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Molecular mechanism of dBigH1 action

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1. INTRODUCTION

1.1. DNA in a living cell

1.1.1. DNA condensation

All eukaryotes contain the genetic information in form of DNA. In the case of humans for example, every single cell of our body contains the whole genetic material inherited by our parents in form of 44 autosomal chromosomes and two sex chromosomes. If one was to align those 46 chromosomes one after the other a DNA strand of about two meters would be the result. On the other hand, an average human cell is about 20 to 100µm in size and the cell's nucleus, where the DNA is inside, only about 6µm. So how can a fibre that long be stored inside a nucleus that small? The answer is that the DNA is not present as a naked molecule but rather associated with a lot of different proteins to form the so-called chromatin. This interaction with proteins allows the DNA to condensate and form higher order structures (Figure 1).

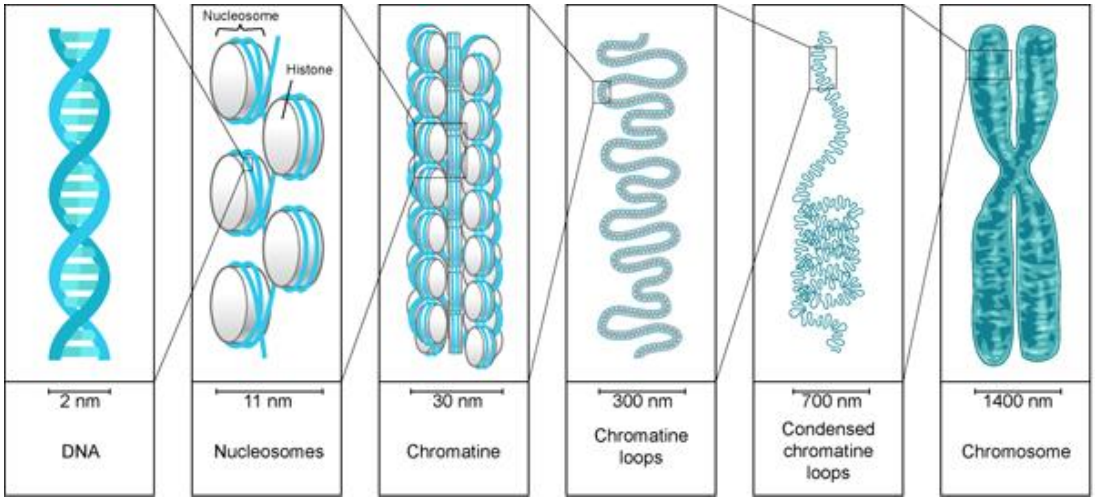


Figure 1. The compaction of DNA into chromatin

The naked DNA molecule gets associated with histone proteins to form the nucleosome. Interaction with additional scaffold proteins allows the compaction into higher ordered structures until the metaphase chromosome that is the most condensed form of chromatin (Shmoop Editorial Team, 2008).

1.1.2. Different types of chromatin

Chromatin is the natural state of DNA in a living cell and is not only important for packaging due to space limiting reasons, but also in the context of gene expression. If we think of the fact that all the genes are present in every single cell of an organism one could ask the question, why aren't all cells the same? The answer lies in the regulation of gene expression. This means that in every cell only those genes are expressed that are important for the cell to maintain its character. At the same time, all other genes must be prevented from transcription. Indeed, this can be achieved by multiple strategies but the simplest and most effective is still the activation or repression of genes by chromatin decondensation or condensation, respectively. As mentioned, the DNA is associated with different proteins that can condense and de-condense the chromatin in a way that the underlying genes on the DNA get accessible for transcription factors or stay in a closed conformation. The relaxed and transcriptionally active state of chromatin is called euchromatin, whereas the condensed and transcriptionally repressed state is called heterochromatin. They are mainly achieved through covalent modifications, mostly by acetylation and methylation of histones (Figure 2).

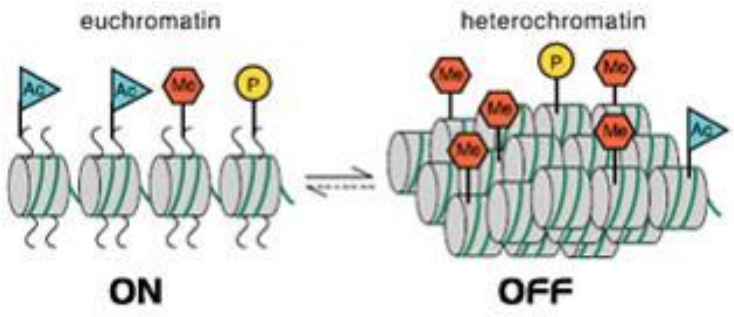


Figure 2. The different states of chromatin

The relaxed and transcriptionally active form of chromatin, the euchromatin, is characterized by a high percentage of histone acetylation and low methylation. Heterochromatin on the other hand, that represents the condensed and transcriptionally repressed form of chromatin, contains very few histone acetylation but more methylation (Jenuwein et al., 2001).

The most important class of proteins that are associated with the DNA and promote chromatin condensation and decondensation are the histones. Together with other factors like chromatin remodelers, histone proteins are responsible for the formation of different chromatin states and thus for the regulation of gene expression. The regulation of chromatin is also crucial for processes like replication and cell division when the chromatin needs to be either relaxed or condensed.

1.1.3. The nucleosome

The basic unit of chromatin is the nucleosome. Each nucleosome is made up by the four core histones H2A, H2B, H3 and H4. Two of each core histones will form the nucleosome octamer around which the superhelical DNA is wrapped 1.7 times (Kornberg, 1977; Richmond et al., 1997). More precisely, the H3 and H4 histones will form heterodimers by hydrophobic interactions, and these heterodimers will then associate to form histone tetramers (Arents et al., 1991). Likewise, H2A and H2B will form dimers, of which two of them will bind opposite the H3-H4 tetramer to form the histone octamer (Eickbush et al., 1978). A linker histone, called histone H1, will bind the nucleosome and the DNA at the DNA entry and exit site forming the so-called chromatosome. This structure allows the compaction of the chromatin into higher order structures (Noll et al., 1977; Thoma et al., 1979). The crystal structure of the nucleosome core particle reveals that the DNA interacts with the core histones mainly by the negatively charged phosphodiester backbone (Figure 3), (Richmond et al., 1997). This principle allows the interaction of the nucleosome with the DNA to be independent of the DNA sequence.

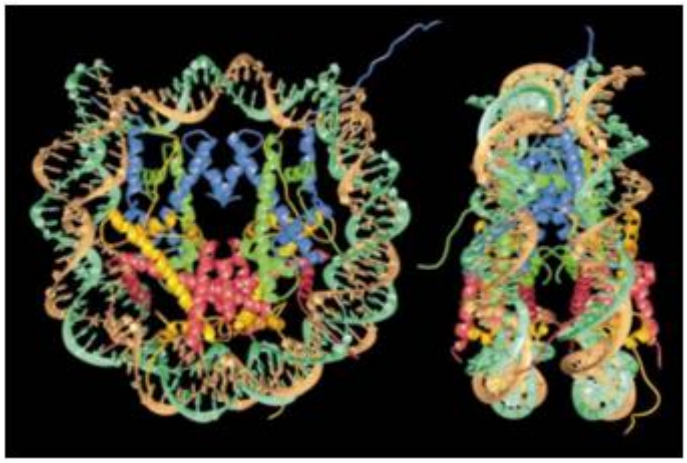


Figure 3. X-ray structure of the nucleosome core particle

Front and side view of the nucleosome core particle. The DNA phosphodiester backbones (green and turquoise) interact with the core histones. H2A, yellow; H2B, red; H3, blue; H4, green, (Richmond et al., 1997).

Nucleosomes were discovered when scientists digested purified chromatin with Micrococcal nuclease and were surprised to find a regular pattern when looking at the fragments on an agarose gel (Noll, 1974; Kornberg, 1974). No matter how long they would let the DNA being digested, the resistant fragments were not getting smaller than around 146bp in length. This suggested that the DNA was protected by a protein complex that doesn't allow the enzyme to cut the DNA. In the same year, a 10nm fibre could be observed by electron microscopy that shows the beads on a string model of chromatin were the beads represent the nucleosomes (Figure 4), (Olins & Olins, 1974).

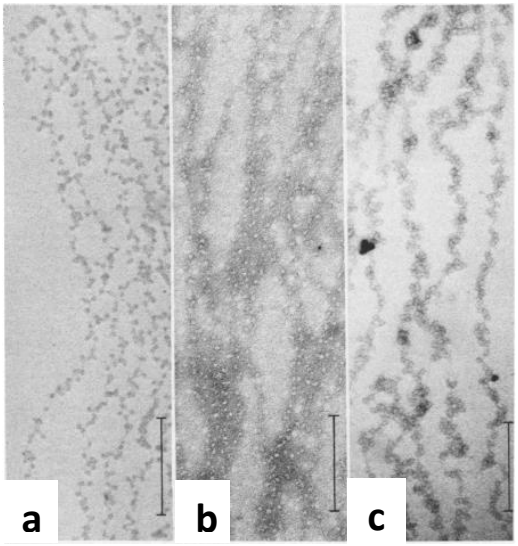


Figure 4. Chromatin fibres showing the beads on a string model

Electron microscopy pictures of rat thymus chromatin stained positively and negatively, respectfully (a & b). Chicken erythrocyte chromatin negatively stained (c). The nucleosomes can be recognized nicely in (c) as beads that seem to be aligned on a string (Olins & Olins, 1974).

1.1.4. The core histones

Histones are evolutionarily very conserved proteins. The core histones, that is H2A, H2B, H3, and H4, are all expressed as multi-copy genes that are clustered on the DNA as repeat arrays. Their expression is connected to DNA replication, which is when they are needed to assemble the newly synthetized DNA into chromatin.

As mentioned the core histones build dimers. They are basic proteins and contain the histone fold domain near the C-terminal region, three α-helices flanked by two loops. This helical structure allows them to interact with other histones in a head-to-tail fashion that is also known as the hand-shake motif (Mariño-Ramírez et al., 2005). This allows them to dimerize and finally form the histone octamer, that together with the DNA forms the nucleosome (Figure 3). The N-terminal tail of the core histones can be modified in different ways.

1.1.5. Core histone modifications

Post-translational modifications of core histones can modify the chromatin from an open state to a more condensed one or the other way around. By changing between the euchromatin and heterochromatin, underlying genes get activated or repressed. Thus, histone modifications are a powerful tool to regulate the expression of genes. Histone modifications include methylation, phosphorylation, acetylation, deimination and ubiquitination. Here we will mention just a few examples of those modifications. The most modified core histone is H3, although all core histones can be modified (Figure 5).

In H3 and H4 histones, lysine methylation for example can promote both, transcriptional activation or repression, depending on the site of the modification. Lysine residues can be either mono-, di- or tri-methylated. The most common gene activating marks are H3K4me1 and H3K4me3, whilst on the other hand H3K9me3 and H3K27me3 are repressive modifications (Greer at al., 2012).

Histone acetylation is normally associated with open chromatin and active transcription. Acetylation is most often occurring at promoter regions. Two common modifications are H3K9ac and H3K27ac (Roth et al., 2001).

Phosphorylation of histones not only affects the activity of genes but also is important in processes like mitosis and DNA repair. For example, H3 phosphorylation of serine 10 (H3phosphoS10) and of threonine 120 of H2A (H2AphosphoT120) are mitotic markers that will lead to the condensation of chromatin to prepare for mitosis (Nowak et al., 2004; Rossetto et al., 2012).

Phosphorylation of H2A.X at serine 139, that thereby becomes γ-H2A.X, happens after DNA double-strand breaks and leads to the recruitment of DNA damage repair proteins. H2A.X replaces the canonical H2A in about 20% of all nucleosomes (Lowndes et al., 2005).

Histone ubiquitylation is mostly found on H2A and H2B. Monoubiquitylation of H2A normally leads to gene silencing, whereas the same mark on H2B leads to transcriptional activation. Ubiquitylation also plays an important role in DNA damage response. Polyubiquitylation of histone H2A/H2A.X provides a

recognition site for DNA repair proteins. Monoubiquitylation of H2A, H2B and H2A.X is also found at DNA double-strand breaks (Cao et al., 2012).

Figure 5. The core histones and its post-translational modifications

The N-terminal region of the core histones can be modified in various ways. The four core histones are shown plus H2A.X, an H2A variant that replaces the canonical H2A in many nucleosomes (Copyright © Abcam, 2011)

1.1.6. Histone variants

Histone variants, that are non-canonical histones, are expressed from single loci in the genome and are moreover under constitutive transcription. Unlike the canonical histones, the non-canonical histones have specific functions.

One example of such a histone variant is CenH3, the eukaryotic centromeric histone variant H3 that, as the name indicates, occupies a special role at the centromeres of chromosomes, where it replaces the canonical histone H3 in the nucleosomes. It plays a crucial role in centromere identity and kinetochore assembly at mitosis. (reviewed in Talbert et al., 2010).

Another example is the mammalian H2A.Z that is also present in yeast. This histone H2A variant is shown to promote transcription by being present in promoter regions and thereby being involved in recruiting RNA Pol ll (Adam et al., 2001; Gévry et al., 2009). Furthermore, it was also described to facilitate DNA repair after breaks by incorporating into nucleosomes flanking the damaged region. It seems as in both cases incorporation of H2A.Z leads to a more open chromatin state that allows for reactions like transcription or repair (reviewed in Ransom et al., 2010).

These different roles of the histone variants are also due to structural differences between them and the canonical histones, that can lead to changes in the nucleosomes from inside the core particle. These changes can then affect for example the association of the DNA with the histone octamer and lead to a more lose conformation, that can alter the accessibility of a gene that lies underneath (Zlatanova et al., 2009).

1.2. Linker histone H1

In *Drosophila*, like the core histones, linker histone H1 is expressed as a multi-copy gene and its expression is coupled to DNA replication. However, in the rest of the species linker histone H1 is expressed as single copy genes, although the different variants can be clustered in groups. Most metazoan species contain several variants of linker histones that can have specific functions, especially during development and differentiation. Mammals have 11 different variants of H1, whereas yeast for example has only one. However, most species contain several linker histone variants, like chicken (7), *Xenopus* (5) or the zebrafish (4), (Figure 6), (reviewed in Izzo et al., 2008). As mentioned above, H1 binds to the DNA entry and exit site of the nucleosome and stabilizes the folding of chromatin into higher order structures (Noll et al., 1977; Thoma et al., 1979). H1 is also important for stabilizing the position of the nucleosomes along the chromatin, not allowing spontaneous sliding of nucleosomes or ATP-dependent remodelling (Hill, 2001; Ramachandran et al., 2003).

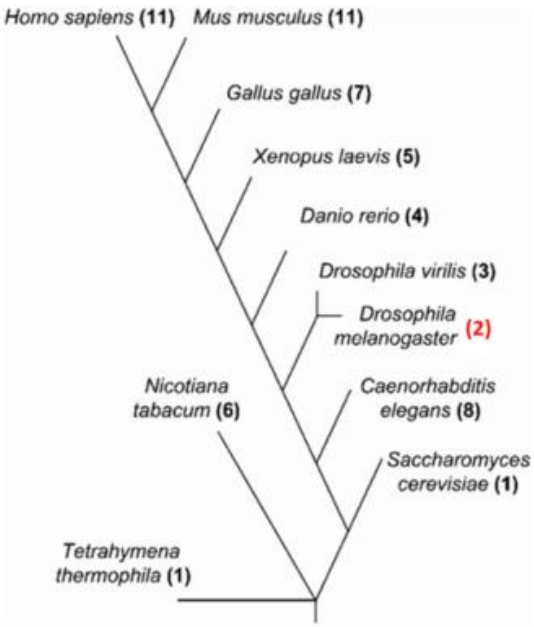


Figure 6. H1 variants in different species

Linker histone H1 variants for different species. The histones are represented on an evolutionary tree. (modified from Izzo et al., 2008).

1.2.1. Linker histone H1 structure

Histone H1 has a tripartite structure that consists of a central globular domain and two variable domains. In contrary to the core histones, it does not contain a histone fold motif. Instead the globular domain contains a winged-helix motif and is evolutionary conserved (Hartman et al., 1976; Cerf et al., 1994). The two variable domains are a short N-terminal tail and a longer C-terminal region, that is rich in positively charged lysine residues (Figure 7). The globular domain and especially the positively charged C-terminal region interact with the negatively charged DNA.

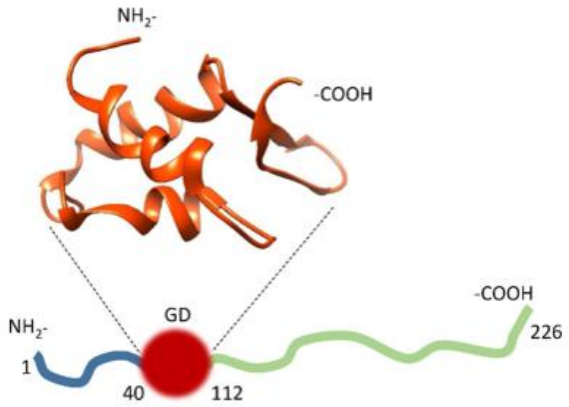


Figure 7. Linker histone H1 structure

Tripartite structure of linker histone H1. The globular domain is flanked by a short NH2 (blue) and a longer COOH (green) variable domain. The indicated amino acids positions are for histone H1.5. The C-terminal region (green) is rich in positively charged lysine residues that can interact with the negatively charged backbone of the DNA (Dimitrov et al., 2015).

The resulting charge neutralization allows higher order chromatin structures (Clark et al., 1990; Subirana, 1990). The N-terminal domain is usually characterized by hydrophobic residues at its extreme part and more basic amino acids near the globular domain (Böhm et al., 1985). Although it is not clearly known what the function of the extreme hydrophobic part of the N-terminal tail is, it has been shown that deletion of the N-terminal domain reduces its binding affinity for chromatin (Allan et al., 1986; Öberg et al., 2012). This suggests that the N-terminal domain plays a possible role in binding of the histone H1 to the

nucleosome. Nevertheless, the C-terminal domain is the primary determinant of the binding affinities of the different H1 variants.

1.2.2. Linker histone H1 in *Drosophila*

As mentioned earlier, most metazoans contain several linker histone H1 variants and amongst them also more than one somatic H1 variant. However, *Drosophila* is somewhat special in this regard as it contains only one somatic H1 variant. In *Drosophila*, the core histones as well as the somatic linker histone H1 are located on the second chromosome (Figure 8). In *Drosophila*, the mRNAs of core histones as well as the somatic histone H1 contain no introns nor do they get polyadenylated. Instead the 3' end forms a stem loop secondary structure that regulates its translation (reviewed in Yang et al., 2008). Although the somatic H1 is located in the core histone repeat, its expression seems to be independent from the others (Nagel et al., 2000; Ryder et al., 2004). In contrast to the core histones, that are expressed during a very short period at the beginning of S-phase, the somatic H1 is expressed throughout the whole S-phase (Gugliemi et al., 2013). The only core histones expressed out of S-phase are single gene core histones variants. (Horard et al., 2015). This independent regulation is possible because the histone H1 gene does not contain the TATA box and uses TRF2 (TBP-related factor 2) instead of the TBP (TAT-binding protein) as the transcription factor to regulate its expression (Isogai et al., 2007).

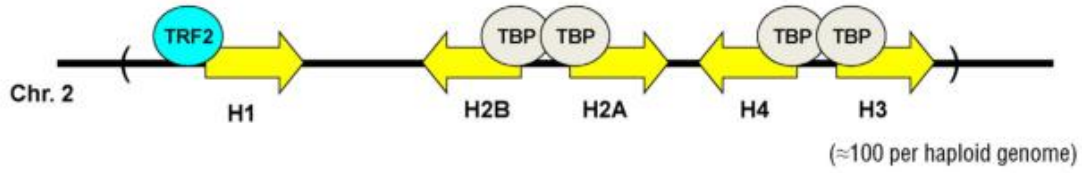


Figure 8. Genomic organization of *Drosophila* histones

The somatic linker histone H1 is present in about 100 copies on the second chromosome together with the core histones. Differently than the core histones, the somatic histone H1 genes do not contain TATA box and uses TRF2 (TBP-related factor 2) to regulate its expression (Isogai et al., 2007).

Another interesting finding is that the half-life of the mRNA of linker histone H1 is much shorter than the half-lives observed for the core histones (Gugliemi et al., 2013). This suggests that there seems to be a need for an uncoupling of the expression of H1 and the core histones. Although one would expect that whenever core histones are needed also H1 is needed, it doesn't seem to be the case. Overexpression of an H1-GFP construct in flies using the UAS/GAL4 system showed that the flies counteract it by reducing the endogenous somatic H1 levels (Siriaco et al., 2015). Again, this shows that too much is no good and that there are versatile mechanisms to regulate it.

1.2.3. Linker histone H1 modifications

Although the linker histone H1 can be methylated, acetylated and ubiquitinated, the most studied histone H1 post translational modification is phosphorylation. H1 phosphorylation is a reversible process and can lead to both, chromatin condensation or decondensation, depending on the site of the modification as well as on the cell cycle context. It is believed that during interphase, just before entering S phase, partial phosphorylation leads to a relaxed chromatin conformation, allowing the activation of transcription (Chadee et al, 1995; Herrera et al., 1996). This relaxation was shown to be induced by structural changes in the C-terminal domain upon phosphorylation (Roque et al., 2008). A second wave of phosphorylation during mitosis (M phase), often referred to as the maximal H1 phosphorylation, leads to chromatin condensation and facilitates the separation of the replicated genome into the daughter cells (Bradbury et al., 1973; Yasuda et al., 1981).

As for methylation, the most important, and as it seems amongst vertebrates very conserved modification, is methylation of lysine 26 of H1.4 in the N-terminal region. Methylation of this residue leads to heterochromatin formation via the recruitment of heterochromatin protein 1 (HP1) and L3MBTL1, two proteins known for their strong ability in heterochromatin formation (Lu et al., 2009; Daujat et al., 2005). The same methylation was found in *Drosophila* as K27me2 as is described in the next section.

1.2.4. Linker histone H1 modifications in *Drosophila*

In *Drosophila*, modifications of the somatic H1 are almost exclusively on the N-terminal region and the globular domain (Figure 9). Mass spectrometry analysis has revealed a large variety of modifications. Again, the most abundant being phosphorylation (Bonet-Costa et al., 2012).

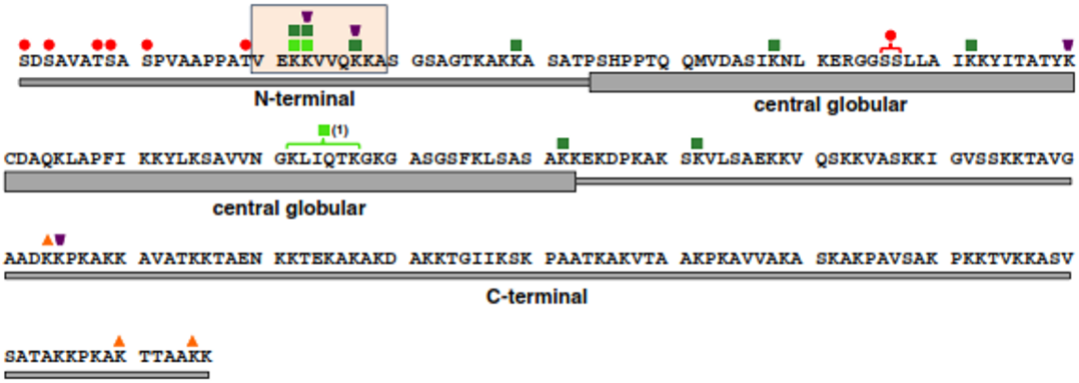


Figure 9. Post translational modifications of *Drosophila* histone H1

The modifications are concentrated on the N-terminal region and the globular domain. The most abundant being phosphorylation (red circles). Other modifications are mono-methylation (light green squares), di-methylation (dark green squares), acetylation (orange triangles) and ubiquitination (purple trapezoids) (Bonet-Costa et al., 2012).

