleosome will depend on the transcriptional activity of a given gene. Highly transcribed genes will be targeted differently to repressed genes.

Proposed experiments

This part of the project will address how different remodelers act in concert to position the +1 nucleosome. Recently published genome-wide remodeler-nucleosome interaction assays of SWI/SNF, RSC, ISW1a, ISW1b, ISW2, and INO80 in Saccharomyces show that each ATP dependent nucleosome remodelers shows a certain degree of specificity to the nucleosomes they target and that the +1 nucleosome is targeted differently from the rest of the nucleosomes [1]. Our approach will be an extension to this study. We combine principles of ChIP-exo [1,42,43] and Mass Spectrometry [44] (ChIP-exo-MS/MS) to identify the chromatin remodelers associating with the +1 nucleosome.

Large scale proteomic analysis in Saccharomyces cerevisiae to identify factors that bind the +1 nucleosome

We propose a large scale proteomic analysis in Saccharomyces cerevisiae to compare the factors that bind to the +1 nucleosome in two levels (1) functionally, averaged across all genes in a functional category and (2) locally, at a single gene.

Gene expression levels of ‘‘growth’’ and ‘‘stress’’ genes follow distinct and conserved pattern in Saccharomyces and this is accompanied by a transition of chromatin organization from a ‘‘growth’’ to a ‘‘stress’’ pattern [27]. Therefore we propose the categorizing of Saccharomyces genes to a functional category and then analyzing each functional category for the factors that

bind to the +1 nucleosome. As in Tsankov et al. [27], we will define ‘‘growth’’ genes by their co-expression with cytoplasmic ribosomal proteins and “stress” genes as those that anti-correlate with the expression of growth genes. A set of genes will then we compiled using for each category (10 genes for each functional category). As a control, we will use 10 precisely positioned nucleosomes from one gene (for each functional category) selected from the list of 10 (referred to as control nucleosomes in the text). We will use the genome-wide nucleosome positioning maps of *Saccharomyces cerevisiae* generated at base pair resolution by Brogaard et al., (2012) [45] to identify the control nucleosomes.

To capture dynamic interactions between chromatin remodelers and nucleosomes we propose an extension to the ChIP-exo approach [1,42,43]. In accordance with ChIP-exo, the interactions between chromatin remodelers and nucleosomes will be ‘frozen’ using formaldehyde. Chromatin pellets will then be sonicated and digested with lambda exonuclease. The exonuclease has a strand-specific 5’-3’ nuclease activity that degrades the DNA strand in the 5’–3’ direction until a cross linking point is encountered. As a result of the strand specific activity of lamda exonuclease, sequences 3’ to the exonuclease block remain intact (figure 2).

We propose to adapt the ChIP-exo approach to enrich for +1 nucleosomes. DNA coupled to beads will be used to capture the remodeler crosslinked nucleosomes from the chromatin sample (Figure 2.A). The bait sequence will be determined by mining through the base pair-resolution genome-wide maps of nucleosome locations in Saccharomyces published in Brogaard et al., (2012)[45]. This will allow us to specify the DNA sequence occupied by the +1 nucleosome and an algorithm will allow for a specific sequence to be used for pull downs. As sequences 3’ to the exonuclease block remain intact, these single stranded sequences will allow for the annealing of the complementary bait sequences. Following enrichment, the samples will then be eluted and analyzed by mass spectrometry. The MS/MS fragment data will be analyzed for known ATP dependent chromatin remodelers using a database search and hierarchical clustering.

Comparison of the proteomic data sets between the +1 nucleosome and the control nucleosomes will enable us to identify the subset of remodelers that bind specifically to the +1 nucleosome. Analyzing differences between the two functional categories will enable us to identify the factors important for the positioning of the +1 nucleosome in, a. active genes and b. repressed genes.

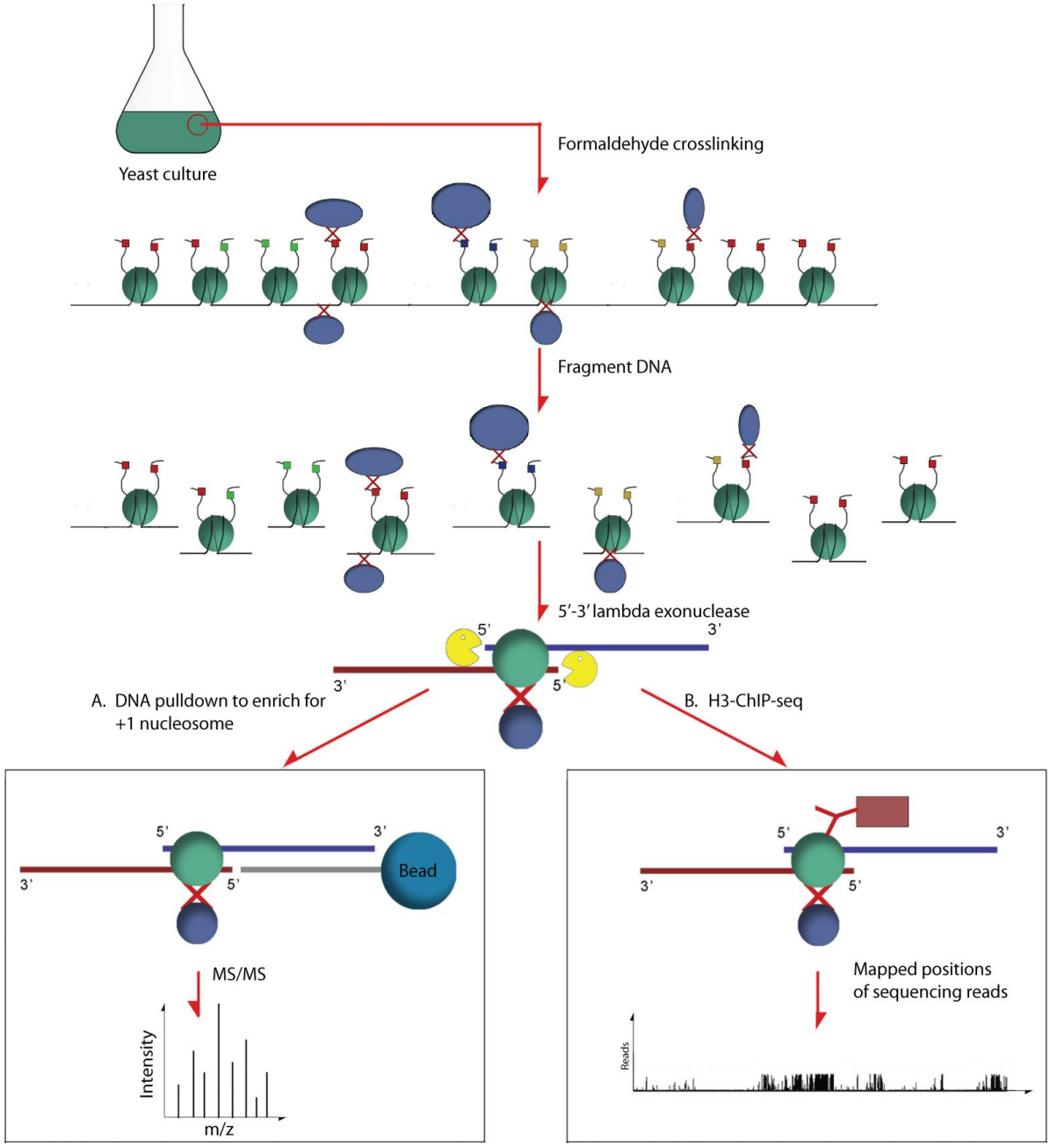


Figure 2: Illustration of the ChIP-exo and proposed ChIP-exo-MS/MS methods. Following formaldehyde treatment, chromatin pellets are sonicated to fragment DNA and digested with lambda exonuclease with 5'-3' exonuclease activity. (A.) DNA pulldowns are performed using complementary sequences to the the intact sequences 3' to the exonuclease block to 'fished out' the +1 nucleosomes. (B.) Histone H3 will be immunoprecipitated and DNA fragments that immunoprecipitated with H3 will used to generate a map of all nucleosomes.