In the same study, one modification was found to be conserved, that is the di-methylation of lysine 27. It seems that it plays a role in promoting heterochromatin organization during mitosis (Bonet-Costa et al., 2012). This modification corresponds to the K26me of H1.4 in vertebrates as described previously.

1.2.5. Functions of linker histone H1

Apart from stabilizing the nucleosome and allowing the formation of a higher order structure, linker histone H1 plays diverse roles. Very early it was believed that H1 is a very potent and dominant inhibitor of transcription (Shimamura et al., 1989). Although for long it was believed that H1 is mainly a structural component and thus constantly associated with chromatin, fluorescence recovery after photobleaching (FRAP) experiments have shown the contrary. In living mouse cells, it was shown that H1 association and dissociation is a highly dynamic process, with H1 molecules entering and exiting the nucleosomes constantly (Misteli et al., 2000).

Histone H1 was shown to be implied in processes like heterochromatin formation, regulation of gene expression, DNA repair and early embryogenesis (Hergeth et al., 2015).

When reducing the normal H1 levels to around 20% by RNAi, the flies fail to develop and die at larval stage. At the same time, there is evidence of failure of heterochromatin formation and uncontrolled gene expression, suggesting that histone H1 is indispensable for proper heterochromatin formation (Lu et al., 2009). Consistently, it was shown that RNAi induced depletion of H1 in *Drosophila* affected above all genes in heterochromatic regions, turning them from a repressed to an active state and showing evidence that H1 is needed for the silencing of transposable elements (Vujatovic et al., 2012).

However, changes in levels of histone H1 can lead to both, up- or down-regulation of genes. In chicken cells, that were successfully depleted for all six H1 variants, many genes were affected, mainly by downregulation (Hashimoto et al., 2010).

The functions of different variants in early embryogenesis will be explained in the next chapter.

1.3. Embryonic and germline specific linker histone variants

Apart from the somatic H1 and its variants, many species have germline specific H1 variants that are expressed during early embryogenesis. Those embryo specific linker histones get replaced by somatic H1 when the zygotic genome gets activated. In the adults, those H1 variants are normally expressed in the germline.

The first species that was discovered to have a germline specific H1 variant was the sea urchin. In 1980 it was shown that the sea urchin had a sperm chromatin specific H1 variant called SpH1 (Strickland et al., 1980). Later it was also shown that the same animal has an early embryo specific linker histone variant called Cs-H1, that replaces the somatic H1 during the first cleavage stages of the embryo and in the female germline (Brandt et al., 1997).

Embryo specific histone H1 variants are deposited by the mother and are needed during the first rounds of DNA replication, when the zygotic genome is still silent and zero or very little transcription is going on. Normally, once the zygotic genome gets activated and transcription starts, the linker histone variant gets replaced quite rapidly by the somatic histone H1. Even in mammals there are embryo specific histone H1 variants. Mice contain the H1oo that gets replaced by somatic H1s after the first cleavages. The same variant was also found to be present in humans (Tanaka et al., 2001, 2003). Humans, mice and rats are also known to share three male germline specific linker histone variants, called H1t, HILS1 and H1T2 (Drabent et al., 1991, 1993; Iguchi et al., 2003; Yan et al., 2003; Martianov et al., 2005; Tanaka et al., 2005). Other examples of species that contain an embryo specific form of H1 are: H1M in zebrafish (Müller et al., 2002), B4/H1M in *Xenopus* (Smith et al., 1988) and of course dBigH1 in *Drosophila*, that is also present in the male and female germline (Pérez-Montero et al., 2013). It seems to be a common trait in metazoans to have specific histone H1 variants that replace the somatic H1 during early embryogenesis and in the germline. Figure 10 shows an overview of some species and their embryo specific histone H1 variant.

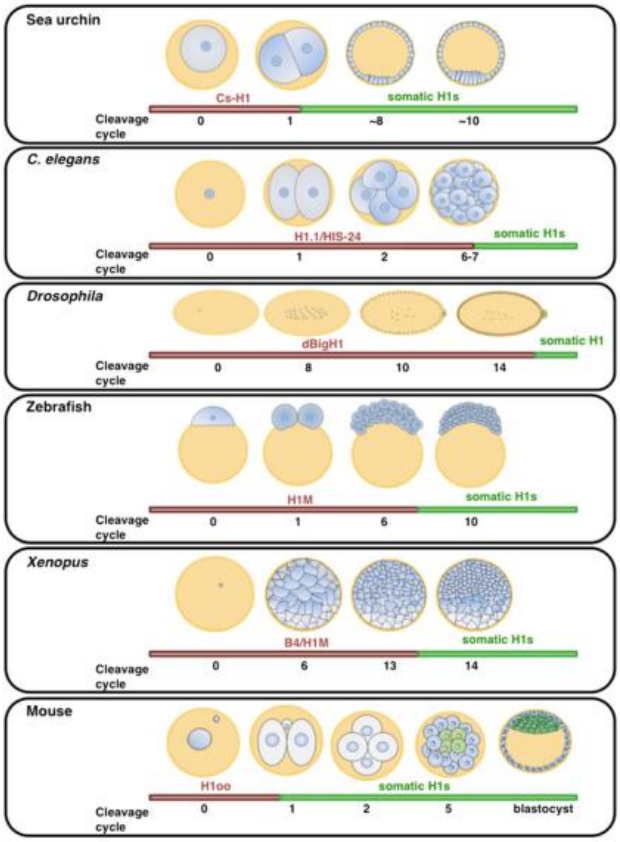


Figure 10. Early embryo specific H1 variants

The histone H1 variant gets deposited by the mother and serves for the first rounds of replication when the zygotic genome is inactive. Once it gets activated the embryonic H1 gets replaced by the somatic H1. Zygotic genome activation can occur very early like in the case of the mouse or the sea urchin but can also occur much later like for example in *Drosophila*. In case of the fruit fly the zygotic genome gets activated after about 14 cleavages, very similar to *Xenopus* (reviewed in Pérez-Montero et al., 2016).

A very interesting study was published in 2011 when it was shown that somatic nuclei could be reprogrammed to pluripotency by incubation in *Xenopus* oocyte extract. They saw that the early embryonic linker histone B4 was incorporated into the chromatin of the reprogrammed nuclei as the somatic H1 was gradually lost. Further they observed that when the incorporation of B4 was impaired by injecting antibody against it or by dominant negative interference, the nuclei wouldn't undergo reprogramming, giving strong evidence that B4 incorporation is a necessary event for pluripotency gene reactivation (Jullien et al., 2015).

1.3.1. Embryonic and germline specific linker histone dBigH1

Until recently, *Drosophila* was an exception to most metazoans as it contained only one linker histone H1 variant, the somatic H1. This was rather surprising as it was known for example that the early stage of embryogenesis in *Drosophila* is transcriptionally silent like it is the case for *Xenopus*. And very much like in *Xenopus*, chromatin reconstituted in vitro using oocyte extract of both species, show reduced transcription. However, the early embryo specific H1 variant in *Xenopus*, B4, had already been discovered long ago (Smith et al., 1988). Since it was long known that the early *Drosophila* embryo does not contain any somatic H1, people were suggesting other mechanisms. In 1994, it was proposed that High-mobility-group-protein D (HMGD), another architectural chromatin binding protein, worked as an embryo specific linker binding protein in *Drosophila* (Jones et al., 1994). However, even before the discovery of dBigH1 this hypothesis was questioned, as it was shown that HmgD null mutant flies showed no detectable defects in chromatin and were perfectly viable (Ragab et al., 2006).

dBigH1 is the so far only discovered linker histone H1 variant in *Drosophila*. dBigH1 was named like this because of its high molecular weight compared to the one of the somatic H1. Nevertheless, it shows the typical tripartite structure of the somatic H1.

When looking at the amino acid sequences of the two histones the first thing that gets apparent is the very large N-terminal tail of dBigH1. The globular domain of dBigH1 has an identity of 57% compared to the somatic H1. The globular domain is usually the domain that is most conserved between the H1 variants. When comparing the C-terminal region it is a bit less conserved (35% identity), but it shows the typical high abundance of positive charged lysine (K) residues that are needed to interact with the negatively charged DNA. As already mentioned the clearest difference between the histones is the very large N-terminal tail of dBigH1. It contains 103 amino acids and hence overtops the 44 amino acids long N-terminal tail of the somatic H1 by far (Pérez-Montero et al., 2013).

1.3.2. The dBigH1 N-terminal domain

Another very interesting feature of the N-terminal region of dBigH1 is its very high content of acidic residues. Very unlike any other histone H1 variant known, the N-terminal tail contains many aspartic (D) and glutamic (E) residues that result in a highly negatively charged region. These acidic residues account for about 38% of all the N-terminal tail. In the sequence, they are highlighted in red (Figure 11).

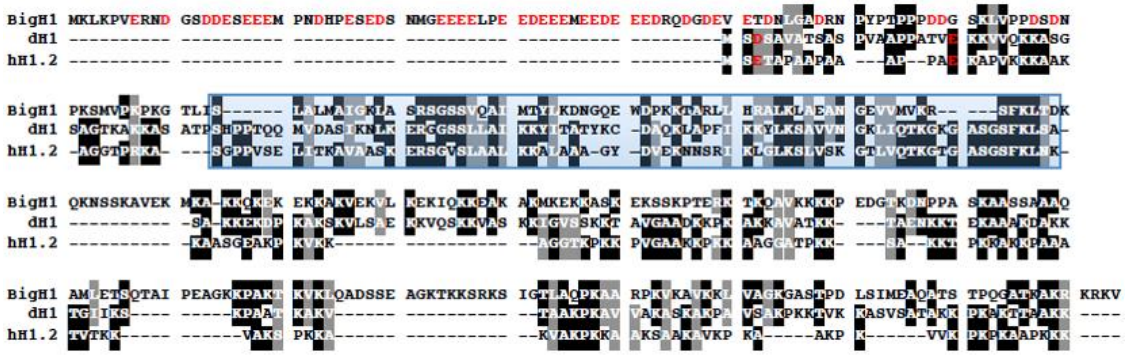


Figure 11. Sequence comparison between dBigH1 and somatic H1

Sequence comparison between the two linker histone variants dBigH1 and H1. dBigH1 contains a much longer N-terminal region (103 amino acids) than the somatic H1 (44 amino acids) and shows only a similarity of about 30% compared to the somatic H1. The negatively charged aspartic (D) and glutamic (E) residues are highlighted in red. The blue box represents the globular domain (Pérez-Montero et al., 2013).

When we compare this very large and acidic N-terminal tail of dBigH1 to the other early embryo specific linker histones that we mentioned above, we can see that it is not a common feature. Figure 12 shows a comparison between the mouse somatic H1.2 and the different oocyte specific linker histones from the different species. What seems to be a common phenomenon is that embryo specific histones have longer C-terminal tails than the somatic H1 and that they contain more acidic residues than the somatic H1. The *Xenopus* B4/H1M for example contains almost 20 times more acidic residues in the C-terminal region than the somatic H1. However, only the *Drosophila* H1 variant shows a clearly prolonged N-terminal tail and such a high abundance of negatively charged amino acid

residues (37.9%). Similarly, the zebrafish and the sea urchin oocyte specific linker histones show an elevated percentage of acidic residues in their N-termini, with 15% and 18.5%, respectively (reviewed in Carbonell et al., 2015).

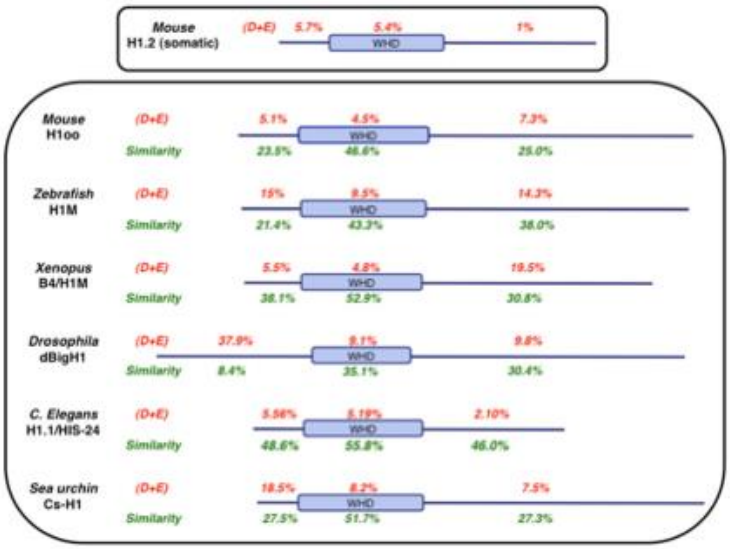


Figure 12. Structure of the different oocyte specific linker histones

Mouse somatic histone H1.2 is showed as a comparison. The globular domain is in the middle containing the winged-helix domain (WHD). The C-terminal region (to the right) tends to be longer than in the somatic H1. The N-terminal regions show approximately the same length except for dBigH1 that is much longer and contains more acidic residues. The similarity of each region to the corresponding region of somatic H1 is shown in green. The percentage of acidic residues is shown in red. Aspartic (D) and glutamic (E) (Carbonell et al., 2015).

1.3.3. dBigH1 gets replaced by somatic H1 at cellularization

dBigH1 is present during early embryogenesis as a maternal contribution and gets replaced by somatic H1 when cellularization starts after 14 rounds of DNA replication. This is also the moment when the zygotic genome gets activated and starts transcribing. The whole process, from when the egg is laid until cellularization takes only about two hours. Thus, dBigH1 can only be detected during this short period. After that, somatic H1 starts appearing and replaces dBigH1 (Figure 13), (Pérez-Montero et al., 2013).

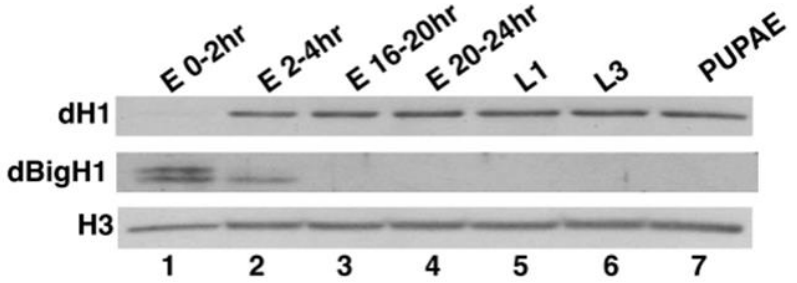


Figure 13. Replacement of dBigH1 by H1 at cellularization

Western blot analysis of dBigH1 abundance. Chromatin was crosslinked at different developmental stages and checked for dBigH1 and H1. Lanes 1-4: Embryos of different ages in hours. Lanes 5 & 6: first and third instar larvae, respectively. Lane 7: Pupae stage. Histone H3 was used as a loading control (Pérez-Montero et al., 2013).

1.3.4. dBigH1 modifications

Another interesting thing that can be observed in Figure 13 is that dBigH1 appears as a double band in the first lane and as a single band in the second lane. This points to a possible dBigH1 posttranslational modification that seems to play a role in its affinity to chromatin.

This hypothesis correlates to a mass spectrometry-based study that performed a large-scale identification of phosphorylation sites of all proteins present in developing *Drosophila* embryos. In this work, they revealed that dBigH1 contains three putative phosphorylation sites. Those are serine 287, 299 and 331. However, according to the study only the serine 299 shows a high probability of being a true phosphorylation site, whereas the other two remain ambiguous (Bo Zhai et al., 2008).

Indeed, when treating with alkaline phosphatase, the slow-migrating band disappears and only the lower dBigH1 band is visible. This fact indicates that the upper band does correspond to a phosphorylated form of the protein (Pérez-Montero et al., 2013).

1.3.5. dBigH1 stays in the primordial germ cells

The only cells that keep expressing dBigH1 after cellularization are the primordial germ cells (PGC), that will give rise to the animal's germline. Immunostaining of early gastrula stage embryos show that dBigH1 is present in the PGC (Figure 14), (Pérez-Montero et al., 2013).

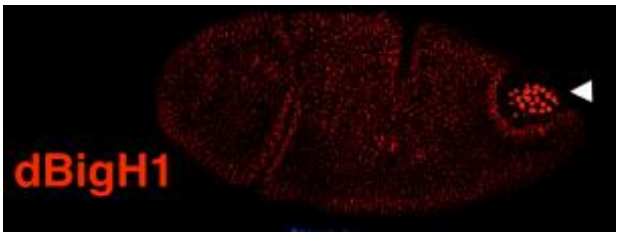


Figure 14. dBigH1 stays in the primordial germ cells

Immunostaining of a *Drosophila* early gastrula stage embryo. dBigH1 is still expressed in the primordial germ cells that will give rise to the adult germline (Pérez-Montero et al., 2013).

1.3.6. dBigH1 represses zygotic gene activation

It is also shown that dBigH1 helps keeping the zygotic genes repressed until cellularization. dBigH1 null mutants show a significantly increased expression of zygotic genes during early embryogenesis than wild type flies, as could be shown by qPCR analysis. Additionally, dBigH1 null mutants show an increased reactivity to αPol IIo^{ser2}, the elongating form of RNA polymerase ll. The higher reactivity was shown by immunostaining with late preblastodermal stage embryos as well as by western blot with whole extract from WT and dBigH1 mutant embryos (Pérez-Montero et al., 2013).

1.4. Chromatin remodelers and histone chaperones

Chromatin remodelers play an essential role in the interplay between the nucleosome and the DNA. The nucleosome is not only acting as a packaging unit but at the same time as a physical barrier, allowing or banning access of proteins to the DNA, no matter if they come to transcribe, repair or replicate. Using ATP as an energy source, chromatin remodelers can slide nucleosomes along the DNA, exchange histones for other variants or simply disintegrate or assemble them at any place to open or close the underlying DNA and thus make it accessible for proteins to bind like for example transcription factors (Figure 15), (Clapier et al., 2009). By regulating the integrity and regularity of nucleosomes, they are very likely to create the necessary structure for higher-order chromatin structures.

Histones are positively charged proteins and can therefore interact with the negatively charged DNA through electrostatic binding and neutralize it. The fact that histones carry a positive charge also makes them insoluble under physiological salt conditions as they can be found in a living cell. In vitro, under these conditions DNA and histones bind non-specifically, leading to a disordered pattern of nucleosomes rather than a regular one (Wilhelm et al., 1978).

Histone chaperones are acidic proteins that bind histones and thus avoid non-specific interactions and at the same time keep them soluble. Chaperones are involved in assembly and disassembly of nucleosomes as well as in histone trafficking, for example from where they are produced to where they are needed (De Koning et al., 2007; Eitoku et al., 2008). During replication, histone chaperones disassemble nucleosomes in front of the replication fork and reassemble them after the DNA is replicated. During DNA damage, they are also known to promote repair by disassembling nucleosomes and making the DNA more accessible for repair proteins. Histone chaperones are also responsible for

exchanging canonical histones with histone variants (reviewed in Ransom et al., 2010).

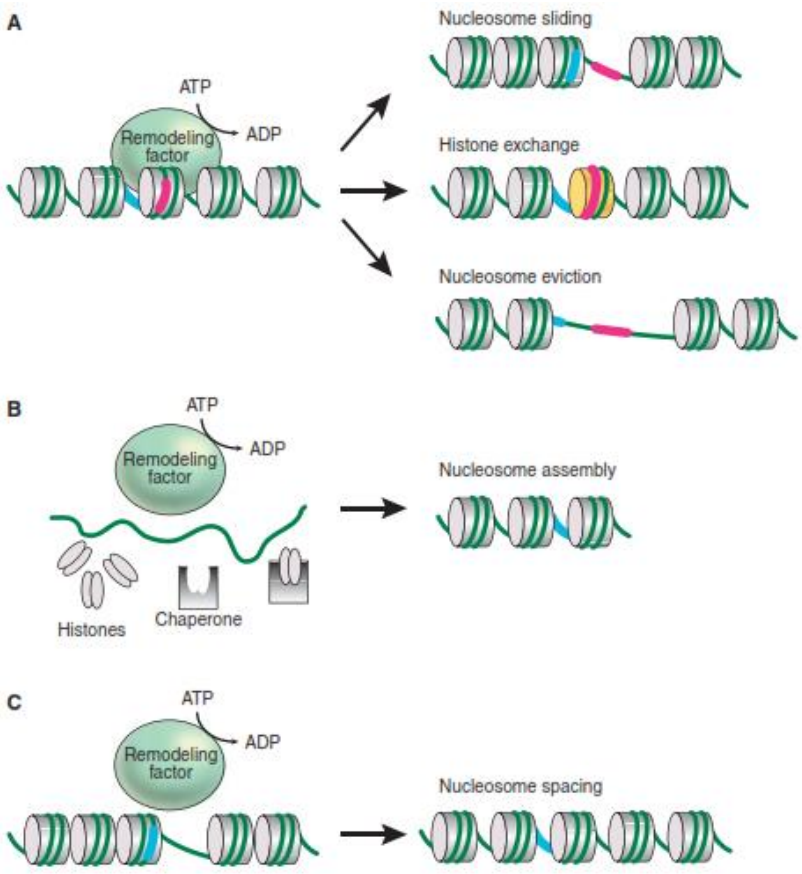


Figure 15. Different outcomes of ATP-dependent nucleosome remodelling

Chromatin remodelers can act in different ways on the chromatin. Sliding of nucleosomes along the chromatin can make a sequence accessible that was previously wrapped around a nucleosome or the other way around (shown by red and blue parts). It can exchange histones to incorporate specific histone variants or it can simply disassemble whole nucleosomes, thus opening a longer region of DNA (A). Another function is the assembly of nucleosomes on a region of DNA that was previously free of nucleosomes. This is usually done with the help of histone chaperones (B). Chromatin remodelling factors may also equilibrate the distances between nucleosomes in case of irregular spacing (C). This can be important in the context of proper chromatin folding into higher order structures (Müller-Planitz et al., 2013).

1.4.1. The chromatin remodeler ACF1

ACF1 is the large subunit of the ATP-dependent nucleosome sliding factors ACF (ATP-dependent chromatin assembly and remodelling factor) and CHRAC (chromatin accessibility complex) and was first purified from *Drosophila* embryos. ACF and CHRAC are both members of the ISWI (imitation switch) family of chromatin remodelers (Ito et al., 1999; Varga-Weisz et al., 1998). ACF in *Drosophila* contains two subunits, the ATPase ISWI and ACF1, whereas CHRAC contains in addition to ISWI and ACF1 two small histone-fold proteins; CHRAC14 and -16 (Ito et al., 1999; Corona et al., 2000). *Drosophila* contains four protein complexes that contain the ISWI subunit: ACF, CHRAC, NURF (nucleosome remodelling factor) and TRF2 (TBP-related factor 2). However, no role in chromatin assembly could be described for NURF or TRF2 (Hochheimer et al., 2002).