1.2 What is the role of the individual remodelers?

Hypothesis:

1. An ATP dependent chromatin remodeler will result in the movement of the +1 nucleosome from its original location.
2. Remodelers work cooperatively to determine the position of the +1 nucleosome.
3. The binding of one remodeler may be dependent on the binding of another remodeler.
4. The depletion of one or more remodelers will lead to a change in the position of the +1 nucleosome or disrupt the precise positioning making the +1 nucleosome more dynamic.

Proposed experiments

The results of 1.1 will be used to create yeast strains where the remodeler of interest will be depleted using anchor away techniques described in Haruki et al., (2008)[46]. This technique will allow for the rapid depletion of a given protein form the nucleus of Saccharomyces cerevisiae, but tethering it to a ribosomal subunit by rapamycin-dependent heterodimerization. The massive flow of ribosomal subunits out of the nucleus will result in the depletion of the tethered target protein from the nucleus in a short amount of time (minutes depending on the protein). The anchor strains will help bypass problems inherent to deletion mutants and temperature sensitive mutants such as physiological adaptations to deletions, leaky responses and temperature shift induced shock.

Anchor away strains will be used in the ChIP-exo-MS/MS approach described in 1.1 to generate mass spec based proteomic data on the remodeler binding to the +1 nucleosome in the absence of one or multiple remodelers. When generating bait sequences for DNA pull downs for ChIP-exo-MS/MS approach, the nucleosomes in the remodeler depleted strains may show a shift from wild type nucleosome positions. This may cause problems when capturing of the +1 nuclesome as new primers will be needed to capture differences in nucleosome positions. To circumvent this potential problem, high-resolution genome wide map of all nucleosome positions will be generated for the anchor strains following rapamycin induced depletion. Maps will be generated at base pair resolution using the chemical mapping approach described by Brogaard et al., (2012) [45]. These will be used when generating bait sequences for the DNA pull downs.

We propose a slight change of order to the ChIP-exo approach [1,42,43] for ChIP-seq. Following lambda exonuclease digestion of sonicated chromatin, histone H3 will be immunoprecipitated and the associated DNA fragments will be detected by deep sequencing to generate a map of all nucleosomes (Figure 2.B). Thus, following our modified ChIP-exo approach, half of the sample will be used for H3-ChIP-seq, while the other half is used for ChIP-exo-MS/MS after enrichment for the +1 or control nucleosomes.

Proteomic data generated will be analyzed as an average over each functional category. Since averaging removes gene specific features, we will also consider differences at single gene level. Depending on the results, we could explore the effects of deleting combinations of remodelers in the positioning of the +1 nucleosome. The changes in remodeler binding to the control nucleosomes will be observed and the comparison will help delineate the events following remodeler binding to the +1 nucleosome. We attempt to answers to questions such as, a. does the binding of one remodeler influence the binding of another, and b. what is the order of remodeler binding events. The answers will also help decipher the combinatorial effects of remodeler binding events on the +1 nucleosome.

Nucleosome movements and directionality observed using genome wide ChIP-seq maps will give additional insight to the functional aspects of each remodeler. We will look at 3 different chromatin parameters, a. Distance between +1 nucleosome and the transcription start site, b. Width of the 5' nucleosome free region, c. Spacing between the +1 and neighboring nucleosomes (+2, +3, +4) (Figure 3) . The results will be compared between the anchor strain at wild type situation and following rapamycin induced remodeler depletion. The nucleosome delocalization will be quantified as a standard deviation of individual tag locations from wild type situation. Applying a t-test between the mutant and wild-type positions will enable us to analyze the nucleosomes that underwent a statistically significant change in position. Results will be averaged over functional category and at single gene level. These results will be used to generate a complete picture of the events leading to remodeler binding and the positioning of the +1 nucleosome.