ISWI containing chromatin remodelers seem to be especially involved in de novo nucleosome assembly rather than nucleosome sliding and spacing. Together with histone chaperones, ACF can promote nucleosome assembly in vitro (Lusser et al., 2005). More detailed, in this study the group of James Kadonaga compared the ability of chromatin reconstitution between ACF and CHD1 (chromo-ATPase/ helicase-DNA-binding protein 1), that has been found to be a chromatin assembly factor. Both complexes were shown to use NAP1 as a histone chaperone but only ACF could incorporate linker histone H1. Interestingly, the ACF reconstituted chromatin showed a larger nucleosome repeat length than chromatin assembled with CHD1. These two facts suggest that ACF is responsible for heterochromatin formation whereas CHD1 promotes the formation of active chromatin (Lusser et. al, 2005). Moreover, chromatin of *Drosophila* flies lacking the ACF1 subunit of ACF and CHRAC show a shorter nucleosome repeat length than wild type flies, again suggesting that ACF and CHRAC are responsible for the formation of repressive chromatin (Fyodorov et al., 2004).

Many chromatin remodelers are unable to move nucleosomes when linker histone H1 is bound, they tend to get stuck in between. Others, especially the

ISWI containing ACF, can move nucleosomes even in the presence of somatic H1 in the chromatin.

In humans, the ACF1 homolog BAZ1A is known to facilitate DNA replication through heterochromatin (Collins et al., 2002). The physiological role of ACF1 in *Drosophila* is still poorly understood and the few things known are due to loss-of-function mutation studies. *Drosophila* flies with a loss of ACF1 show a so-called “semi-lethality” during their development from larvae to pupae stages. This phenotype can be assigned to the reduction of regularity of nucleosome arrays, that can lead to a less repressive chromatin and thus to uncontrolled gene activities. Flies lacking ACF1 fail to form heterochromatin as effectively as wild type flies and show defects in polycomb-dependent silencing (Chioda et al., 2010; Fyodorov et al., 2004). In mice, the ACF1 homolog BAZ1 is expressed in the early embryo and stays high only in male testis. In accordance to the previous study, it is also shown that male mice lacking BAZ1A suffer from sterility due to a mis-regulation of gene expression in the germ cells. More precisely, a massive up-regulation of genes in spermatocytes and spermatids can be observed (Dowdle et al., 2013).

Another observation in ACF1 deficient flies is a shorter S phase during the cell cycle, that can lead to erroneous replication. At the same time, it supports the theory that ACF is responsible for heterochromatin formation, as mentioned above (Fyodorov et al., 2004). Very much like dBigH1, expression of ACF1 in *Drosophila* is highest during embryogenesis and stays at high levels throughout the life of the fly only in neuroblasts and primordial germ cells. A constitutive expression of ACF1 under the α-tubulin promoter was shown to be lethal (Chioda et al., 2010).

1.4.2. The histone chaperone NAP1

Nucleosome assembly protein 1 (NAP1) is a homodimeric histone binding protein. Although its function in vivo remains elusive, it is known that it acts as a chaperone for H2A-H2B dimers in *Drosophila* and mammalian cells. More exactly, it is responsible for the transport of the dimer from the cytoplasm to the nucleus (Fuji-Nakata et al., 1992; Ito et al., 1996).

In accordance to that, NAP1 was proposed to facilitate transcription by removing the H2A-H2B dimers from the nucleosomes to allow RNA polymerase to proceed (Levchenko et al., 2004). In yeast, NAP1 was shown to be present in complexes with SWR1, a protein complex that catalyses the exchange of H2A-H2B dimers for the variant H2A.Z-H2B (Mizuguchi et al., 2004). In addition, also in yeast it was observed that NAP1 itself can exchange H2A-H2B dimers for H2A.Z-H2B in nucleosomes (Park et al., 2005). Again, this proposes a role for NAP1 in regulation of transcription, as the incorporation of H2A.Z in yeast was shown to promote transcriptional activation in vivo (Redon et al., 2002).

In vitro, NAP1 can assemble nucleosomes under physiological conditions. Apart from the H2A-H2B dimer it also binds the $(H3\text{-}H4)_2$ tetramer with almost the same affinity (Fujii et al., 1992; Andrews et al, 2008). However, the exact mechanism by which NAP1 assembles nucleosomes is unknown.

During spermatogenesis, small positively charged proteins called protamines replace about 90% of all histones in the DNA. This is necessary to achieve an extraordinary high compaction of the sperm DNA and thus making it transcriptionally and enzymatically inactive (Ward et al., 1991; Balhorn et al., 1982; Balhorn et al., 2007). After fertilization, the oocyte possesses all the factors needed to convert the condensed sperm chromatin such that it becomes transcriptionally active. An important step in that procedure is the removal of the deposited protamines and the deposition of the core histones as well as the linker histone to form nucleosomes (McLay et al., 2003). Recently a work was published that shows that NAP1, together with other chaperons, plays a role in sperm chromatin remodelling at fertilization. More exactly, NAP1 was shown to remove protamine A from sperm DNA of the male pronucleus (Emelyanov et al., 2014).

NAP1 was shown to be histone chaperone for the *Xenopus* linker histone H1M/B4 (Shintomi et al., 2005). In 2015, it was confirmed that NAP1 acts as a chaperone for the embryonic histone H1 variant H1M/B4 in *Xenopus* egg extracts. Moreover, when depleting the egg extract of NAP1 they see a significant reduction of H1M/B4 binding to mitotic chromosomes causing chromosome decondensation (Miller et al., 2015).

NAP1 was also shown to remove somatic H1 from chromatin fibres in HeLa cells when incubated with the chaperone, proposing a role for NAP1 in chromatin decondensation by histone H1 removal. Thus, NAP1 could have effects on overall gene expression (Kepert et al., 2005).

Together these findings point to a diverse role of NAP1 in the cell. It has the capacity to assemble nucleosomes in vitro and plays different roles in vivo, being a chaperone not only for the core histones dimer H2A-H2B but also for histone like proteins during fertilization as well as embryonic specific linker histones.

1.5. *Drosophila Melanogaster* as a model organism

Already in the 19th century Gregor Mendel and Charles Darwin made use of model organisms to gain knowledge of basic biologic principles. In the case of Gregor Mendel, he used peas to study the mechanisms of genetics of heredity. Charles Darwin made famous the Darwin's finches, which he was studying on the Galapagos Islands to gain insights to his idea of natural selection. However, an important step was made at the very beginning of the 20th century, when the first model organisms entered the research laboratories.

The fruit fly *Drosophila melanogaster* was one of the first animal models used in laboratories, if not the very first one. Thomas Hunt Morgan, who is usually referred to as the father of *Drosophila melanogaster*, continued in some way the work of Gregor Mendel. He defined genes and matched them to chromosomes. At that time, DNA was still not recognized as the genetic material. In 1933 he won the Nobel Prize in Physiology and Medicine for his discovery that chromosomes play an important role in heredity. Hermann Joseph Muller, an undergraduate student of Hunt Morgan, won the Nobel Prize in the same field in 1946 for discovering that x-ray irradiation produces mutations in the genes. This illustrates the very successful start that *Drosophila* had as a model organism, important biologic principles were discovered with it.

Ever since the fruit fly has conquered many more laboratories all around the world and was used in a countless of research studies. Its genome was fully sequenced in 2000, shortly before the human genome was sequenced (Adams et al., 2000). The most important reasons why *Drosophila* is such a good model system are the following (Stocker et al., 2008):

- It is easy to handle, occupies little space and it is cheap to maintain.
- Short life cycle. It takes just approximately 10 days from the laid egg to the adult fly.
- Each female can lay up to 100 eggs per day.

- It has only four pairs of chromosomes.
- *Drosophila* males do not undergo recombination during meiosis. This makes it easier for genetic studies.
- Its genome is easy enough to understand but complex enough for the discovered information to be utile for higher organisms like humans. In fact, about 75% of all known human disease genes have a corresponding match in the genome of *Drosophila* (Reiter et al., 2001). This makes it a legitim model organism also for medical research.

1.5.1. The *Drosophila* life cycle

As already mentioned, it takes just about 10 days for an embryo to develop into an adult fly. The *Drosophila* life cycle is divided into four stages: egg, larva, pupa and adult fly. The larva stage is further divided into first, second and third instar larva during which it eats and grows to prepare to become enter the pupa stage (Figure 16).

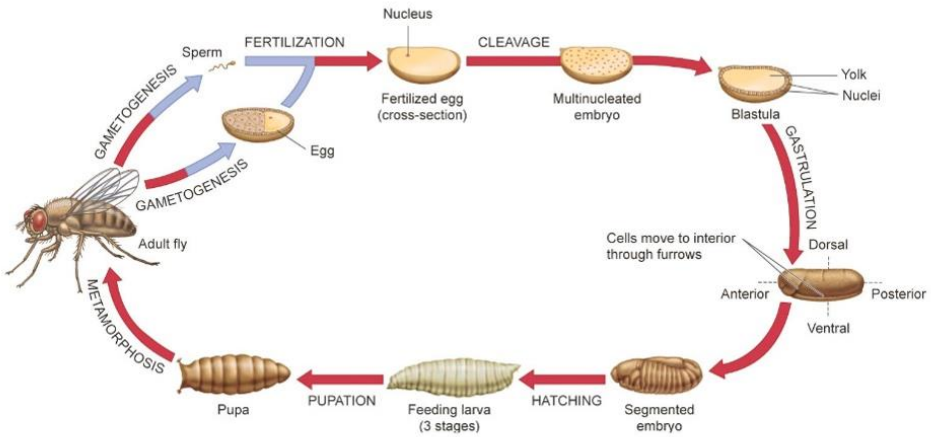


Figure 16. The *Drosophila Melanogaster* life cycle

When keeping the flies at 25°C the life cycle takes only about 10 days. After fertilization, the egg goes through gastrulation to produce the larva that is characterized by three stages: the first, second and third instar larva. After the larval stage the fly enters the pupa stage that through metamorphosis will develop into the adult fly (Purves et al., 1998).

1.5.2. The syncytial blastoderm

A very interesting stage in the life of *Drosophila*, especially in the context of this work, is the syncytial blastoderm stage that takes place only during the first two to two and a half hours after the egg is laid. The zygote nucleus starts dividing mitotically in the centric region of the egg. These divisions take place every nine minutes approximately. After eight divisions, when the egg contains 256 nuclei, they start to migrate towards the membrane of the egg (Figure 17). At the same time, a few nuclei will migrate to the posterior part of the embryo to form the pole cells. These cells will later give rise to the germ line of the adult fly. The nuclei that migrated towards the periphery of the egg will undergo five more divisions, but at a slower rate than before. The embryo is called syncytial blastoderm during this stage because all the nuclei are inside the same cytoplasm, which is the egg. After 13 divisions cellularization starts and the nuclei that are now all at the periphery will get enclosed by a plasma membrane forming cells. Cellularization of the embryo is usually completed after division 14 (Zalokar et al., 1976; Foe et al., 1983; Karr et al., 1986).

Another important fact is that during the syncytial blastoderm stage and prior to cellularization, the zygotic genome is inactivated and transcriptionally silent. During this period gene expression depends primarily on maternal deposited products. These products involve amongst others histone proteins and chromatin remodelers, important for the formation of chromatin of the newly replicated DNA. At division 9, when the nuclei start migrating to the periphery of the egg, maternal-to-zygotic transition (MZT) takes place. During this time the zygotic genome gets gradually activated and the maternal deposited products are being degraded. This process is completed at cellularization of the embryo (reviewed in Benoit et al., 2009).

Figure 17. The syncytial blastoderm stage of *Drosophila*

Confocal microscopy picture of DAPI stained chromatin. After fertilization, the zygotic nucleus divides in the centric region of the egg (1-8). After that the nuclei start migrating towards the periphery and few nuclei will migrate to the posterior part to form the pole cells (9-10). Cellularization of the replicated nuclei is finished at stage 14. The zygotic genome gets gradually activated from stage 9 and is completed at cellularization (Kotadia et al., 2010).

1.6. Spermatogenesis in *Drosophila*

In the *Drosophila* testis, we can find two different types of stem cells; the germal stem cells (GSCs) and the somatic cyst stem cells (CySCs), that divide once every 24 hours (Sheng et al., 2009).

Drosophila spermatogenesis is a tightly regulated process that involves a series of cellular morphological changes (Figure 18). The process begins in the apical region of the testis that is also referred to as the germinal proliferation center. It is the region where hub cells and the two types of stem cells (germal stem cells and somatic cyst stem cells) are located. The GSC divides to produce another GSC and a daughter cell, the Gonialblast (GB). A somatic cyst stem cell will also divide and produce another CySC and a cyst cell. Two cyst cells will surround the GB cell. The GB will undergo four rounds of mitosis with incomplete cytokinesis, giving rise to 16 cells that are interconnected by intercellular bridges. This population of interconnected cells are called spermatogonia and are enclosed by two cyst cells forming structures called cysts. After this fourth round of mitosis, each of these 16 cells inside the cyst stops mitosis and differentiates to a spermatocyte.

The primary spermatocytes enter an extended G2 phase that lasts for about three days. During this time, they grow and undergo extensive gene expression. At the end of the primary spermatocyte growth, most transcription is shut down and the 16-primary spermatocyte undergo two meiotic divisions to produce 64 haploid spermatids, again with incomplete cytokinesis and connected by intercellular bridges. The spermatids will start elongating within the syncytial cyst. The cyst becomes polarized, giving the spermatids an orientation so that all nuclei are pointing to the same side of the cyst whilst the tails point in the other direction. The spermatids will further differentiate until they eventually develop into mature sperm (Fuller et al., 1993; Hime et al., 1996; Fabian et al., 2012).

It is during the transition from spermatids to mature sperm, a process called individualization, that the nuclei become smaller and the chromosomes reach their highest condensation (Tokuyasu, 1974).

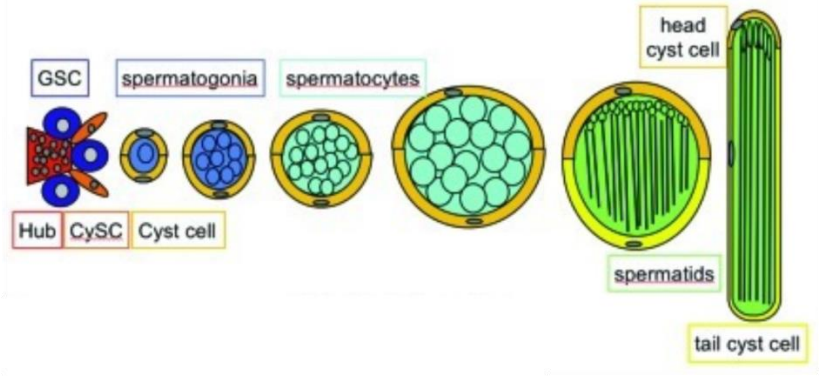


Figure 18. Spermatogenesis in *Drosophila*

Schematic representation of spermatogenesis in *Drosophila*. Germal stem cells divide asymmetrically to generate another GSC and a gonialblast (GB) daughter cell. The somatic cyst stem cell will divide, producing another somatic cyst stem cell and a normal cyst cell. The GB get surrounded by two cyst cells and complete four rounds of mitosis to produce 16 spermatogonia with incomplete cytokinesis so they stay connected between each other. They differentiate into spermatocytes that undergo two meiotic divisions to become spermatids (Demarco et al., 2014).

As already mentioned, the two chromatin remodelers ACF and CHRAC in *Drosophila* share the common subunit ACF1 (Ito et al., 1999). ACF1 is present in *Drosophila* at high levels during embryogenesis. After that, high levels of ACF1 expression can only be found in undifferentiated cells, such as neuroblasts and primordial germ cells (Chioda et al., 2010).

This pattern, that resembles the one of dBigH1, allows us to speculate about a possible interaction between these two proteins.

1.6.1. The fusome

The fusome is a germ line specific organelle that consists of membrane skeletal proteins and vesicles. It is believed that it plays an important role in germ cell differentiation and spermatogenesis in general. Mutations in structural genes of the fusome lead to improper cyst cell formation and sterility in males (Leon et al., 1999). In males, the fusome starts as a very condensed, dot-like organelle in germal stem cells and gonialblast cells. During the spermatogonia stage the fusome starts to branch, and the further spermatogenesis proceeds the more branched it will appear. The more a cell is differentiated into a spermatocyte, the clearer will the fusome be branched. It continues branching during the two meiotic divisions that form the spermatids (Hime et al., 1996). Adducin is a cytoskeletal protein that is abundant in the fusome of the *Drosophila* germ line and can be used as a marker to detect the state of branching of the fusome (Yue et al., 1992; Lin et al., 1994) (Figure 19).

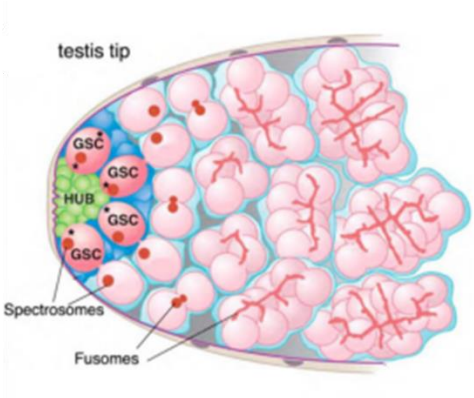


Figure 19. The testis tip and the fusome

Germal stem cells divide and become another germal stem cell and a daughter cell that becomes a spermatogonia cell. Cyst cells will start to grow around the spermatogonia and spermatocyte stages. As they move to the posterior part of the testis the fusome starts to grow and branches. Aduccin is a marker of the *Drosophila* fusome, the more branched it is the more probable it is that the enclosed cells are spermatocytes (Lighthouse et al., 2008)

1.7. Transcription in DREX

The first cell-free system for chromatin reconstitution under physiological conditions was described in 1977 using *Xenopus* egg extract (Laskey et al., 1977). It was then when it became possible to study processes like chromatin formation and its relationship to other fundamental processes like transcription and replication. The extract was prepared from unfertilized frog eggs. Earlier it was postulated that such an extract would contain all the necessary histones for chromatin assembly. This assumption was made because it had been shown that in *Xenopus* early embryos DNA synthesis was occurring at a higher rate than histone synthesis (Adamson et al., 1974). For many years, the *Xenopus* cell-free system was the most used one for chromatin assembly in vitro under physiological conditions and eventually lead to the development of a very similar system using *Drosophila*.

Based on the fact that early *Drosophila* embryos replicate their DNA very rapidly and contain all the histones and chromatin assembly factors needed in form of maternal contribution, the group of Peter Becker introduced a new cell-free system for in vitro chromatin reconstitution (Becker et al., 1992). Although another cell-free system for nucleosome assembly from *Drosophila* embryos was described earlier (Nelson et al., 1979), it never got widely adapted and apparently showed serious difficulties in its preparation.

The newly developed *Drosophila* preblastodermic extract (DREX) could reconstitute chromatin with regularly spaced nucleosomes. However, to do so an energy regenerating system in form of ATP and creatine phosphate must be added to the reaction (Becker et al., 1992).

In the same study, they also performed first transcription experiments with chromatin assembled in DREX. They used two different DNA templates carrying the heat-shock-promoter 70 and the fushi tarazu promoter, respectively. To their surprise, transcription was highly repressed and there was almost no difference when adding recombinant histone H1 to the reaction. The results for the DNA template with the hsp70 promoter are shown in figure 20 (Becker et al., 1992).

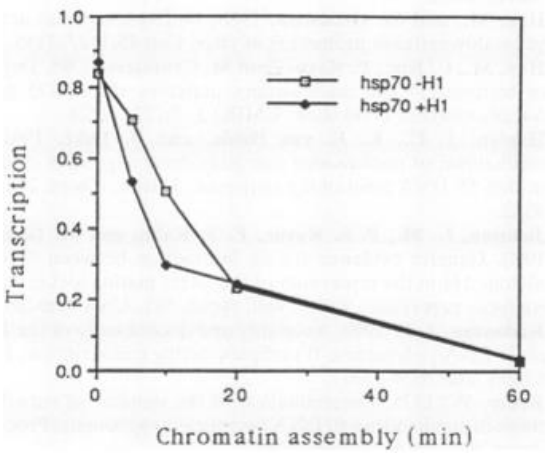


Figure 20. One of the first transcription experiments performed in DREX

A DNA template containing the hsp70 promoter was incubated with or without recombinant histone H1. A difference in transcription can only be observed when the template is not given almost any time to assemble into chromatin. The purified RNA products were analysed by primer extension (Becker & Wu, 1992).

Only two years later, in 1994, the same group published another article where they confirmed that chromatin assembled in DREX is transcriptionally silent and that adding somatic linker histone H1 does not have any additional effect (Sandaltzopoulos et al., 1994). It had been shown earlier, that transcription of chromatin assembled with purified histones and chromatin remodelers can be repressed when adding linker histone H1 to the reaction (Laybourn et al., 1991). Also, after chromatin assembly in *Xenopus* extract transcription could be further repressed by adding somatic H1 (Shimamura et al., 1989). However, previous results of transcription experiments in *Xenopus* extract had shown the opposite. Addition of somatic H1 did not have an additional effect on transcriptional repression (Shimamura et al., 1988). First results of transcription experiments in DREX pointed to the same direction, that H1 was not able to decrease transcription (Becker et al., 1992; Sandaltzopoulos et al., 1994). These contradictory results led to doubts about the widely-accepted certainty that H1 was the dominant repressor for transcription. In fact, it opened questions about an unknown transcriptional repressor. With what we knew about BigH1 at this point, it seemed very likely that it could play a role in the mentioned repression.

1.7.1. In vitro transcription using HeLa nuclear extract

HeLa is the oldest human immortal cell line to exist and still one of the most used ones to date. The cells were extracted from a cervical cancer in 1951 from a woman called Henrietta Lacks, shortly before she died (Bang et al., 1952). One of its uses in the beginning was the development of vaccines against the polio virus (Scherer et al., 1953). HeLa cells are widely used in cancer research. Like many other cancer cells, they divide very rapidly, and they contain an active form of telomerase, which means that during cell division the telomers do not get shorter, an indispensable feature for any immortal cell line (Ivankovic et al., 2007).

The best characterized cell-free system for in vitro transcription of DNA templates is the one using HeLa nuclear extracts (Dignani et al., 1983). Besides the fact that it contains all the factors needed for transcription it is very poor in factors like RNases, proteases and other inhibitory activities, which allows a very large storage time of the extract. Proteins present in HeLa nuclei are essential transcription factors like RNA Polymerase II, TFIIA, -IIB, -IID, -IIE, -IIF, -IIH, as well as more specific factors and cofactors like Sp1, Oct-1, NF-κB, USF, ATF, p300 and others (Sawadogo et al., 1985; Kadonaga et al., 1986; Reinberg et al., 1987; Maldonado et al., 1990).

In the transcription experiments performed in this study, we use the *Drosophila* preblastodermic extract to reconstitute chromatin, and HeLa nuclear extract for transcription of the reconstituted chromatin templates.

2. MATERIALS & METHODS

2.1. Materials

2.1.1. Antibodies

In western blots:

Antibody	Species & Dilution	Origin
α-dBigH1	Rabbit, 1:5000	Generated in the lab
α -H1	Rabbit, 1:10’000	Provided by Dr J. Kadonaga
α -H3	Rabbit, 1:5000	Cell signaling 9715S
α -HP1	Rat, 1:10’000	Font-Burgada et al., 2008
α -ACF1	Rat, 1:50	Provided by Dr. P. Becker
α -NAP1	Rabbit, 1:4000	Provided by Dr. J. Kadonaga

In immunostainings:

Antibody	Species & Dilution	Origin
α -dBigH1	Rabbit, 1:400	Generated in the lab
α -ACF1	Rat, 1:2	Provided by Dr. P. Becker
α-β-galactosidase	Mouse, 1:500	Promega Z-3781
α -Aduccin	Mouse, 1:100	Abcam 54985

Secondary antibodies:

WB: Western Blot, IS: Immunostaining

Secondary antibody	Dilution	Origin
α-rabbit Cy3	1:400 (IS)	Jackson (111-165-144)
α-rabbit Cy5	1:400 (IS)	Jackson (111-175-144)
α-mouse Cy2	1:400 (IS)	Jackson (115-225-146)
α-rat Cy5	1:400 (IS)	Jackson (112-175-143)
α-rabbit HRP	1:10'000 (WB)	Jackson (111-035-144)
α-mouse HRP	1:10'000 (WB)	Jackson (715-035-150)
α-rat HRP	1:10'000 (WB)	Jackson (712-035-150)

2.1.2. Fly lines

Genotype	Characteristics	Origin
White^{-} (w118)	White gen mutated, used as control	Bloomington *Drosophila* Stock Center
Acf1^{2}	Flies mutant for ACF1 by deletion of the promoter region	Provided by Dr. D. Fyodorov
Nos-Gal4	Expresses Gal4 under the control of the nanos promoter	Bloomington *Drosophila* Stock Center
35575 RNAi-ACF1	Expresses ds RNAi for ACF1 under UAS control	Bloomington *Drosophila* Stock Center
Acf1^{7}	Flies mutant for ACF1 by deletion of 3098bp of the gene	Provided by Dr. P. Becker

Acf1-fosmid	Flies have an extra copy of ACF1 fused to GFP	Provided by Dr. P. Becker

2.1.3. Primers

Delta ½-Nt	5’ – GTC GAG ACA GAT AAT CTC GG – 3’
Delta Nt	5’ – TCCCTAGCTCTTATGGC – 3’
Vector Nt	5’ – GTCGTCGTCGTCGGTAC – 3’
T7 forward (for sequencing)	5’ – TAATACGACTCACTATAGGG – 3’
Ct (for sequencing)	5’ – ATCGGTGAGTTTAAAGGAT – 3’

2.1.4. Plasmids

Name	Use	Origin
pAc5.1-V5-His A	Contains the promoter *Drosophila* actin 5C gene. Used for the actin-GFP construct.	Invitrogen
pEGFPN1	Used for the actin-GFP construct.	Clontech
pET-30b(+)-dBigH1	Expression of recombinant dBigH1. Making and expression of dBigH1-Δ1/2-Nt and dBigH1-Δ-Nt	Novagen, dBigH1 cloned by Dr. Alex Vaquero
pEt-29b(+)-H1	Expression of recombinant H1	Novagen, H1 cloned by Dr. Jordi Bernues

2.2. Methods

2.2.1. Sodium dodecyl sulphate (SDS)-Page

A polyacrylamide gel separates proteins according to their molecular mass. The samples are dissolved in SDS-buffer containing β-Mercaptoethanol, as this denatures the protein and provides it with a negative charge. The gel is made of two components, a first upper gel, called the stacking gel, that contains 4% of acrylamide and serves to gather the proteins, and a lower gel that usually consists of 10% acrylamide and serves to separate the proteins. The samples are heated at 95°C during 10 minutes before loading. The chamber is filled with 1xSDS running buffer and a current is applied such that the negatively charged proteins will migrate towards the electric force of the positive charge. Normally it takes about 90 minutes when run at 120 volts.

SDS-Page stacking gel:

- 5.6 ml ddH2O
- 2.3 ml Tris HCl, pH 6.8 (0.5M)
- 680µl BAA (2%)
- 1.28 ml acrylamide (40%)
- 50µl SDS (20%)
- 100µl APS (10%)
- 5µl TEMED

SDS-Page separating gel:

- 7.1 ml ddH_2O
- 5 ml Tris HCl, pH 8.8 (1.5M)
- 2.6 ml BAA (2%)
- 5 ml acrylamide (40%)
- 100µl SDS (20%)
- 200µl APS (10%)

- 10µl TEMED

1x running buffer:

- 2 g SDS
- 12 g Tris HCl
- 57.6 g Glycine
- Adjust total volume to 1 L with ddH_2O

2.2.2. Western Blot

The SDS-gel gets applied on a nitrocellulose membrane and packed between layers of blotting-paper and sponge-like sheets. The running buffer gets replaced by SDS-transfer buffer, commonly known as blotting buffer. The chamber gets again energized at 100 volts for 60 minutes. During this time the proteins get blotted onto the nitrocellulose membrane. After blotting the membrane gets incubated for one hour with 5% milk PBS-Tween. This step blocks all the target site of the membrane which were not occupied by proteins. The membrane gets washed in PBS-T and incubated with the first antibody for an hour at room temperature. After another washing step the secondary antibody gets applied, usually also during one hour at room temperature. In the end the membrane gets wetted western blotting detection reagent, that is the substrate for the enzyme on the secondary antibody. The proteins can now be made visible with autoradiographic films.

SDS-transfer buffer:

- 5.8 g Tris HCl
- 2.9 g Glycine
- 0.17 g SDS
- 200 ml Methanol
- Adjust total volume to 1 L with ddH_2O

PBS-Tween (0.05%):

- 100m ml 10x PBS
- 900 ml ddH_2O
- 0.5 ml Tween

2.2.3. Transformation & Mini-preparation of plasmid DNA

The extraction and purification of plasmid DNA from bacteria is a very useful tool. It can be divided in four main steps: transformation of the bacteria, growth of the bacterial colony, harvesting and lysing of the bacteria and purification of the plasmid. The most used bacteria for plasmid preparation is E. Coli. In this study, we used DH5α, a competent E. Coli strain especially designed for cloning. The plasmids usually carry a resistance gene that gets expressed in the bacteria. This allows for the selection of only those bacteria that have taken up the plasmid successfully. The transformation of the bacteria includes the following steps:

- Between 1 and 10µg of plasmid, usually coming from a ligation, is added to 100µl of competent DH5α cells that were thawed on ice.
- The mixture is incubated for 20 min on ice
- Incubate during exactly 2 min at 42°C
- Add 1 ml LB medium and incubate 30 min at 37°C
- Spin down at 9000 rpm and take out the supernatant leaving about 100µl of liquid
- Resuspend and plate on agar plates that contain the appropriate antibiotic
- Incubate overnight at 37°C

The next day, colonies are picked and added to tubes containing 4 ml of LB medium plus the antibiotic needed. The tubes are incubated in a shaker overnight at 37°C.

The next day, the actual mini-preparation is taking place. From each tube 1 ml is taken out and the DNA is extracted and purified. The protocol is the following:

- Spin down 1 min at 9000 rpm
- Take out supernatant
- Resuspend pellet in 100µl GTE (25 mM Tris-HCl pH 8.0, 10 mM EDTA). Before using add 3µl of RNase to 1 ml of GTE to prevent RNA in the sample.
- Add 200µl lysis buffer (0.2 M NaOH, 1% SDS). Invert tubes and leave 5 min on ice.
- Add 150µl of 3 M sodium acetate. Invert tubes and leave 5 min on ice.
- Spin down 3 min at full speed
- Transfer supernatant to a new tube
- Add 400µl of Phenol/Chloroform. Invert tubes and spin down at full speed for 2 min. The Phenol/Chloroform extracts the DNA by dissolving proteins and lipid contaminants. The DNA stays in the upper aqueous phase.
- Transfer upper phase into a new tube
- Add 1ml of cold 100% ethanol. This precipitates the DNA.
- Incubate 20 min on ice
- Spin down 5 min at full speed, remove the supernatant.
- Wash pellet by adding 300µl of 70% ethanol, spin down at full speed for 2 min.
- Remove supernatant and dry pellet for 10 min at room temperature.
- Resuspend in 30 - 50µl of water or TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA).

The plasmid is then ready to be analyzed either by digestion using restriction enzymes or by DNA sequencing if one needs to know the exact sequence of the plasmid.

2.2.4. Maxi-preparation of plasmid DNA

A maxi-preparation of a plasmid is basically the same as a mini-preparation but in larger volumes. If the mini-prep gives a positive result and one is interested in producing the plasmid in large amounts, the rest of the 4ml tube from the day before can simply be added to an Erlenmeyer containing 500 ml of LB medium and the bacteria can be grown again in a shaker overnight at 37°C. The next day, the maxi-preparation can be done. The protocol is essentially the same. The rule of thumb amount of plasmid DNA obtained by a mini-preparation is about 50 – 100 µg, whereas a maxi-preparation can give yields of up to 900 µg.

2.2.5. Expression of recombinant proteins

The overexpression of the recombinant proteins H1 and dBigH1 as well as the truncated constructs dBigH1-Δ-Nt and dBigH1-Δ1/2-Nt was done in E. coli BL21-LysS competent cells. The BL21-LysS carry a plasmid encoding the T7 lysozyme that lowers the background of bacterial expressed proteins, but it does not interfere with genes induced by IPTG. However, for the cells to become transformed chloramphenicol must be added to the agar plates prior to their use. The purification was done using nickel-charged agarose resins (Ni-NTA, Qiagen), that bind the histidine tag of the protein.
Here, I describe the protocol that was used from the transformation of the cells until the purified protein. The protocol contains some changes compared to the standard one. These changes are the result of its optimization to adapt to the needs of our recombinant proteins. The protocol is subdivided by days.

Day one

- Thaw BL21-LysS competent cells on ice
- Add 5µl of the plasmid coming from the Maxi-Prep to 100µl BL21 cells
- Mix carefully with the pipette

- Leave 20 min on ice for the transformation
- Incubate the Eppendorf at 42°C during 2.5 min
- Add 1ml of LB medium
- Incubate one hour at 37°C to express the resistance gene
- Centrifuge 1 min at 9000 rpm and take out 1ml of medium
- Resuspend the pellet and plate it equally on two agar plates with kanamycin resistance. Remember to add chloramphenicol to the agar plates (25 μg/ml).
- Incubate overnight at 37°C

Day two

- Check that there are colonies on the agar plates
- Take four small tubes with 4ml LB medium each and add 8μl of kanamycin to each
- Pick one colony to each tube
- Incubate in a shaker overnight at 37°C

In the meantime, prepare one big Erlenmeyer with 450ml ddH_2O + 150ml LB medium (4x) and keep them at 4°C.

Day three

- Take out the Erlenmeyer and let them reach room temperature
- Add 1.2ml of kanamycin
- Add three tubes from the incubation to the Erlenmeyer
- Incubate at 37°C whilst shaking for about three hours
- Take out 1ml and measure the absorbance in a photo spectrometer. It should reach 600nm compared to LB medium alone.
- Add 0.1M Isopropyl-β-D-1-thiogalactopyranoside (IPTG) to induce expression of the protein
- Incubate in a shaker at 20°C overnight

Day four

- Centrifuge everything during 15 min at 4000g, discard the supernatant
- Resuspend the pellet in 10ml lysis buffer
- Sonicate the sample, use an amplitude of 30% and sonicate it about 10 - 15 times with sonication periods of 10 seconds each.
- Transfer the sample to an ultracentrifuge tube and centrifuge it for one hour at 20'000rpm.
- Separate the supernatant from the pellet
- Keep at -80°C if continuing the next day

Day five (Work at 4°C to prevent protein degradation)

- Add 0.5ml of Ni-NTA into a column (Biorad)
- Equilibrate with 10ml lysis buffer
- Apply the sample and let it pass through three times to allow binding of the protein to the matrix
- Wash with 10ml lysis buffer
- Wash with 5ml buffer D
- Pre-elute with 3ml buffer D containing 20mM imidazole (PE1, PE2, PE3)
- Elute with 5ml buffer D containing 100mM imidazole (E1, E2, E3, E4, E5)
- Elute with 5ml buffer D containing 500ml imidazole (E6, E7, E8, E9, E10)

The protein will usually elute in the E1-E5 fractions. Expression and purity was checked by coomassie staining and western blot against the protein.

Lysis buffer:

- 0.5 M NaCl
- 20 mM Hepes, pH 7.9
- 1 mM EDTA
- 20 mM ß-mercaptoethanol
- 1 mM PMSF
- 0.1% NP-40
- 20% Glycerol

Elution buffer D:

- 50 mM KCl
- 20 mM Hepes, pH 7.9
- 0.2 mM EDTA
- 0.1 mM PMSF
- 0.5 mM DTT
- 20% Glycerol

2.2.6. Cloning and functional analysis of the Actin-GFP construct

The plasmid for the reconstitution experiments was obtained using the two vectors pEGFP-N1 and pAc5.1-V5-His (Figure 21). The GFP reporter gene was cut out of the pEGFP-N1 vector using the restriction enzymes EcoRI and NotI. EcoRI is an endonuclease and recognizes the G/AATTC sequence and creates sticky ends with a 5' end overhang. NotI is also an endonuclease that recognizes the sequence GC/GGCCGC. Just like EcoRI it creates 5' end overhangs. The reaction was the following:

- 1 µl plasmid (1µg/µl)
- 0.5 µl EcoRI
- 0.5 µl NotI
- 4 µl 10x Tango buffer for double digestion
- 14 µl ddH_2O

Incubate for one hour at 37°C.

The 770bp large fragment containing the GFP reporter gene was then cloned into a pAc5.1-V5-His vector, that was previously cut with the same two enzymes. The pAc5.1-V5-His vector is especially designed for the use in *Drosophila* cells for overexpression of recombinant genes. It contains the active promoter of the

Drosophila actin 5C gene. The ligation of the vector and the insert was performed overnight at 18°C using the T4 DNA ligase kit from Thermo Scientific.

- 5 µl Insert (GFP)
- 1 µl vector
- 2 µl 10x T4 DNA ligase buffer
- 1 µl DNA ligase
- 11 µl ddH_2O

Incubate on the bench overnight. The plasmid was then transformed into DH5α competent cells and plated for subsequent maxi-prep preparation.

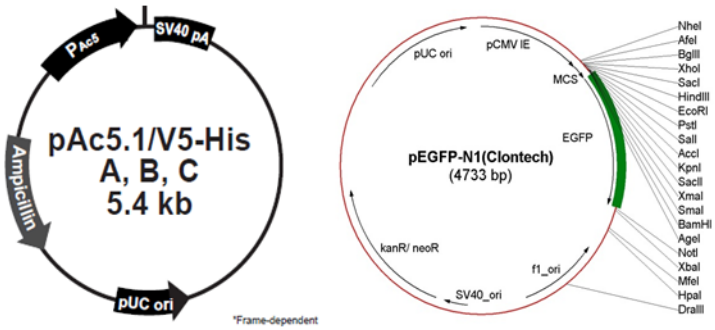


Figure 21. The two vectors used to obtain the Actin-GFP reporter plasmid

Both, the pAc5.1-V5-His (left) and the pEGFP-N1 vector (right) were cut with the endonucleases EcoRi and NotI. Ligation was done overnight at 18C using the Thermo Scientific ligation kit.

To check whether the constructed plasmid was functional, we decided to do a transient transfection into *Drosophila* S2 cells and check the expression of the GFP gene by fluorescence microscopy. S2 stands for Schneider 2, a cell line that was derived from a primary culture of *Drosophila* embryos of about 20 hours of age, just before the 1st instar larvae stage.

Transfection of the plasmid into S2 cells was done as followed: S2 cells were grown on small plates (35mm) overnight in Complete Schneider's *Drosophila* Medium until they reached a density of about 2 to 4 x 10^6 cells/ml. The next day the cells were transfected using 20ug of DNA in a total volume of 300ul containing

0.25M calcium phosphate. This solution was mixed with 300ul of 2xHEPES pH 7.9 and incubated at room temperature for 30 minutes. The final solution was then added dropwise to the cells. The cells were incubated at 25C and the expression of the construct was checked the next day with a fluorescence microscope.

The expression of the reporter gene is clearly visible as a green signal in approximately 30% of all cells (Figure 22). This indicates that the plasmid was well constructed and functional to be used in further experiments.

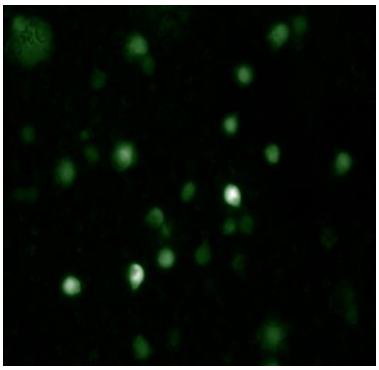


Figure 22. Transfection of *Drosophila* S2 cells

Drosophila S2 cells were transfected with the previously constructed Actin-GFP plasmid. Expression could be detected in approximately 30% of cells using a fluorescence microscope.

2.2.7. Construction of the truncated constructs

The dBigH1-Δ1/2-Nt and the dBigH1-Δ-Nt constructs were both made starting with the full-length protein. dBigH1 was cloned into the pET-30b(+) vector (Novagen) by Dr. Alex Vaquero, a former member of the laboratory. dBigH1 was cloned in such a way that it contains a N-terminal histidine tag. The cloning and expression region of pET-30b(+) is shown in figure 23. dBigH1 is cloned in the EcoR V site. The vector contains a T7 promoter and a kanamycin coding sequence.

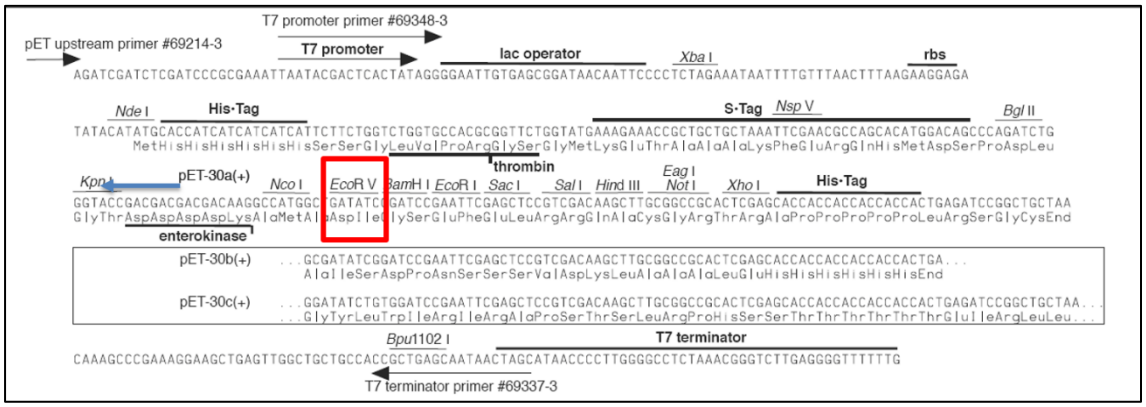


Figure 23. Cloning and expression region of pET-30b(+)

dBigH1 was cloned into the EcoR V site (red box) and contains a N-terminal histidine tag. The vector has a T7 promoter and a kanamycin coding sequence. The blue arrow indicates the region where the Vector-Nt primer is located. The other two primers used, ΔNt and Δ1/2-Nt are located within the dBigH1 region and will amplify the gene and the plasmid in the other direction, thus creating a sequence that lacks the whole or half the N-terminal region.

The dBigH1-Δ-Nt construct lacks the whole 103 amino acids of the N-terminal tail and therefore consists only of the globular domain and the c-terminal region. Since most of the negatively charged residues are in the outer part of the N-terminal region, we decided to make another construct that we called dBigH1-Δ1/2-Nt. This construct lacks the first 59 residues of the N-terminal tail and therefore resembles the somatic H1 in size and polarity. A schematic representation of the constructs in comparison with the full-length protein is shown below (Figure 24).

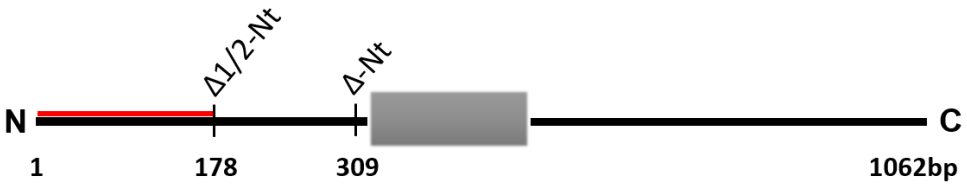


Figure 24. Schematic representation of the truncated constructs

The Δ-Nt construct lacks the whole N-terminal region and consists only of the globular- and the C-terminal domain. The Δ1/2-Nt construct lacks the first 59 amino acids of the N-terminal tail where the clear majority of negatively charged residues are located, represented with a red bar.

The actual procedure how the truncated dBigH1 constructs were made is explained in the following section.

2.2.7.1. Construction of dBigH1-Δ-Nt

In the case for dBigH1-Δ-Nt we designed primers that would amplify the whole vector including dBigH1 apart from the 309 base pairs of the N-terminal tail. One primer, called ΔNt, starts at the border of the globular domain of dBigH1 and moves away from the N-terminal region, amplifying the globular domain and the C-terminal region. The other primer, called Vector-Nt, is located near the start of dBigH1 but moves away from it, thus amplifying the vector. The exact procedure was as following:

Primer phosphorylation

The primers were phosphorylated to create the 5' phosphates on the amplified region that is needed for ligation.

- 1μl primer (100 pmol)
- 2.5μl 10x PNK buffer
- 1μl T4 polynucleotide kinase
- 2.5μl ATP (10 mM)

- 18µl ddH_2O

Incubate one hour at 37°C.

The PCR was done as following:

- 10µl phosphorylated primer ΔNt
- 10µl phosphorylated primer Vector-Nt
- 4µl dNTPs (2.5 mM)
- 1µl plasmid (100ng)
- 5µl 10x buffer expand
- 19µl ddH_2O

Incubate for 5 min at 95°C to allow denaturation, then add:

- 1µl Expand polymerase

29 cycles:

- 1 min at 95ºC (denaturation)
- 1 min at 55ºC (annealing)
- 6 min at 68ºC (extension)

10 min at 72°C and pause at 4°C.

The DNA was precipitated by adding 3 volumes of cold 100% ethanol (150 µl), 5 µl of 3M sodium acetate and incubating it for 30 minutes on ice. After 10 minutes centrifugation at maximum speed, the pellet was resuspended in 18 µl of ddH_2O.

- Add 2µl of 10x T4 DNA buffer (Böhringer buffer)
- 1µl T4 DNA polymerase
- Incubate exactly 2.5 min at 37°C

The T4 DNA polymerase will cut 3’ extensions using its 3’ exonuclease activity.

- Add immediately 2µl of dNTPs (2.5 mM) and incubate for 10 min at 37°C

Now that dNTPs are present, the T4 DNA polymerase will refill the resected strand leaving blunt ends ready to ligate. Stop the reaction by adding one volume of Phenol/Chloroform and centrifuging. Separate the phases and precipitate the DNA as usual with 100% ethanol.