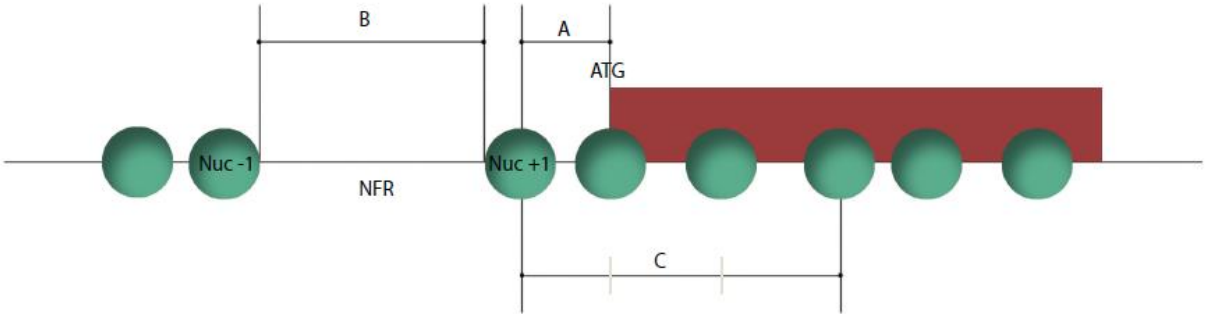


Figure 3: Chromatin parameters. A Schematic drawing of a gene (brown), the promoter (black line) and associated nucleosomes (green) are shown. (A) Distance between +1 nucleosome and the transcription start site (ATG) (B) Width of the 5' nucleosome free region (C) Spacing between the +1 and neighboring nucleosomes (+2, +3, +4).

Experimental setup: Research project 2

This part of the project is based on uncovering the factors and the mechanisms form a link between the stability of the +1 nucleosome and its positioning.

2.1 What is the link between the stability of the +1 nucleosome and its positioning?

Hypothesis:

1. There is a correlation between the stability of the +1 nucleosome and its precise positioning.
2. Histone H3 turnover rates can be related to the stability of the +1 nucleosome as it signifies an event of nucleosome eviction.
3. Chromatin remodelers may have an effect in stabilizing or destabilizing the +1 nucleosome and thereby effect its positioning
4. The passage of RNA polymerase II is involved in evicting the +1 nucleosome, and thereby may have an effect on its positioning.
5. DNA binding factors may have an effect in the positioning and the stability of the +1 nucleosome
6. The presence of histone variant H2A.Z in the +1 nucleosome may affect its stability.

Combining ChIP-exo and the RITE assays to study the correlation between the stability of the +1 nucleosome and its precise positioning

We propose to address these issues using an assay that enables study of replication independent histone replacement and allows for the simultaneous mapping of nucleosome positions. Histone turnover rates in yeast signify an event of nucleosome eviction, whereby histones are removed and replaced. Thus, studying histone turnover at various time points will allow for a representative map of nucleosome stability over time. We propose to achieve this by using a combination of the RITE assay [47] and the described ChIP-exo-MS/MS method. While the RITE assay will allow the measuring of histone turn over, ChIP-exo-MS/MS method will enable us to restrict our analysis to a single nucleosome by enriching for the +1 nucleosome.

A potential problem in studying transcription induced histone eviction in yeast is the transient dissociation of histones from the maternal genome during replication. This is especially important as yeast cells double in approximately 90 min in rich medium [48]. Thus, to study histone exchange, cells need to be arrested in G1, to prevent their passage into the S-phase. We propose the use of the previously described recombination-induced tag exchange (RITE) assay [47] where a RITE cassette, containing two C-terminal epitope tags, HA and T7, are placed downstream of one of the histone H3 genes, while the other is deleted. The RITE cassette places the T7 tag between two Lox P sites. Hormone-induced activation of Cre recombinase, causes a permanent epitope-tag switch resulting in the exchange of the "old" HA tag by the "new" T7 tag. As described earlier [47], switching will be performed on nutrient starved cells at Go. The cells will be released into fresh media containing α-factor to arrest the cells in G1. These G1 arrested Saccharomyces cerevisiae contain nucleosomes with both the "old" HA tag, and new T7 tag. To measure turnover rates, cells will be arrested at G1 for varying amounts of time and analyzed for tag exchange.

The HA tags and T7 tags are then used to generate nucleosome arrays at single base pair resolution using the chemical mapping approach [45]. However, before chemical mapping, we will first enrich for the +1 nucleosome using the approach described in our modified ChIP-exo-MS/MS method. Following 5'-3' lambda exonuclease digestion, of fragmented and cross linked chromatin, the resulting DNA strand 3' of the nuclease block is used as a platform for DNA pull

downs to enrich for the +1 nucleosome (figure 2). Thus, we will be able to generate data on the stability and its influence on the positioning of the +1 nucleosome in a time dependent manner.

2.2 The factors influencing the stability and positioning of the +1 nucleosome

2.2.1 Involvement of Chromatin remodelers in the stability and positioning of +1 nucleosome

The assay described in 2.1 can also be used to generate proteomic data on the factors that selectively bind to the new and old histones using ChIP-exo-MS/MS. We propose the use of anchor away strains to study the involvement of chromatin remodelers on histone stability and positioning. The approach allows for remodelers to be depleted in cells at Go prior to tag exchange. The effect of the remodeler depletion at proteomic level will be compared with the degree of histone exchange and positioning over time.

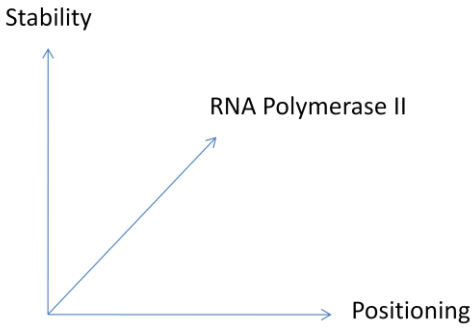
2.2.2 Involvement of RNA polymerase II in the stability and positioning of +1 nucleosome

In order to decipher the affect RNA polymerase passage on the stability and positioning of the +1 nucleosome, we propose to use the techniques of proposed in 2.1 combined with an assay that can modulate RNA polymerase II activity. We propose to enrich for RNA polymerase II on stress genes (repressed genes) and deplete it at growth genes or highly transcribed genes.

The effect of RNA polymerase II on repressed genes can be studied by tethering the holoenzyme of RNA polymerase II to the promoters of the selected repressed "stress" genes. We use the methods described in Gaudreau et al., (1997)[49] where the DNA-binding domain of a gene of interest is fused to a component of the RNA polymerase II holoenzyme resulting in gene activation. We propose fusion of the DNA-binding domain of a gene of interest to the Gal 11 component of the RNA polymerase complex, which will recruit the holoenzyme of RNA polymerase II to DNA, activating the repressed gene. RNA polymerase II passage on highly expressed "growth" genes can be studied by anchor away strains that would deplete the large subunit of RNA polymerase II (RPB1) by the addition of rapamycin induced depletion. The results will be compared with the wild type situation.

These assays will be combined with simultaneous assessment of histone H3 turn over and mapping of the +1 nucleosome, as described above. The degree of histone exchange and

deviation of nucleosome positions between the wild type (genes at their original repressed state) will be analyzed as an effect of RNA polymerase passage and will be presented analyzed as below.



2.2.2 Involvement of general DNA binding proteins in the stability and positioning of +1 nucleosome

We proposed the use of assays described in 2.1 to analyze the affect of DNA binding proteins on the positioning of the +1 nucleosome. As in 2.1, anchor away strains will be generated for known DNA binding proteins and its effect on nucleosome stability and positioning of the +1 nucleosome will be analyzed. Additionally, ChIP-exo-MS/MS will allow for the collection of proteomic data of factors binding to the +1 nucleosome in the absence of the DNA binding proteins.