10x T4 DNA buffer (Böhringer buffer):

- 0.33 M Tris acetate, pH7.9
- 0.66 M Potassium acetate
- 0.1 M Magnesium acetate
- 5 mM DTT

Resuspend the DNA in 45 µl ddH_2O and add 5 µl of 10x ligase buffer and 1 µl of T4 DNA ligase. Incubate at room temperature for three hours and transform into competent DH5α cells for mini-prep preparation. The mini-preps were analyzed by restriction enzyme digestion and sequencing using the T7-forward primer, which is the primer for the T7 promoter on the vector. Positive constructs were then amplified by maxi-prep.

2.2.7.2. Construction of dBigH1-Δ1/2-Nt

the generation of the dBigH1-Δ1/2-Nt resulted to be more complicated for reasons we never quite understood. In the end, we managed to make the construct in the following way.

The first step of the procedure was exactly as described for dBigH1-Δ-Nt, except that instead of the Δ-Nt primer the Δ1/2-Nt primer was used. In the end, when the PCR had blunt ends as made by the T4 DNA polymerase, the product was not ligated but digested with the restriction enzyme XhoI. This left the dBigH1-Δ1/2-Nt coding region with an additional vector sequence at its C-terminal tail of about 50 base pairs, giving the fragment a length of about 935 base pairs. It was run on an agarose gel, cut out and purified from the gel using the Gel Band Purification

kit from GE Healthcare. The other fragment, the rest of the vector, had a size of about 5200 base pairs.

On the other hand, the vector was digested with Bgl II, leaving sticky ends. The product was then treated like described to make blunt ends. After that the product was digested with XhoI, thus leaving one blunt end and one sticky end, just like the insert. It was run on an agarose gel and the vector was purified from the gel.

Both parts were resuspended in 30µl of water and ligation was done with 5µl of vector and 15µl of insert. The plasmid was inserted into competent DH5α cells for mini-prep. The mini-preps were analyzed by restriction enzyme digestion and sequencing using the Ct primer. We called this primer Ct because it lies in the C-terminal region of dBigH1. Positive constructs were then amplified by maxi-prep.

2.2.7.3. Expression of the recombinant proteins

The two truncated dBigH1 constructs as well as the full-length dBigH1 and the somatic dH1 were expressed in E. coli BL21 cells and purified by Ni-NTA agarose column (Figure 25).

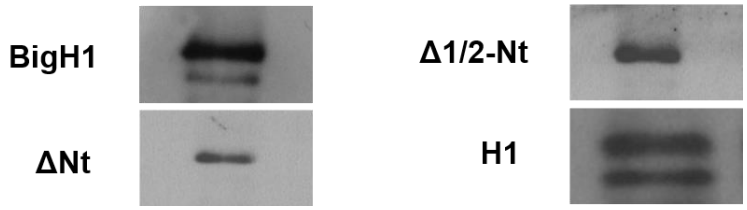


Figure 25. Proteins used in transcription assays

Western Blots of the four recombinant proteins that were used in the experiments. All of them were expressed in E. coli BL21 cells and purified by Ni-NTA agarose column using the His-Tag of the protein.

2.2.8. Preblastodermic extract (DREX) preparation

The *Drosophila* preblastodermic extract (DREX) used for chromatin reconstitution is prepared from fly embryos between 0 and 90 minutes of age. Since the embryo is naturally very small at this stage, a very large number of flies is needed to prepare a reasonable amount of DREX. Special cylinders are needed that are about 40cm in diameter and 60cm in length. It takes about 6 weeks to grow a big enough fly population to prepare the extract. About 10ml of extract can be prepared from a population of approximately 150’000 – 200’000 flies. Once the population is ready, the DREX can be prepared in one to two days. In each of the cylinders, six in total, a tray with fly food is put inside. The flies will start eating and at the same time laying eggs in the food. After 90 min, the trays are removed, and the embryos are washed out of the food using special sieves. The used trays are replaced by new ones and again after 90 minutes the embryos are washed and kept at 4°C to stop the development of the embryo. This procedure is repeated usually between six and eight times, depending on the amount of DREX needed and on how many eggs are being laid.

Once enough embryos are collected the actual extract preparation protocol can begin. After the dechorionation of the embryos, they are centrifuged to remove the membrane and other cell debris. The sample is washed a few times and then applied to an ultracentrifuge where the nucleic acids are getting removed. The detailed protocol from the point the embryos are collected looks like the following (adapted from P.B. Becker et al. Methods in Molecular Biology, Vol.119: Chromatin Protocols):

Harvesting of the *Drosophila* Embryos

1. Collect embryos laid on apple juice plates with yeast paste during a 90-min time window.
2. Rinse the embryos off the plates with tap water and using the paint brush, collect the embryos in the 0.125 mm mesh sieve.

3. Using a squeeze bottle with EW buffer rinse the embryos from the sieve into a clean beaker. Store the suspension on ice until extract preparation. Pool the embryos of 4–6 successive collections in EX buffer on ice.
4. Aspirate the cold EW and replace it for fresh EW at room temperature (RT) and allow embryos to settle.

Collection of Dechorionated Embryos

1. Remove the supernatant and adjust the volume with fresh EW to 200 ml.
2. Add 60 ml of 13% hypo chloric acid (RT) and stir vigorously for 3 min.
3. Pour the embryos back into the fine collection sieve (mesh diameter 0.125 mm) and rinse vigorously with cold tap water.
4. Transfer the dechorionated embryos to a 500-mL cylinder and wash the embryos with 500 ml EW (RT) and let the embryos settle again.
5. Aspirate off the EW.
6. Wash the embryos with 500 ml 0.7% NaCl (RT) and let them settle again.
7. Aspirate off the 0.7% NaCl.
8. Wash the embryos with 500 ml EX-buffer (at 4°C) and let them settle again. From now on keep embryos on ice and perform all manipulations at 4°C with precooled buffers.
9. Aspirate off the EX-buffer as much as possible, without losing embryos, add additional 100µl 1M DTT and 100µl 0.2M PMSF to the embryos.
10. Transfer the embryos to the homogenizator vessel on ice and allow them to settle for at least 15 min.
11. Aspirate off the EX-buffer as much as possible, leaving behind the packed embryos.

Extract Preparation

1. Stir up packed embryos to resuspend and homogenize them with one complete stroke at 3000 rpm and 6 strokes at 1500 rpm.
2. Measure the volume of the homogenate and add 1M $MgCl_2$ to a final concentration of 6.5mM. Mix the $MgCl_2$ immediately with an additional homogenization stroke at 1,500 rpm.

4. Centrifuge the extract for 5 min at 10.000 rpm (17,000g) in a chilled (4°C) HB4 rotor, using COREX tubes.
5. Collect the turbid cytoplasmic extract.
6. Transfer the extract to an ultracentrifugation tube.
7. Centrifuge for 2 hours in a chilled (4°C) SW55.1 Ti-rotor at 45,000 rpm 190,000g.
8. Isolate the clear extract with a needle and a syringe.
9. Flash freeze aliquots in liquid nitrogen and store the aliquots at -80°C.

Extract (EX) buffer:

- 10 mM HEPES-KOH, pH 7.6
- 10 mM KCl
- 1.5 mM MgCl
- 0.5 mM EGTA-KOH, pH 8.0
- 10% glycerol
- 10 mM ß-glycerophosphate
- 1 mM DTT
- 0.2 mM PMSF

Embryo wash (EW) buffer:

- 0.7% NaCl
- 0.05% Triton X-100

2.2.9. Depletion of dBigH1 from DREX

Depletion of dBigH1 from the *Drosophila* preblastodermic extract (DREX) was done using protein A magnetic beads (Novex) and IgG from rabbit serum immunized with dBigH1.

In a normal depletion, 320µl of DREX were depleted using 150µl of beads. The yield would be around 250µl, the rest was usually lost between the changes of the beads and the tubes. The exact protocol used is as following:

dBigH1 depletion Protocol:

- Wash the beads 3x with 200µl wash buffer (10mM Hepes pH8, 8% glycerol)
- Block the beads in wash buffer + 0.5% BSA during 1 hour on the rotating wheel at room temperature
- Wash 2x and resuspend beads in 200µl wash buffer
- Add IgG (3ul/10ul beads). Incubate 1 hour on the rotating wheel at room temperature
- Wash 2x with wash buffer
- Wash 2x with Hepes only, to get rid of the glycerol in the wash buffer
- Resuspend in 10 mM Hepes and split in five Eppendorf
- Add DREX and incubate at 4°C on the rotating wheel. Transfer the DREX every 45 min to the next tube
- In the end, transfer DREX to a new Eppendorf and make sure not to carry over any beads

2.2.10. Chromatin reconstitution in DREX

600 ng of the circular DNA plasmid was reconstituted as following (adapted from P.B. Becker et al., Methods in Molecular Biology, Vol.119: Chromatin Protocols):

- 84µl Exb50
- 40µl DREX
- 14µl McNap
- 2ul plasmid (300 ng/ µl)

The mixture was then incubated during 4 hours in a water bath at 26°C.

Exb50:

- 10 mM Hepes, pH 7.6
- 50 mM KCl
- 1.5 mM $MgCl_2$
- 0.5 mM EGTA, pH 8.0
- 10 mM β-Glycerophosphate
- 10% Glycerol

McNap:

- 3 mM MgCl2
- 2 mM DTT
- 30 mM ATP (in 0.1 M Hepes, pH 7.6)
- 300 mM Creatine phosphate (CP)
- 0.02 µg Creatine kinase (CK)

The CP and CK are needed as an energy recreating system for ATP-dependent chromatin remodelers. The CP provides a pool of phosphate groups and the CK can use them and convert ADP to ATP.

2.2.11. Micrococcal Nuclease (MNase) assay

To check the quality of the assembled mini-chromosomes and if there is a regular nucleosome spacing, the chromatin is digested with Micrococcal nuclease, a bacterial-derived nuclease that cuts the DNA un-specifically. The reaction was as following (adapted from P.B. Becker et al. Methods in Molecular Biology, Vol.119: Chromatin Protocols):

- 35µl of assembled chromatin from the reconstitution in DREX
- 18µl MNase mix:
    - 90µl Exb0
    - 6µl $CaCl_2$ (6mM)
    - 4µl MNase (0.2 units/ µl, diluted in 0.1% BSA)

Buffer Exb0:

- 10 mM Hepes, pH 7.6
- 1.5 mM $MgCl_2$
- 0.5 mM EGTA pH 8.0
- 10 mM β-Glycerophosphate
- 10% Glycerol

The MNase digestion is performed at 37°C and starts when the MNase mix is added to the chromatin. At every chosen time point, 51µl are taken out and transferred into an Eppendorf that contains 20µl of 100 mM EDTA to stop the reaction. Then:

- Add 1µl RNase and incubate for 30 minutes at 37°C
- Add 2µl of 10% SDS and 6µl of Proteinase K (10mg/ml)
- Adjust volume to 200µl with RNase-free water
- Incubate for one hour at 45°C

After this step the DNA gets extracted by adding 200µl Phenol/Chloroform. 20 µl of sodium acetate are added and the DNA is precipitated overnight at -20°C by adding 3 volumes of 100% ethanol.

The next day the samples are centrifuged during 30 minutes at maximum speed in a table centrifuge and the pellet is washed with 500µl 70% ethanol. After another centrifugation of 10 minutes the pellets are resuspended in 17µl of water and 3.4µl of Orange G DNA loading buffer (6x) is added.

The samples are run on a large 1.5% agarose gel at about 100 volts for five hours. The gel is soaked with water containing 10ug/100ml ethidium bromide and the bands are made visible using a UV-illuminator.

2.2.12. Transcription in HeLa nuclear extract

After chromatin reconstitution, the mini-chromosomes were precipitated using 15mM of $MgCl_2$ and resuspended in 62µl Exb50, the same volume that was used to precipitate the chromatin.
The transcription mix was as following:

- 12µl minichromosomes
- 10µl Buffer-C_{90}
- 20µl Rxn mix
- 14µl HeLa nuclear extract

This was incubated at 30°C for 60 minutes. The transcription reaction was stopped by adding 100ul of 2x Stop-mix and incubating for 15 minutes at 39°C. The RNA was extracted with 1x volume Phenol/Chloroform and precipitated with 3 volumes of 100% ethanol overnight at -20°C. The next day the samples were spun down during 30min at maximal speed in a table centrifuge and the samples were resuspended in 100µl RNase-free water.

Buffer C_{90}:

- 20 mM Tris HCl, pH 7.8
- 1 mM EDTA
- 1 mM DTT
- 10% Glycerol
- 90 mM NaCl

Rxn-mix:

- 20mM Tris (1M), pH 8.3
- 5 mM $MgCl_2$
- 3 mM DTT
- 25mM rNTP mix
- 6.25% PEG 8000

Stop-mix (2x):

- 200 mM NaCl
- 20 mM EDTA
- 1% SDS
- 12µl glycogen
- 12µl Proteinase K (10mg/ml)
- Adjust with RNase-free water to 1 ml

2.2.13. RNA purification

The precipitated RNA was further purified, and any DNA remainders were removed by DNase digestion using the QIAGEN RNeasy Mini Kit.
The purified RNA was dissolved in 30µl of RNase-free water and used in reverse transcription to generate cDNA.

2.2.14. Retro-transcription of mRNA into cDNA

Transcriptor First Strand cDNA Synthesis Kit (Roche) was used. Reverse transcription was done using PCR tubes and a PCR machine for incubating the reactions.

1. Resolve the precipitated RNA in 30µl of RNase-free water
2. Set up the following mix:

   - 2µl of total RNA
   - 1µl oligo $(dT)_{18}$
   - 10µl RNAse-free water

3. Incubate at 65ºC for 10min for denaturation of the template-primer mixture
4. Add the following components in this order:

   - 4µl 5x Reaction Buffer
   - 0.5µl Protector RNase Inhibitor
   - 2µl dNTPs
   - 0.5µl Transcriptor RT (enzyme)

5. Mix gently (do not vortex) and incubate using the following program:

   - 50ºC for 1 hour
   - 85ºC for 5min
   - Pause at 4ºC

6. Dilute the products of the retro-transcription 1:5 in RNase-free water.
Note: The direct samples that were used for qPCR without transcription were diluted 1:100 in RNase-free water.

7. Store the samples at -20ºC or continue directly with qPCR.

2.2.15. Quantitative PCR (qPCR)

The qPCR is performed using 96-well plates and a Light Cycler 480 machine (Roche). Relative expression levels are calculated using the standard curve method.

To normalize the results for the transcription samples we also performed qPCR of the same samples but without the transcription step. For this, after chromatin assembly and precipitation with $MgCl_2$, but prior to transcription, we retained some material that we used directly in qPCR with the same primers for GFP. Those numbers were then used to normalize the results from transcription and be able to compare them between the different conditions.

1. Prepare oligo/SYBR Green master mixes (given are the amounts required per 10µl reaction).

- 0.5µl forward primer [10 µM]
- 0.5µl reverse primer [10 µM]
- 5µl 2X SYBR Green I Master (Roche)

2. Set up 10µl PCR reactions in a 96-well plate. Use the 1:5 dilutions of the RT products and the direct samples that are diluted 1:100.

- 6µl oligo/SYBR Green master mix
- 4µl template (cDNA or controls)

3. The following standard PCR program is used:

- 5min at 95ºC

45 cycles:

- 30sec at 95ºC (denaturation)
- 30sec at 60ºC (annealing)
- 30sec at 72ºC (extension)

2.2.16. Preparation and Immunostaining of *Drosophila* testis

Testis from *Drosophila* males were dissected and incubated with antibodies to analyze them under the confocal microscope. The protocol was as following:

1. Dissect the testis in cold PBS.

2. Fix tissues in 4% paraformaldehyde during 15-20 min at room temperature

3. Wash shortly with PBS-0.3%-Triton X-100 (3 times).

4. Wash with PBS-0.3%-Triton X-100, 20 min.

5. Wash the tissues 10 min with fresh PBS-0.3%-Triton X-100-2%-BSA (3 times).

6. Incubate O/N at 4°C with primary antibody in PBS-Triton X-100-2%-BSA on the rotating wheel. Use 500µl Eppendorfs.

7. Wash the tissues shortly with fresh PBS-0.3%-Triton X-100 (3 times).

8. Wash 10 min with fresh PBS-0.3%-Triton X-100-2%BSA (3 times).

9. Incubate 2h at room temperature with secondary antibody (1:400 in PBS-0.3%-Triton-2%BSA) wrapped in aluminium foil.

10. Wash shortly with fresh PBS-0.3%-Triton X-100 (3 times)

11. Incubate with DAPI (600 µl PBS-0.3%Triton X-100 + 6 µl DAPI 2ng/µl) 25 min at room temperature.

12. Wash 5 min with fresh PBS-0.3%-Triton X-100 (3 times).

13. Wash 10 min with PBS without Triton X-100.

14. Mount testis in Mowiol onto a glass slide and cover with a cover slip.

15. Let harden at 4°C for at least one hour before going to the microscope.

2.2.17. Microscopy & Image analysis

The preparations were looked at under a Leica SPE confocal microscope, and the images were processed and analysed with the open source imaging software ImageJ. It is a Java-based program for displaying, editing, processing and analysing, and was specifically designed for scientific use from the National Institutes of Health in Maryland, USA (Collins, 2007).

3. OBJECTIVES

1. To study the molecular mechanism of dBigH1 action.

2. To study the factors that regulate dBigH1 deposition.

4. RESULTS

4.1. Chromatin reconstitution in DREX

Early *Drosophila* embryos replicate their genomes once every nine minutes and the subsequent assembly of the replicated DNA into chromatin depends fully on factors deposited by the mother (Foe et al., 1983). This fact, and being inspired by the already existing in-vitro chromatin assembly system using *Xenopus* egg extract (Shimamura et al., 1988), drove scientists to develop a similar system in *Drosophila* (Becker & Wu, 1992).

The in-vitro reconstitution system using preblastodermic *Drosophila* extract (DREX) represents a very powerful system for the study of the assembly of cloned DNA into chromatin. It contains all the necessary core histones and their carriers as part of the maternal contribution. However, it does not contain somatic histone H1. (Becker & Wu, 1992).

As explained in the materials and methods section, chromatin can be reconstituted in-vitro and digested by Micrococcal nuclease to get a typical nucleosome ladder pattern (Figure 26).

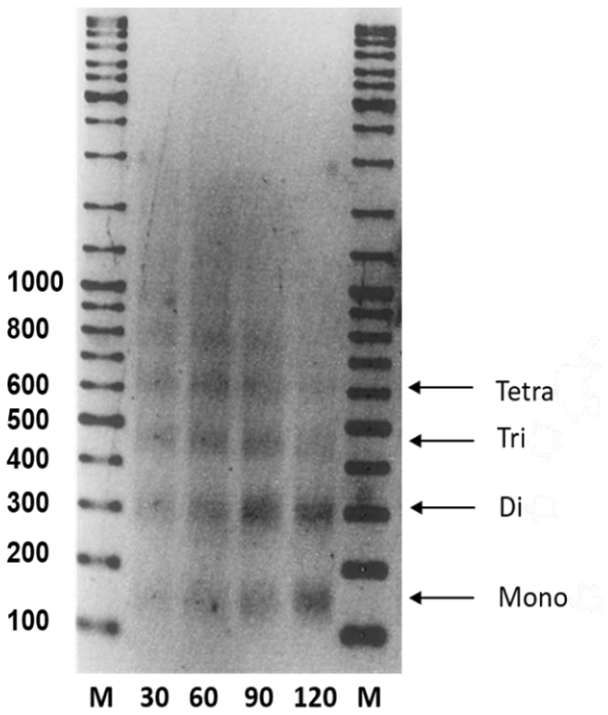


Figure 26. Chromatin reconstitution in *Drosophila* preblastodermic extract

Chromatin nucleosome ladder. The Actin-GFP plasmid was incubated as described in the methods in DREX during four hours at 26°C. The reconstituted chromatin was digested with Micrococcal nuclease during 30, 60, 90 and 120 seconds and loaded on an 1.5% agarose gel. The mono-, di-, tri- and tetra-nucleosomes are indicated with arrows. The molecular weight marker (M) in steps of 100bp.

4.1.1. Analysis of the incorporation of dBigH1 into assembled minichromosomes

DREX is naturally lacking somatic histone H1, although it can be present in very little amounts. However, it contains the embryonic H1 variant dBigH1 (Figure 27).

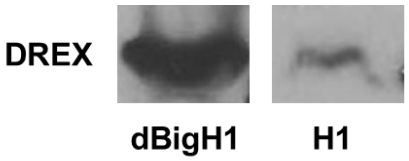


Figure 27. The presence of dBigH1 and H1 in DREX

WB analysis of DREX against dBigH1 and H1 show that dBigH1 is easily detectable whereas H1 seems to be present only in very little amounts.

Therefore, we asked whether the endogenous dBigH1 that is present in the DREX gets incorporated into the reconstituted chromatin. To test this, we performed four standard chromatin reconstitutions experiments and mixed them together after the 4h of incubation at 26°C. This was necessary as previous attempts with only one or two reconstitutions failed to give visible bands when performing a WB. The reconstituted chromatin was then separated from the reaction by precipitation with 15 mM $MgCl_2$ (Schwarz et al., 1994). To ensure that dBigH1 was not precipitated by $MgCl_2$, we performed as a control experiment the same four reconstitutions but without adding the DNA in the beginning. Instead the DNA was added after the 4h of incubation, in order not to give it any time to reconstitute into chromatin. Precipitation was equally done using 15 mM $MgCl_2$. After precipitation, the samples were analysed by agarose gel and WB. Figure 28 shows a scheme of the experimental setup.

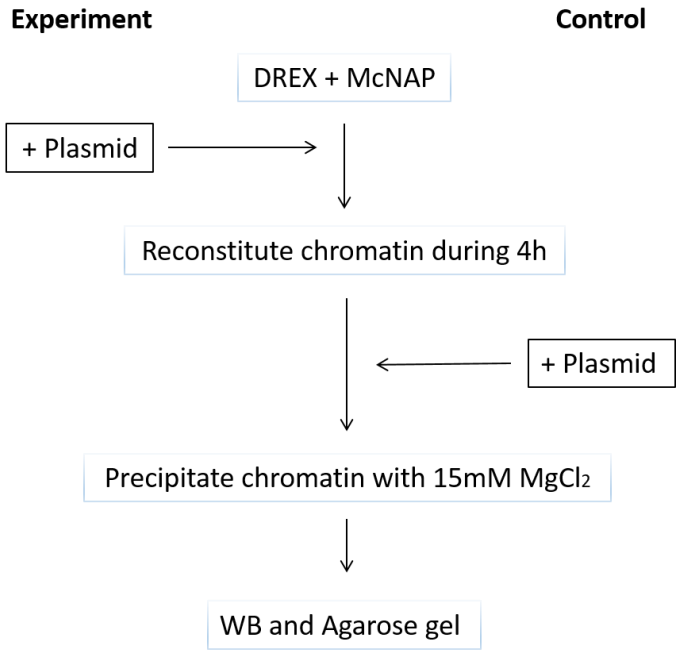


Figure 28. Experimental setup to check the incorporation of dBigH1

To check whether the endogenous dBigH1 gets incorporated into the assembled chromatin we performed four reconstitutions and precipitated the chromatin with 15mM $MgCl_2$. To ensure that dBigH1 was not precipitated directly by $MgCl_2$ we included a control where we added the plasmid just before the precipitation and therefore not giving it time to assemble into chromatin. The McNAP buffer contains the creatine phosphate and the creatine kinase, which are needed as an energy producing system for ATP-dependent chromatin remodelers.