2.3 Does H2A.Z enrichment at the +1 nucleosome lead to instability and increased positioning?

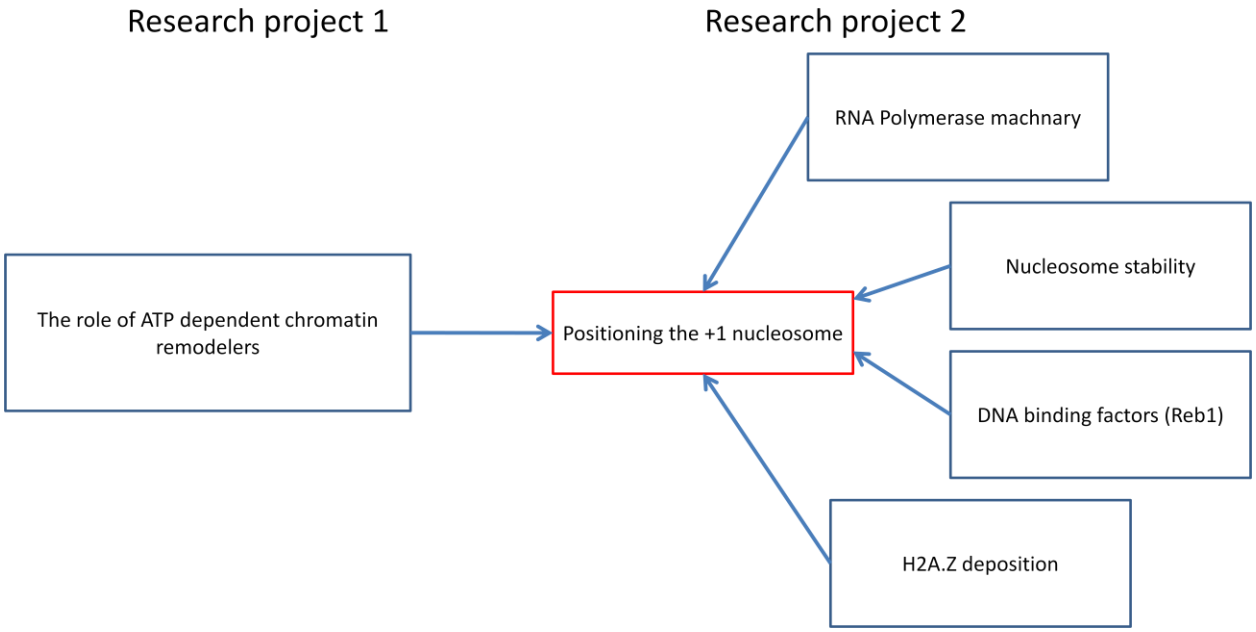
Hypothesis:

1. Deposition of H2A.Z leads to its decreased stability of the +1 nucleosome
2. Deposition of H2A.Z histone variant functions to erase histone modifications
3. These nucleosomes are thus refractory to chromatin remodeling complexes and nucleosome mobility influenced by chromatin remodeling complexes is reduced.

We propose to experimentally test these hypotheses by creating anchor away strains of Swr1 protein, the catalytic component of the SWR1 complex [36,39], responsible for the deposition of H2A.Z at the +1 nucleosome. Depletion of SWR1 will reduce the incorporation of H2A.Z, thereby influencing the stability the +1 nucleosome. Apart from generating data on nucleosome

stability and positioning of the +1 nucleosome as described in 2.1, the possibility of enriching for the +1 nucleosome and generation of proteomic data through ChIP-exo-MS/MS will also support further study of the role of histone H2A variants in the positioning of the +1 nucleosome.

Significance and Conclusions



The main goal of the proposal is to understand the factors involved in positioning of the +1 nucleosome. The proposal contains two main projects. ATP dependent chromatin remodelers are known to treat the +1 nucleosome differently from the other histones. The first project will identify the role of individual ATP dependent chromatin remodelers in the precise positioning of the +1 nucleosome. The second project will allow integration of these results into a model that seeks to identify the effects of the transcription machinery, DNA binding factors and incorporation of histone variants in the stability of the +1 nucleosome and its positioning.

We propose a novel approach to achieve these goals by using a modified version of the ChIP-exo approach by Yen et al. (2012). The ChIP-exo-MS/MS approach provides a method of enriching for any nucleosome of interest and thereby allows a single nucleosome to be followed and analyzed over time. Mass spec based proteomic data generated as well as the models that would emerge as a result of the proposed research will help identify the factors that cooperatively act to position the +1 nucleosome.

Time Table

Time table	Year 1	Year 2	Year 3	Year 4	Year 5
1. Research project 1					
1.1 Identify the Multiple remodelers acting on the +1 nucleosome	→	→			
1.2 What is the role of the individual remodelers?		→	→		
2. Research Project 2					
2.1 Factors affecting the stability and positioning of the +1 nucleosome			→	→	→
2.2 H2A.Z incorporation and histone stability and positioning				→	→

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