In the agarose gel (Figure 29, A) only the reconstituted chromatin is precipitated by $MgCl_2$ and not the naked plasmid. WB analysis shows that dBigH1 is getting incorporated into the reconstituted minichromosomes as it is present in both the pellet (P) and the supernatant (SN) fraction, whereas in the control experiment dBigH1 can only be detected in the SN fraction (Figure 29, B).

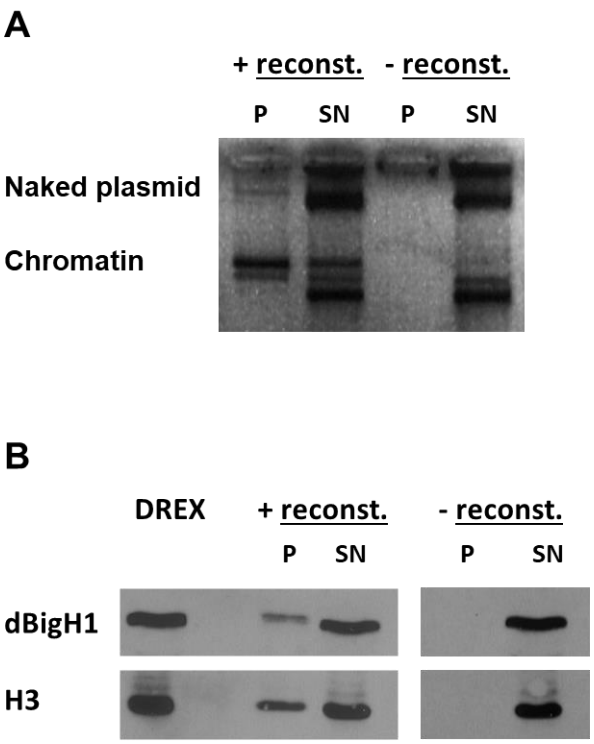


Figure 29. dBigH1 gets incorporated into reconstituted chromatin

(A) Four separate chromatin reconstitutions were performed in parallel and mixed together after incubation. After precipitation with 15 mM $MgCl_2$ and separating the pellet (P) and the supernatant (SN), the samples were loaded onto an agarose gel. In the left (+) is where the DNA was added as usual at the beginning. A clear band, representing the reconstituted chromatin can be seen in the pellet fraction. In the right is the control experiment (-) where the DNA was added just before precipitation and therefore nothing was precipitated, only the naked plasmid is visible in the supernatant fraction. **(B)** Western blot analysis of the precipitated samples. In the reconstituted sample (+) dBigH1 is present both in the pellet and in the supernatant fractions. In the control sample (-) dBigH1 is only present in the supernatant fraction. Histone H3 was used as loading control and total DREX was used for comparison.

It was shown that several somatic H1 variants in mammals (H1.1-H1.5, H1.0 and H1x) can be incorporated into the assembled minichromosomes using DREX when being added as a recombinant protein (Becker et al., 1992; Clausell et al., 2009). Thus, we addressed the question whether recombinant dBigH1 can be incorporated into chromatin when added to the chromatin assembly reaction. We also tested if the dBigH1-Δ-Nt construct, which misses the entire N-terminal domain, would also be capable of incorporating into chromatin.

We performed two chromatin assemblies, one with adding recombinant dBigH1 and one with adding the truncated dBigH1-Δ-Nt construct. After assembly, we precipitated the chromatin with 15 mM $MgCl_2$. Indeed, we could detect the recombinant dBigH1 and dBigH1-Δ-Nt in the reconstituted chromatin (Figure 30, first two lanes).

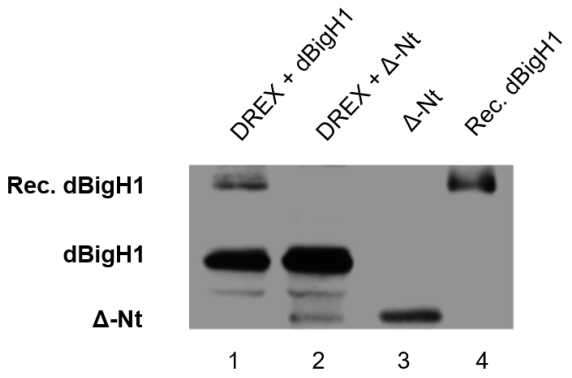


Figure 30. Recombinant dBigH1 and dBigH1-Δ-Nt get incorporated into reconstituted chromatin

Two chromatin assemblies were performed after 20µ of recombinant dBigH1 or dBigH1-Δ-Nt were added to DREX. After incubation, the corresponding tubes were mixed together. Chromatin was precipitated with 15 mM $MgCl_2$ and the samples were analysed by WB (lanes 1 and 2). Due to its additional His-Tag, the recombinant dBigH1 protein is detected above the endogenous one, at about 65 kD. The dBigH1-Δ-Nt runs at about 41 kD. The corresponding recombinant proteins were used as control (lanes 3 and 4).

4.1.2. Chromatin assembly with dBigH1 depleted DREX

Next, we depleted dBigH1 from DREX using whole αdBigH1 antiserum. IgG's were coupled to protein A magnetic beads and then incubated with DREX for five rounds of 45 min each at 4°C. After the incubation, depletion of dBigH1 was determined by WB. The mock sample was treated the same, except that it was incubated with non-specific rabbit antiserum (Figure 31).

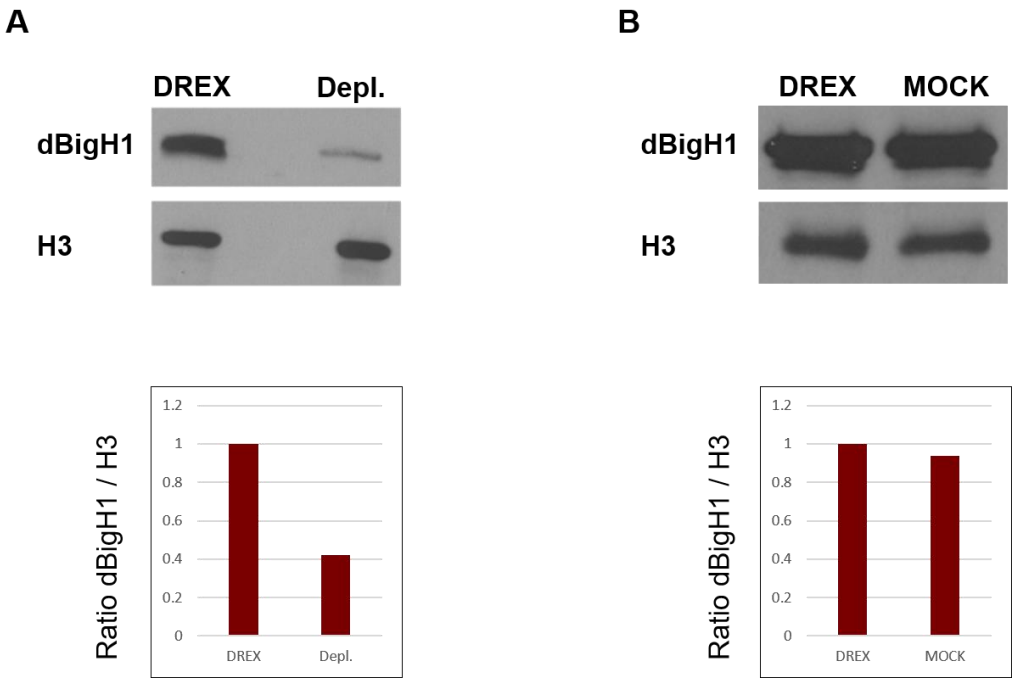


Figure 31. Depletion of dBigH1 from DREX

dBigH1 was depleted from DREX using protein A magnetic beads coupled to IgG serum of a dBigH1 inoculated rabbit. Histone H3 was used as the control. (A) The quantification of the western blot shows a significant decrease of dBigH1 protein levels after depletion. (B) The MOCK treated sample shows no significant change in dBigH1 levels.

Next, we tested if dBigH1-depleted DREX was able to reconstitute chromatin. We performed a standard chromatin assembly reaction and determined that the dBigH1-depleted DREX was functional to reconstitute chromatin, as can be seen after Micrococcal nuclease digestion of the assembled chromatin (Figure 32, A). However, the nucleosome repeat length (NRL) of the chromatin reconstituted with dBigH1-depleted DREX was slightly shorter by about 10bp than NRL of chromatin reconstituted in complete DREX (Figure 32, B). In case of the normally assembled chromatin the NRL was about 166bp, whereas the dBigH1 depleted sample had an NRL of about 156bp. It has been shown that reducing H1 content results in reduced NRL and a more open chromatin state (Rodriguez-Campos et al., 1989, Sandaltzopoulos et al., 1994, Fan et al., 2005)

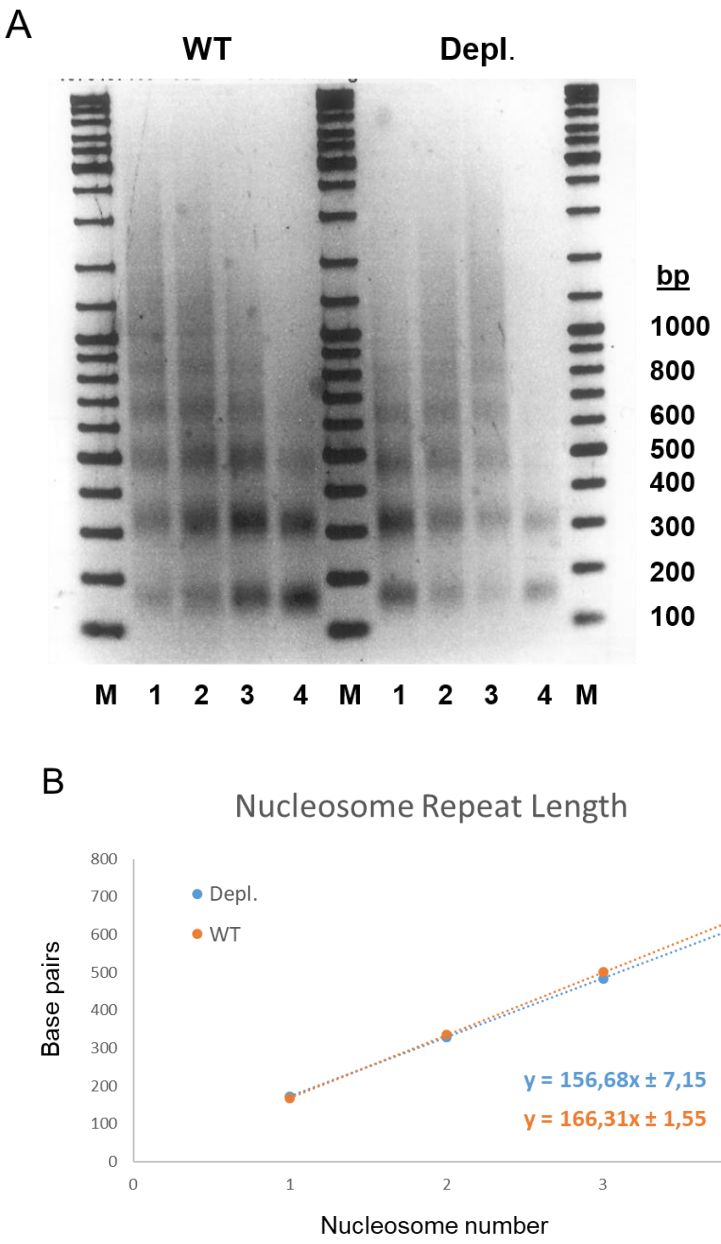


Figure 32. Chromatin reconstitution in dBigH1-depleted DREX

(A) Chromatin was reconstituted using the dBigH1-depleted DREX along with complete DREX and subjected to Micrococcal nuclease digestion for 30 (lanes 1), 60 (lanes 2), 90 (lanes 3) and 120 (lanes 4) seconds. Lanes M correspond to the marker in base pairs (bp) as indicated. (B) To determine the average NRL of chromatin reconstituted in dBigH1-depleted DREX (light blue) and complete DREX (orange), the sizes in bp of mono-, di-, tri- and tetra nucleosomes were plotted as a function of the nucleosome number. The slopes of the graphs correspond to the average NRL.

4.2. Analysis of the effect of dBigH1 on transcription

To analyse the effect of dBigH1 on transcription, a DNA template that carries a GFP gene under the control of a *Drosophila* Actin5C promoter (see 2.2.6) was used for chromatin assembly using complete DREX and dBigH1-depleted DREX. After reconstitution, chromatin templates were precipitated with 15mM $MgCl_2$ and subjected to transcription in HeLa nuclear extract as described in Materials & Methods (2.2.12.). The resulting RNA was reverse transcribed into cDNA that was then used in quantitative real-time PCR with specific primers for GFP (2.2.15.). To compare the results of the different conditions, we determined the concentration of the template, which is the reconstituted chromatin, by qPCR. The amount of in vitro transcription product (GFP mRNA) determined by RT-qPCR was then expressed as a function of the amount of template subjected to transcription. This allowed us for an accurate relative comparison of transcription between the samples.

We observed an increase in transcription of chromatin reconstituted in the dBigH1-depleted DREX compared to the one assembled in complete DREX, since we detected about 2.5 times more GFP mRNA product. Reconstitution with a mock-treated DREX incubated with non-specific rabbit antiserum showed no significant effect (Figure 33). Furthermore, the increased transcription observed when chromatin was assembled in dBigH1-depleted DREX was significantly reduced upon the addition of recombinant BigH1 to the reaction (Figure 33).

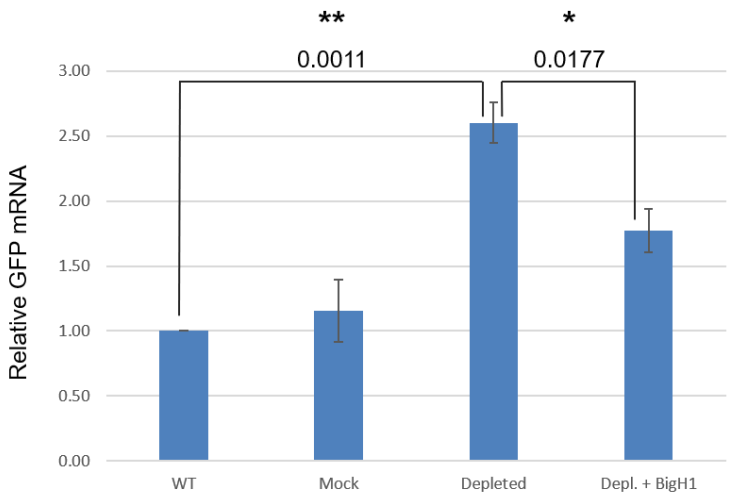


Figure 33. dBigH1 depletion increases transcription in DREX

Chromatin templates were assembled either in complete (WT), mock-treated and dBigH1-depleted DREX, with or without the addition of 10µl of recombinant dBigH1 (Depl. + dBigH1). After reconstitution, chromatin templates were subjected to transcription in HeLa nuclear extract for one hour at 30°C. The amount of GFP mRNA product was determined by RT-qPCR and normalized to the amount of chromatin template determined by qPCR prior to transcription. Results are the average of 2 independent experiments (*, p-value < 0.05; **, p-value < 0.01).

The increase in transcription in dBigH1-depleted DREX compared to the WT, and the significant reduced transcription when adding back recombinant BigH1 to the depleted extract, suggest that dBigH1 is the responsible factor for inhibiting transcription in the early *Drosophila melanogaster* embryo.

4.2.1. Comparison of the effect of dBigH1 and somatic dH1 on transcription inhibition

Next, we compared the inhibition of transcription associated with dBigH1 with that of somatic H1. It had been shown earlier that transcription of chromatin, assembled with purified histones and chromatin remodelers, can be repressed when adding linker histone H1 to the reaction (Laybourn et al., 1991). However, first results of transcription experiments in DREX suggested that addition of

purified H1 was not able to decrease transcription (Becker et al., 1992; Sandaltzopoulos et al., 1994).

To get more clarity in this issue, we performed transcription in dBigH1-depleted DREX and we added increasing amounts of recombinant dBigH1 and dH1. Both histone H1 variants inhibit transcription in a dose-dependent manner (Figure 34). However, dBigH1 inhibits transcription more effectively than dH1, as inferred from the slopes of the corresponding graphs (Figure 34).

4.2.2. Analysis of the contribution of the N-terminal domain of dBigH1 to transcription inhibition

The most notable and most important difference between dBigH1 and somatic dH1 resides in the N-terminal domain. The N-terminal domain of the *Drosophila* somatic histone dH1 is only about 40 amino acids long and is rich in positively charged lysine (K) residues. On the other hand, the N-terminal domain of dBigH1 is much longer, 103 amino acids, and enriched in negatively charged amino acids aspartic (D) and glutamic (E) acid residues (Pérez-Montero et al., 2013). When comparing the sequences between the two histone variants we can also see that clear majority of these negatively charged residues are in the outer 60 amino acids of the N-terminal domain (see Figure 11 in the introduction).

Given the differences between dBigH1 and somatic dH1, we addressed whether the large and acidic N-terminal tail was responsible for the increased inhibitory effect of dBigH1 in comparison to dH1.

For this purpose, we performed dose-dependent transcription experiments similar to those described in Figure 34 but adding increasing amounts of the truncated dBigH1-Δ-Nt and dBigH1-Δ1/2-Nt, which are missing the whole N-terminal domain and the half N-terminal domain, respectively. We could observe that both dBigH1 truncated constructs inhibit transcription in a dose-dependent manner (Figure 35). As can be deducted from the slopes of the corresponding graphs, both constructs show clearly less inhibition than full-length dBigH1.

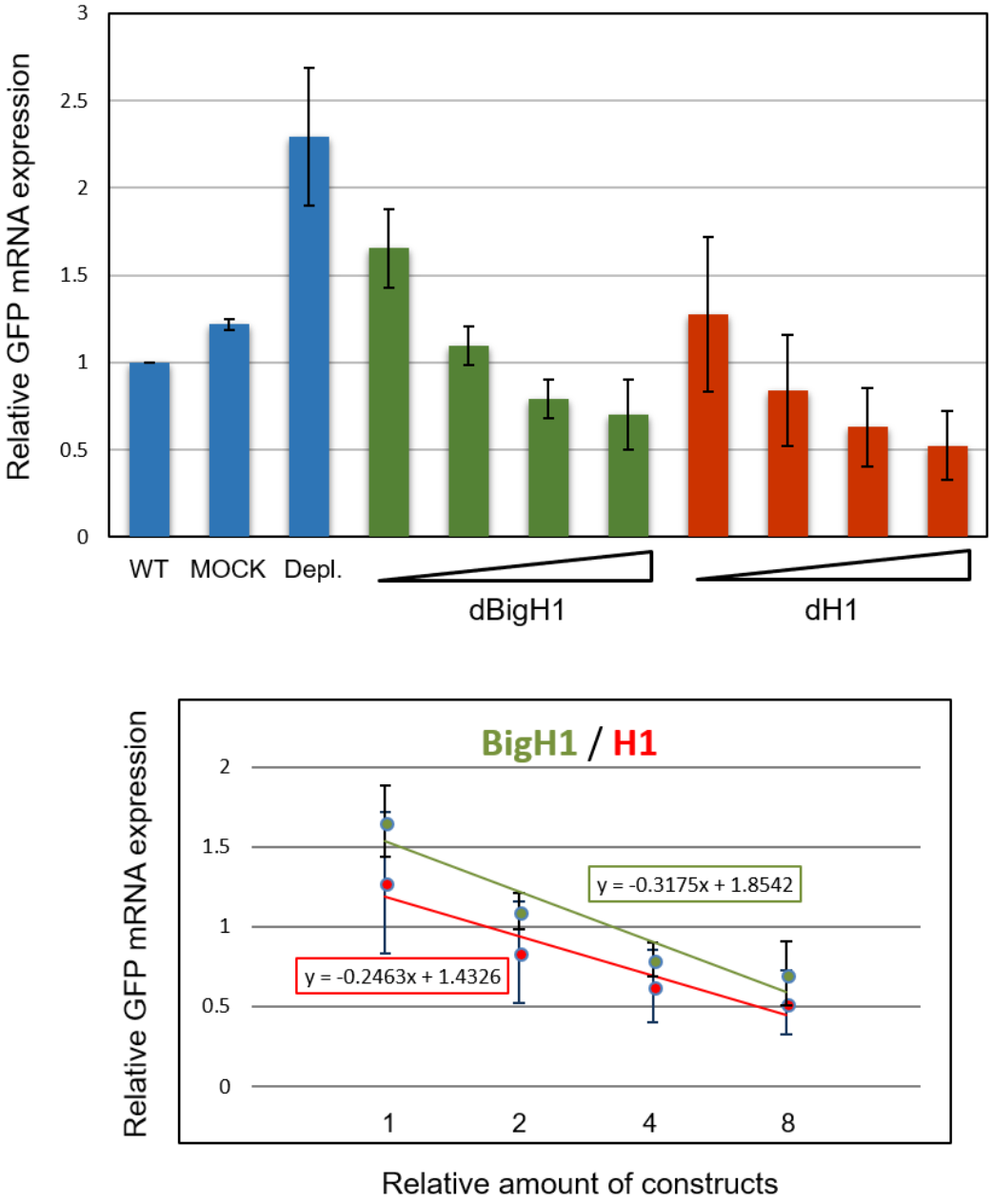


Figure 34. Comparison of the transcription inhibition effect of dBigH1 and dH1

Increasing amounts of recombinant dBigH1 and dH1 were added to a chromatin reconstitution in dBigH1-depleted DREX. Reconstituted chromatin templates were subjected to transcription using HeLa nuclear extract and GFP mRNA levels were determined by RT-qPCR as in Figure 31. The results corresponding to reconstitutions performed in complete (WT) and mock treated DREX are included for comparison. Quantification of the results are shown in the bottom. The r values are: dBigH1 = -0.949, dH1= -0.956. There is a strong negative correlation between the amount of protein added and the inhibitory effect on transcription. Results are the average of 3 independent experiments.

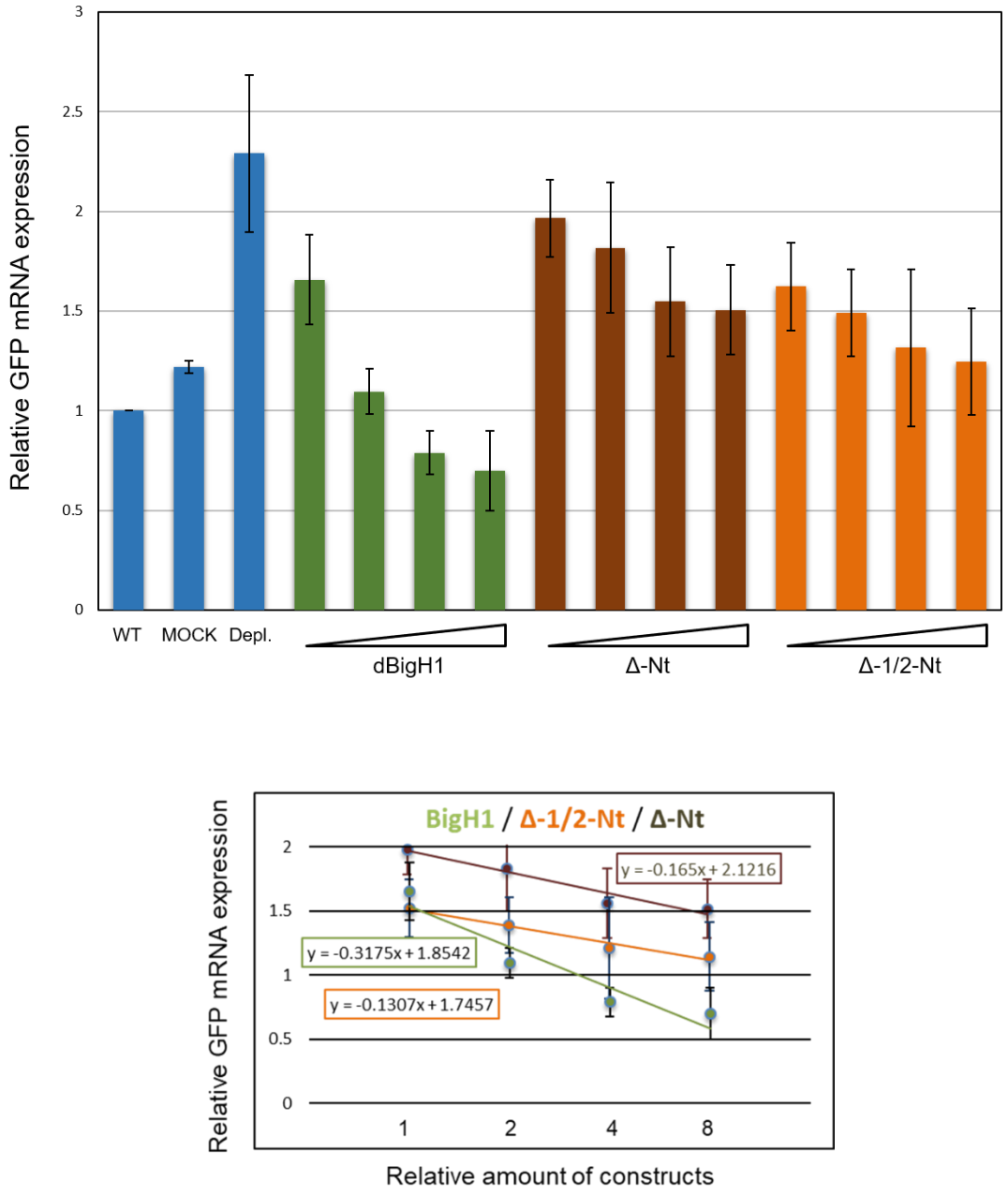


Figure 35. Comparison of the transcription inhibition effect of dBigH1 and truncated dBigH1-Δ-Nt and dBigH1-Δ1/2-Nt forms

Increasing amounts of recombinant dBigH1, Δ-Nt, and Δ1/2-Nt proteins were added to a chromatin reconstitution in dBigH1-depleted DREX. Reconstituted chromatin templates were subjected to transcription using HeLa nuclear extract and GFP mRNA levels were determined by RT-qPCR as in Figure 31. The results corresponding to reconstitutions performed in complete (WT) and mock treated DREX are included for comparison. Quantification of the results are shown in the bottom. The r values are: dBigH1= -0.949, Δ-Nt= -0.969, Δ1/2-Nt= -0.988, suggesting a strong negative correlation between the amount of protein added and the inhibitory effect on transcription. Results are the average of 3 independent experiments.

4.3. Analysis of the contribution of ACF1 and NAP1 to dBigH1 incorporation

As mentioned in the introduction, the histone chaperone NAP1 has diverse roles. It is known to assemble chromosomes *in vitro* (Fujii et al., 1992; Andrews et al, 2008), act as a histone chaperone for the embryonic histone H1 variant H1M/B4 in *Xenopus* (Miller et al., 2015) and being responsible for removal of protamine A of the sperm chromatin in *Drosophila* (Emelyanov et al., 2014).

On the other hand, the chromatin remodeler ACF1 seems to be important in fly development as loss-of-function mutants fail to form heterochromatin that leads to a less repressive state during early embryogenesis and thus to uncontrolled gene activities (Fyodorov et al., 2004). Like dBigH1, expression of ACF1 in *Drosophila* is highest during embryogenesis and stays high throughout the life in the germline (Chioda et al., 2010).

Thus, we decided to check if ACF1 and NAP1 are playing any important role in the behaviour of the linker histone dBigH1. Figure 36 shows that both proteins are present in DREX.

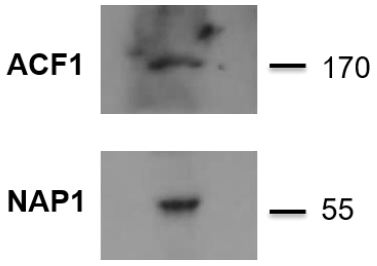


Figure 36. ACF1 and NAP1 are present in DREX

WB analysis of DREX using aACF1 and aNAP1 specific antibodies. The predicted molecular weights are about 175kD for ACF1 and 45kD for NAP1.

Next, we performed chromatin reconstitutions in DREX adding antibodies against ACF1 or NAP1, respectively. By this we tried to neutralize the function of the chromatin remodelers. Interestingly, when neutralizing both ACF1 or NAP1 we observe more dBigH1 incorporation into the assembled chromatin (Figure 37).

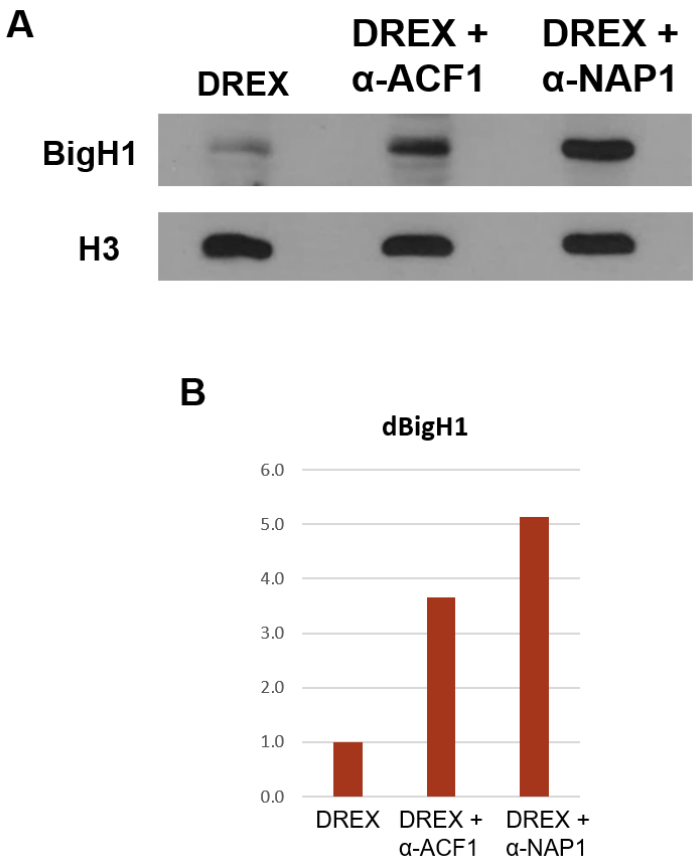


Figure 37. Neutralization of ACF1 or NAP1 leads to more dBigH1 incorporation

Chromatin was reconstituted in DREX after addition of αACF1 or αNAP1 antibodies. (A) WB against dBigH1 and H3 as a control. (B) For the quantification, the bands for dBigH1 were normalized to the corresponding band of H3 using ImageJ. The measured increase was 3.6 times higher when adding antibodies against ACF1 and 5.1 times higher when the histone chaperone NAP1 was neutralized. The results represent a single experiment.

To see if the increased incorporation of dBigH1 influenced chromatin structure, we performed at the same time micrococcal nuclease digestion of the assembled chromatin to check whether we can see differences in the NRL upon NAP1 and

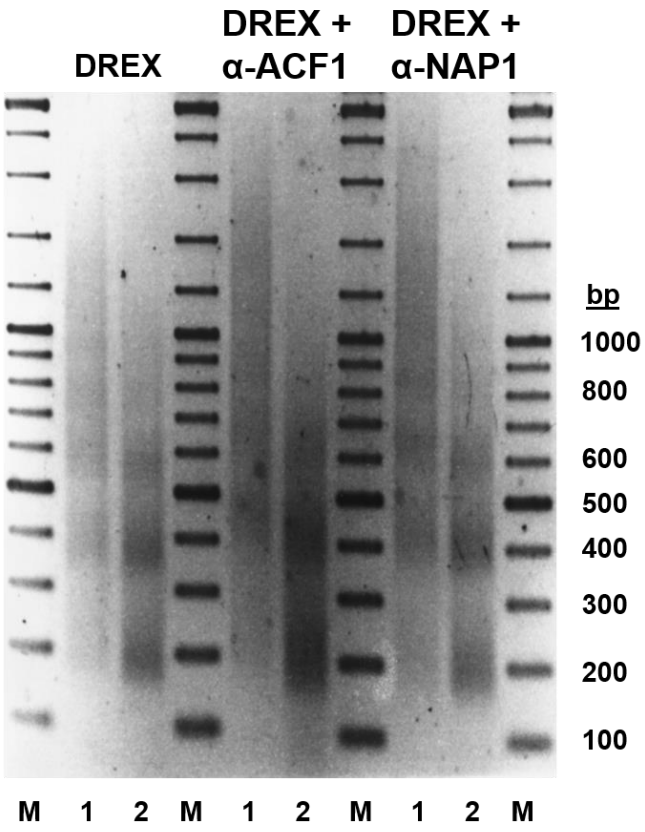
ACF1 neutralization. We observed a more diffuse nucleosome pattern, especially for the ACF1 neutralized sample (Figure 38, A).

To make it more visual, we analysed the picture of the gel using ImageJ (Figure 36, B). The peaks represent the nucleosomes. The wild type condition is shown in red whilst the ACF1 and NAP1 neutralized conditions are shown in green and violet, respectively. Overall, the first impression is that the nucleosome spacing is more diffuse comparing to the wild type conditions. When looking at the mono-, di-, and tri-nucleosomes, one can appreciate the shift of the NAP1-neutralized condition compared to the wild type one (Figure 38, B). Especially for the mono-nucleosome the shift is very clear. The larger distance between the first and the second peak in the NAP1-neutralized sample compared to the wild type condition indicates a larger distance between the nucleosomes.

In the case of ACF1 there is not a clear change in the nucleosome repeat length. The gel is more diffuse, and the pattern looks less regular, accordingly the peaks are less pronounced. However, when looking at the peaks the nucleosome spacing is slightly shifted between the mono- and di-nucleosomes.

It was published that mutation of ACF1 reduces chromatin assembly activity in *Drosophila* embryos, that leads to a shorter NRL and a reduced nucleosomal periodicity. (Fyodorov et al., 2004). This affirms our observation of a more diffuse pattern when neutralizing ACF1.

A


DREX
DREX + α-ACF1
DREX + α-NAP1
bp
1000
800
600
500
400
300
200
100
M 1 2 M 1 2 M 1 2 M

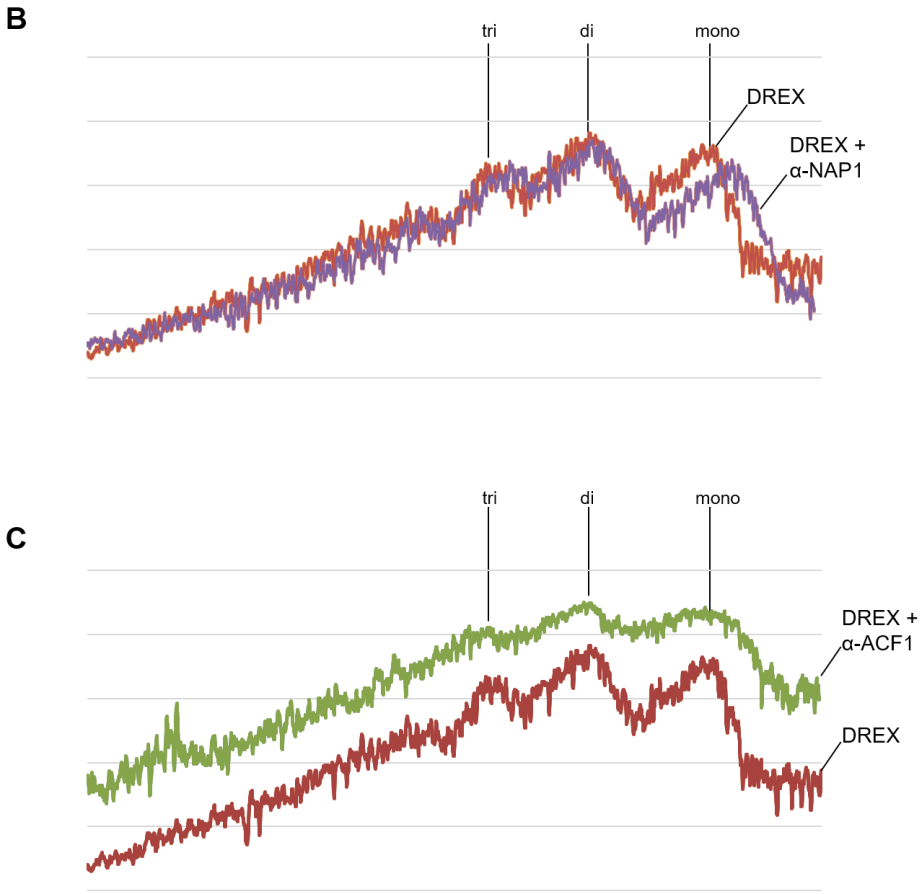


Figure 38. Chromatin assembly under ACF1 and NAP1 neutralizing conditions

(A) Chromatin was assembled under ACF1 and NAP1 neutralizing conditions by adding specific antibodies against each protein. The chromatin was digested by micrococcal nuclease during 60 (lanes 1) and 90 (lanes 2) seconds, along with a control (DREX). The digested samples were run on an 1.5% agarose gel. A slight shift in the nucleosome spacing can be noted. The picture of the gel was further analysed by ImageJ. (B) In the case of NAP1 neutralization there is a shift of the peak representing the mono-nucleosome (mono) compared to the wild type. This increase in distance between the first and the second peak represents a larger distance between the nucleosomes in the chromatin. (C) In ACF1 neutralizing conditions the nucleosome spacing seems to be more diffuse and less regular compared to the control.

4.4. Analysis of the role of ACF1 in the regulation of dBigH1 deposition

As we showed above, ACF1 might play a role in the regulation of dBigH1 deposition into chromatin. To test this hypothesis, we decided to perform experiments *in vivo*, using the *Drosophila* testis, where both, ACF1 and dBigH1, are expressed.

As shown in Figure 39, in the *Drosophila* testis, ACF1 is present in germal stem cells (GSCs) and proliferating spermatogonia. Towards the end of the spermatogonia stage its expression goes down and abolishes in the spermatocytes stage. On the other hand, dBigH1 is present in the GSCs and the spermatocytes, but is not expressed during the spermatogonia stage.

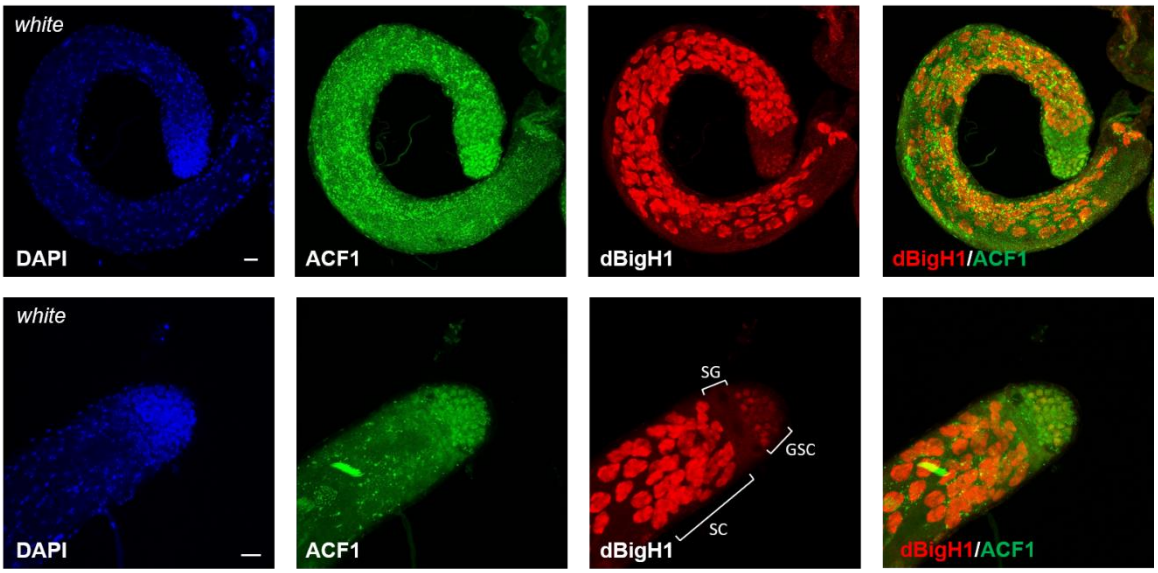


Figure 39. Expression pattern of ACF1 and dBigH1 in *Drosophila* testis

Immunostaining of *Drosophila* testis. ACF1 is present in the GSCs and the proliferating spermatogonia (SG) stage (green). dBigH1 is present in the GSCs and the spermatocytes (SC), but not in the spermatogonia (red). In the second row a magnification of the testis tip is shown. Scale bars correspond to 25 µm.

Next, we tested whether ACF1 influences the pattern of dBigH1 expression in testis. For this purpose, we performed immunostaining experiments with two ACF1 mutant lines, *Acf1²* and *Acf1⁷*, generated by imprecise excision. *Acf1²* contains an 871bp deletion spanning the promoter and 5' transcribed region (Fyodorov et al., 2004), while *Acf1⁷* carries a 3098 bp deletion spanning both intron- and exon regions (Figure 40) (Börner et al., 2016).

Immunostainings of testis from *Acf1²* and *Acf1⁷* flies showed that the expression of dBigH1 is diminished to a significant extent in GSCs (Figure 41), suggesting that ACF1 is required for dBigH1 incorporation into chromatin in the GSCs. On the other hand, we detected normal dBigH1 levels in spermatocytes (Figure 41), where ACF1 is not expressed and, thus, cannot contribute to dBigH1 deposition.

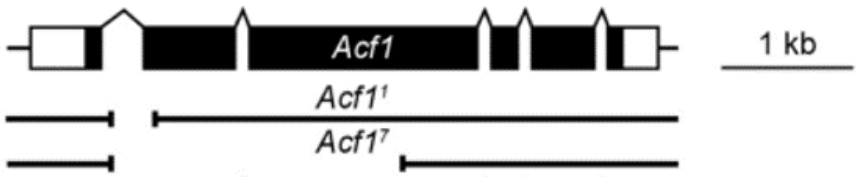


Figure 40. Schematic representation of the *Acf1⁷* constructs

The *Acf1⁷* construct carries a 3098 bp deletion that covers the first intron and part of the third exon. White and black boxes show translated and untranslated exons, respectively. (Börner et al., 2016).

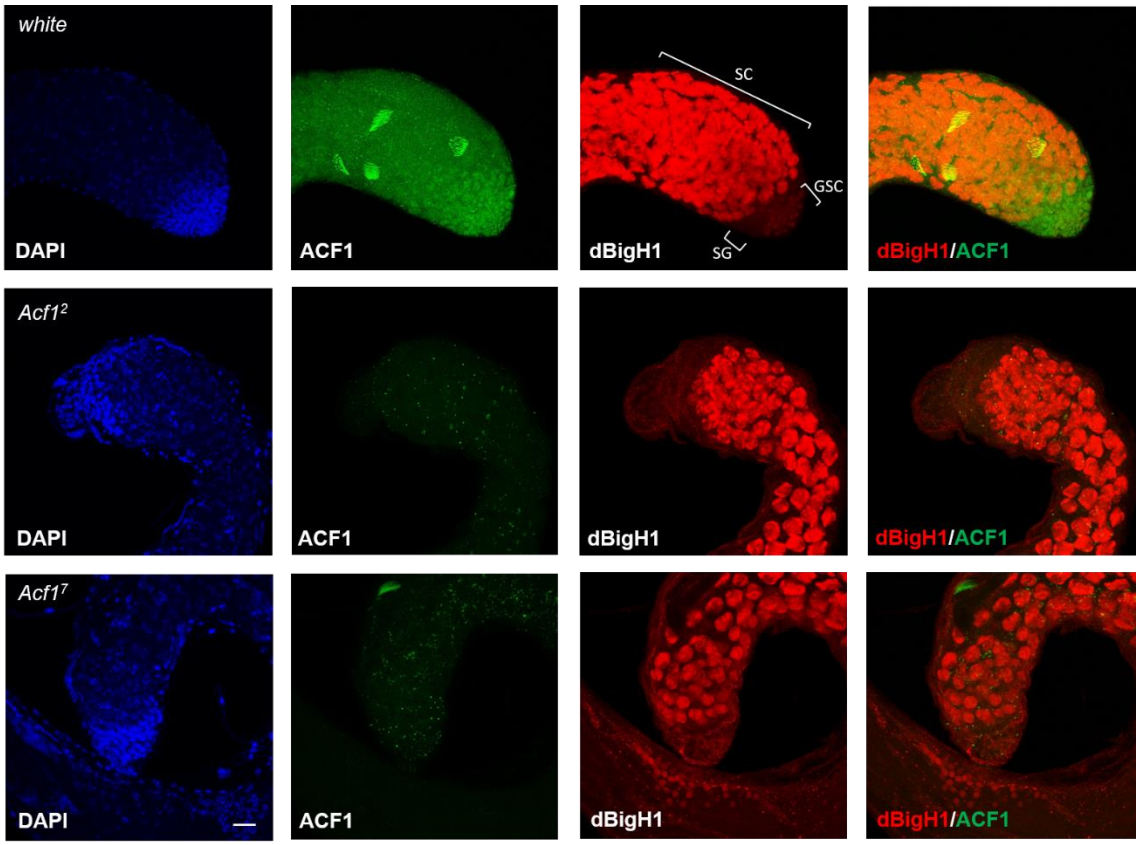


Figure 41. ACF1 mutant testis show an impaired expression of dBigH1 in the GSCs

Immunostainings of testis using the homozygous *Acf1²* and *Acf1⁷* mutant fly lines. dBigH1 can be observed in the spermatocytes region but not in the germal stem cells at the testis tip. Scale bar corresponds to 25 μm.

To further confirm our results, we used an RNAi fly line against ACF1 (stock number 35575) that was crossed to a *nanos*-GAL4 driver line to deplete ACF1 in GSCs and spermatogonia, where *nanos* is specifically active (Asaoka-Taguchi et al., 1999; Gilboa et al., 2004). As a control, we crossed the *nanos*-GAL4 line to a UAS-GFP-RNAi line, to discard the possibility of any effect of the driver line on the phenotype. As with the *Acf1²* and *Acf1⁷* mutant fly lines, we observed that depletion of ACF1 reduced dBigH1 levels in GSCs (Figure 42).

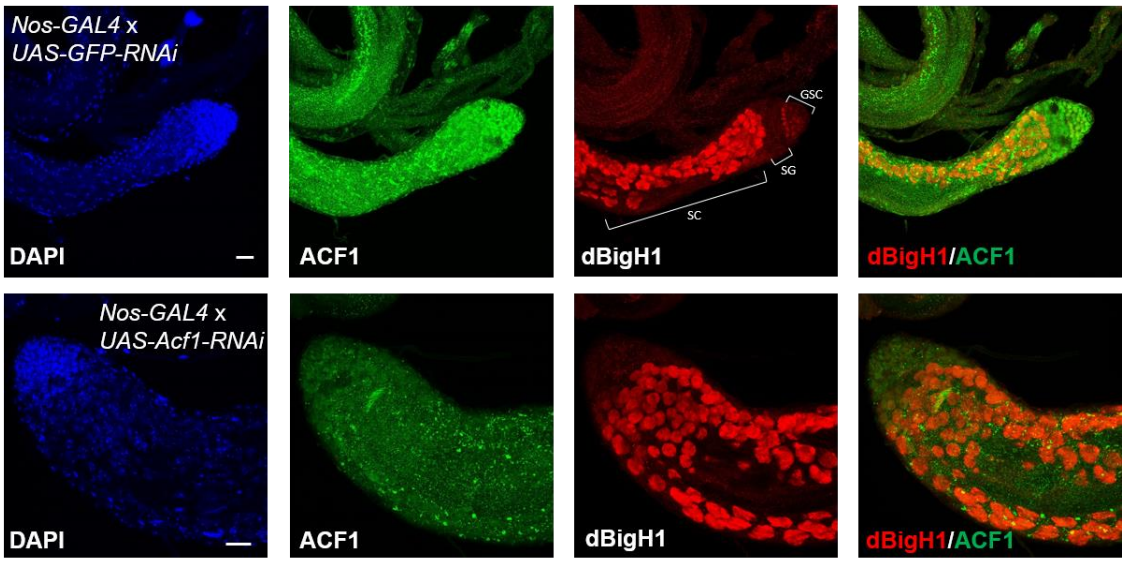


Figure 42. RNAi against ACF1 diminishes dBigH1 expression in the GSCs

A UAS-ACF1-RNAi line was crossed to a Nanos-GAL4 driver line. Nanos is expressed in germal stem cells and spermatogonia, like ACF1. dBigH1 seems to be suppressed in the germal stem cells (lower lane). As a control, a UAS-GFP-RNAi line was crossed to the same driver line (upper panel), and dBigH1 and ACF1 expression are normal. Scale bars correspond to 25 µm.

Next, we tested whether ACF1 overexpression had also an effect on the pattern of dBigH1 expression. For this purpose, we used an *Acf1*-fosmid line, which carries an additional copy of the ACF1 gene fused to a C-terminal GFP (Figure 43) (Börner et al., 2016). This construct is introduced into the fly in a fosmid vector (pFlyFos) that is used for direct transgenesis and mimics an endogenous gene expression of the inserted protein (Ejsmont et al., 2009). Thus, the *Acf1-fos* line has four instead of two copies of the ACF1 gene that are all expressed under the endogenous promoter.

Figure 43. Schematic representation of the *Acf1-fos* construct

The Acf1-fosmid construct carries a C-terminal GFP fused to the normal ACF1 gene. Flies carrying this construct will thus contain four copies of the ACF1 gene (Börner et al., 2016).

As shown in Figure 44, the *Acf1*-fos line showed increased ACF1 expression in comparison to control wild type flies and, concomitantly, there is also an increased dBigH1 expression. We also observed that the regions corresponding to GSCs/GBs is extended, suggesting an increased number of GSCs and GBs.

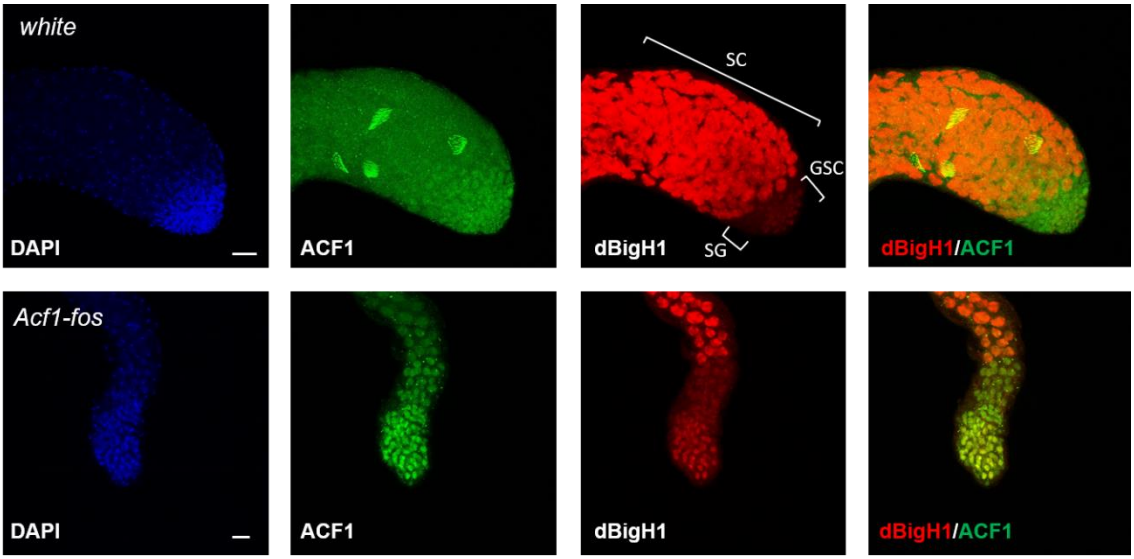


Figure 44. Immunostaining of the *Acf1-fos* line

The *Acf1-fos* flies show a clear extension of ACF1 into the testis. dBigH1 is expressed normally in the tip. The spermatogonia region seems to be highly extended as it is populated by ACF1 but not dBigH1. The DAPI staining also supports the hypothesis that those are proliferating cells. Scale bars correspond to 25 µm.

To confirm the nature of those cells in the extended regions, we decided to cross the *Acf1-fosmid* line with a *Drosophila* strain that carries an *Escargot*-lacZ construct, which marks GSCs and GBs cells. Escargot (esg) is a transcription factor expressed in the GSCs and GBs cells that maintains stem cell behaviour and suppresses differentiation (Korzelis et al., 2014). By crossing the *Acf1-fos* line with the *Escargot-lacZ* line we should be able to differentiate GSCs and GBs cells from spermatogonia and spermatocytes using antibodies against β-galactosidase. Due to recombination *Escargot-lacZ*; *ACF1-fosmid* flies carry only one additional copy of ACF1 instead of two, which could result in a weaker phenotypic outcome.

However, despite of carrying only one additional copy of ACF1 we can see that ACF1 is still overexpressed compared to control flies (Figure 45).

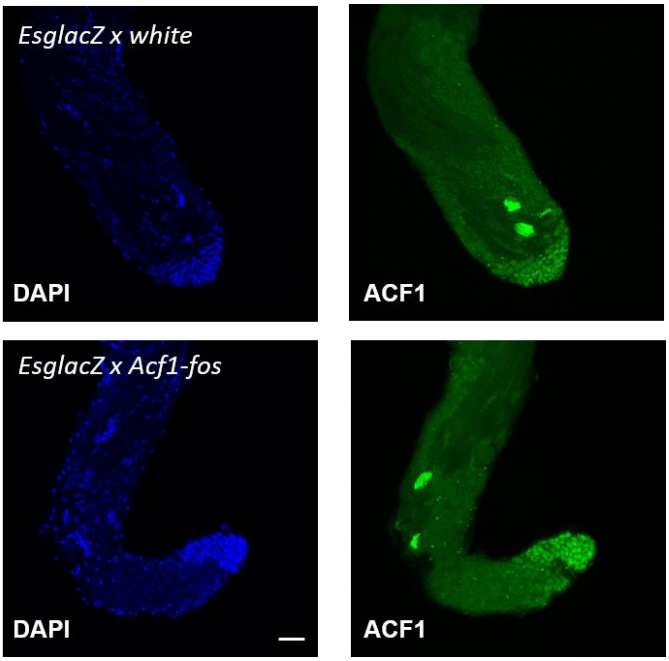


Figure 45. ACF1 overexpression in heterozygous Escargot-lacZ / ACF1-fosmid testes

Due to recombination, the *Escargot-lacZ* / *ACF1-fosmid* flies carry only one additional copy of ACF1 instead of two, which could result in a weaker phenotypic outcome. However, the DAPI and the ACF1 staining are stronger and more elongated (lower panel) than in the control (upper panel). Scale bar corresponds to 25 µm.

The β-galactosidase staining for escargot-positive cells in the same cross suggests more GSCs and GBs in the *Acf1-fos* flies (Figure 46). Overexpression of ACF1 seems to have a positive effect on the abundance of GSCs and GBs.

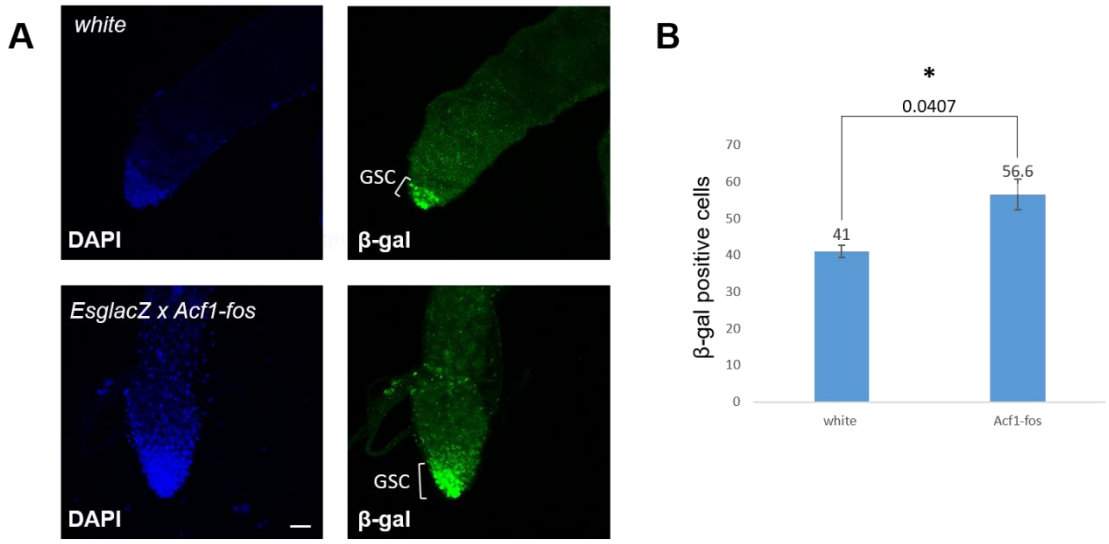


Figure 46. ACF1 overexpression leads to more GSCs

(A) The DAPI staining seems more elongated in the Escargot-β-gal / *Acf1-fos* crossed flies compared to the control, pointing to an increase of proliferating cells. The β-gal signal, that marks escargot, is stronger and more extended. This supports the idea that these cells are indeed GSCs and GBs. Scale bar corresponds to 25 μm. (B) The Acf1-fos flies show significantly more proliferating cells than the control. The cells were counted using different Z projections to ensure no cells were avoided (n=3) (*, p-value < 0.05).

5. Discussion

5.1. dBigH1 represses transcription *in vitro*

Already before developing the *Drosophila* cell-free system for *in vitro* chromatin reconstitution it was known that the early *Drosophila* embryo did not contain any histone H1. After developing the system, the authors showed that recombinant somatic histone H1 could be incorporated when added to the reaction (Becker et al., 1992). Later, using the same system, all seven known somatic H1 variants in mammals were successfully incorporated when added as yeast recombinant proteins (Clausell et al., 2009).

Interestingly, when performing first transcription experiments in DREX, they observed that the chromatin was in an already repressed state, despite the fact of not containing any somatic H1. Adding recombinant somatic H1 did not significantly repress the basal transcription, which was not expected (Becker et al., 1992; Sandaltzopoulos et al., 1994).

These results led to doubts about the widely-accepted certainty that H1 was a dominant repressor for transcription (Shimamura et al., 1989). In fact, it opened questions about an unknown transcriptional repressor possibly present in DREX. Of course, researchers back then didn't know of the existence of the linker histone variant dBigH1.

In 2013, when dBigH1 was discovered, it was also shown that it is the repressing factor *in vivo*. In dBigH1 mutant embryos, overall zygotic gene expression was elevated significantly compared to wild type embryos (Pérez-Montero et al., 2013).

In our experiments, we have shown that dBigH1 gets incorporated into chromatin reconstituted using the *Drosophila* preblastodermic system. This observation let us believe that we had possibly found the repressing factor that was postulated in earlier studies.

Using serum from dBigH1 immunized rabbits we depleted dBigH1 from the extract to a high extent (Figure 31), and we showed that the dBigH1 depleted DREX was still able to reconstitute chromatin. Interestingly, after micrococcal

nuclease digestion separation, we observed a changed nucleosomal pattern between the two conditions. Indeed, the chromatin assembled in dBigH1 depleted DREX showed a shorter nucleosome spacing than the normally assembled one, 156bp compared to 166bp. This observation was in line with the fact that transcriptionally active chromatin shows a shorter repeat length than repressed one (Thomas et al., 1977; Villeponteau et al., 1992). Additionally, it was demonstrated before that when reducing H1 content, chromatin of mouse embryonic stem cells shows a reduced NRL (Fan et al., 2005).

With chromatin reconstituted in dBigH1-depleted DREX, we saw a clear up-regulation of the GFP-reporter plasmid when compared to chromatin assembled in normal DREX (Figure 33). The mock depletion did not show any significant difference in transcription.

If dBigH1 was the repressing factor for in vitro transcription, it should be possible to decrease the elevated transcription of chromatin assembled in dBigH1 depleted DREX by adding back recombinant dBigH1. Indeed, we show that when adding dBigH1 to the depleted DREX at the beginning of the reconstitution, transcription goes down (Figure 33).

Moreover, we show that the decrease of transcription when adding back recombinant dBigH1 happens in a dose-dependent manner. This confirms our hypothesis of dBigH1 being the factor for transcriptional repression *in vitro*.

Additionally, to showing that dBigH1 can repress transcription on *in vitro* reconstituted chromatin, we also demonstrate that somatic H1 does so as well. When adding back recombinant somatic H1 to the depleted DREX, transcription goes down in a dose-dependent manner like for dBigH1, although the effect of repression seems to be slightly weaker in comparison to dBigH1.

Our findings strongly suggest that somatic H1 is a potent inhibitor of transcription as it was postulated before (Shimamura et al., 1989; Laybourn et al., 1992; Vujatovic et al., 2012), At the same time, we provide an answer to the discussion about a possible repressor of transcription in DREX as was suggested earlier (Sandaltzopoulos et al., 1994).

Taken together, our results demonstrate that dBigH1 is the repressive factor in transcription for in vitro reconstituted chromatin. Using the approach of depleting dBigH1 from DREX is a useful tool for future experiments. Effects on transcription of different H1 variants and their competitivity between them, as well as the impact on chromatin structure, could be addressed with this system.

5.2. The effect of the dBigH1 N-terminal region on transcription

Metazoans generally have early embryonic and germline specific linker histone variants. Examples are the Cs-H1 in the sea urchin (Brandt et al., 1997), B4/H1M in *Xenopus* (Smith et al., 1988), H1oo in mammals (Tanaka et al., 2001), H1.1/HIS-24 in *C. Elegans* (Vanfleteren et al., 1988) and dBigH1 in *Drosophila* (Pérez-Montero et al., 2013).

All the germline specific variants show the typical H1 tripartite structure with a conserved globular domain that contains a winged-helix domain and two less conserved unstructured tails. Apart for the H1.1/HIS-24 of *C. elegans*, which resembles most to the somatic H1, all variants contain a larger C-terminal region than the somatic H1. Also, the C-terminal tail of the variants carries significantly more acidic residues compared to the canonical histone H1 (reviewed in Carbonell et al., 2015).

The N-terminal tail of dBigH1 is considerably longer than the one of other variants and shows only an 8.4% similarity to the somatic H1 N-terminal region. It is rich in aspartic and glutamic amino acids (37.9% of total residues) and is therefore very acidic and negatively charged (Pérez-Montero et al., 2013). Although dBigH1 is the only early embryonic and germline variant known that contains such a prolonged and acidic N-terminal tail, we considered it could be the responsible region for the increased transcriptional repression associated with dBigH1.

Indeed, our transcription experiments suggest that the N-terminal tail plays an important role in transcriptional repression by dBigH1.

The dBigH1-Δ-Nt construct lacks the whole N-terminal tail and, although is still able to repress transcription, it does so to a much lesser extent than full length dBigH1 or somatic dH1. The fact that the dBigH1-Δ1/2-Nt construct, which misses only the acidic domain, represses transcription to a similar extent than the dBigH1-Δ-Nt, suggests that the acidic residues are indeed responsible for the increased transcriptional repression associated with dBigH1. As mentioned

before, the majority of acidic residues are located on the outer part of the N-terminal tail and are missing in the Δ1/2-Nt construct. To be precise, from the 39 acidic residues of the full-length protein, only seven remain in the Δ1/2-Nt construct and none in the dBigH1-Δ-Nt.

Germline specific H1 variants show lower chromatin binding affinities than the somatic histone H1. *Xenopus* B4/H1M was shown to have a low chromatin binding affinity compared to the somatic one, and this is most probably due to its much lower content of positive charges in the C-terminal tail (Ura et al., 1996; Nightingale et al., 1996). It was suggested by FRAP experiments that human H1oo is more mobile than somatic H1, probably also due to a lower affinity to DNA (Godde 2009; Meshorer et al. 2006; Teranishi et al. 2004). Taken together, this points to a more dynamic behavior of early embryo specific H1 variants compared to somatic H1 in general. Since almost all germline variants have less positively charged C-terminal tails, and the C-terminal domain is the most important part for DNA binding, this is probably true for all of them. Although we have no data for dBigH1, a similar behavior is probable.

The dBigH1-Δ1/2-Nt construct has the highest sequence similarity to somatic H1 compared to the other constructs. However, when comparing to somatic H1 it shows a lower degree of transcriptional repression. This supports the hypothesis that the acidic residues located in the distant part of dBigH1 play a crucial role in the repression of transcription, and that due to the lack of them repression is weaker. dBigH1 shows the highest degree of transcriptional repression, compared to the truncated constructs and to somatic dH1. Another factor could be a lower affinity to DNA due to its less positively charged C-terminal domain. Consistently, its average residence time on the nucleosome is likely to be shorter compared to somatic H1.

Summarized, our results imply that the N-terminal domain is the responsible region for increased transcriptional repression by dBigH1. Moreover, the dBigH1-Δ1/2-Nt construct shows a similar transcriptional repression to the dBigH1-Δ-Nt, suggesting that the acidic residues located on the outer part of the domain are responsible for the repression in transcription. As mentioned, the possible lower

binding affinity of the truncated constructs could be another reason that the repression in transcription is not higher.

5.3. A possible mechanism for dBigH1 action

One of the first things that stands out when looking at dBigH1 is its highly negatively charged N-terminal region, and it makes sense to assume that this region does not interact with the also negatively charged DNA but is most probably pointing away from it.

Most genes transcribed by RNA polymerase ll contain a TATA-box and an initiator sequence, these two elements define where the initiation complex will be assembled. This complex consists of RNA Pol ll, the TATA-box binding protein (TFllD) and other general initiation factors like TFllA and TFllB. Activator proteins bind to regulatory sequences near the core promoter and enhance the transcriptional rate up to several hundred-fold, they can do so via their activator domain (AD) (reviewed in Brown et al., 2002). The exact mechanism by which activator proteins enhance transcription is not fully understood, but it is thought that the AD can interact with components like the TATA-binding-protein (TBP) subunit of TFllD, TFllB and TBP associated proteins (Stringer et al., 1990; Dynlacht et al., 1991; Lin et al., 1991).
Activator domains vary a lot in their composition and sequence. Acidic activators were identified in yeast, one of the first examples was the activator GAL4 (Ma et al., 1987). Another example is the herpes simplex virus trans activator VP16 (Triezenberg et al., 1988), which is known to interact with TFllB, TFllD and TFllH (Lin et al., 1991; Xiao et al., 1994). The mammalian tumor suppressor protein p53 carries the activator domain on the N-terminal region (Fields et al., 1990), which is known to directly interact with TFllD and TFllH (Liu et al., 1993; Xiao et al., 1994).

Another study suggests that TFllB is the general transcription factor for binding of acidic activator domains. Using affinity chromatography and HeLa nuclear extracts, a specific and direct interaction between TFllB and an acidic activating region was shown, suggesting that acidic activators enhance transcription by recruiting TFllB on to the pre-initiation complex (Lin et al., 1991).

These examples demonstrate that DNA binding proteins can use acidic domains to recruit and bind transcription factors.

We have shown strong evidence that dBigH1 mediates its transcriptional repression through the N-terminal domain. At the same time, we know that this domain is highly enriched in acidic residues and therefore negatively charged. A possible scenario would be one where dBigH1 interacts by its N-terminal tail with TFIIB or another essential transcription factor and sequesters it, thereby preventing its association with RNA Pol II and thus inhibiting the initiation of transcription.

In a study analyzing global RNA polymerase II occupancy in *Drosophila* embryos, it was shown that about 12% of all genes contained stalled RNA Pol II around their transcription start site (Zeitlinger et al., 2007). Those genes were mostly inactive but had RNA Pol II bound and ready: a feature well known from *Drosophila* heat-shock genes, allowing a fast activation of transcription when needed (Lis et al., 1993). They also observed that stalled RNA Pol II enzymes were highly enriched at developmental genes, many of them being poised for activation in a subsequent developmental stage (Zeitlinger et al., 2007).

dBigH1 is found to be uniformly distributed along the genome (Pérez-Montero et al., 2013). However, a scenario where dBigH1 plays a role in retaining those polymerases near the transcription start site, thereby keeping them ready for fast activation, cannot be excluded.

dBigH1 has potential binding possibilities of transcription factors through its N-terminal domain. Transcriptional repression could be achieved either by sequestering single transcription factors and thereby preventing the formation of the pre-initiation complex, or by retaining the already formed complex from elongation, or a combination of both.

5.4. The role of the histone chaperone NAP1 and the chromatin remodeler ACF1 in the regulation of dBigH1 deposition

We showed that when neutralizing ACF1 or NAP1 in DREX, more dBigH1 gets incorporated into the chromatin. At the same time, we observed a more diffuse nucleosome spacing. Especially in the NAP1 neutralized condition, we detect a larger nucleosome repeat length (NRL).

Initially, NAP1 was shown to be a histone chaperone for H2A-H2B dimers in vivo, shuttling the dimers from the cytoplasm to the nucleus (Ishimi et al., 1991; Ito et al., 1996). However, it is also known that, in vitro, NAP1 is also able to bind the $(H3\text{-}H4)_2$ tetramer with almost the same affinity and to promote nucleosome assembly (Fujii et al., 1992; Andrews et al, 2008).

In 2005 it was shown that NAP1 can bind H1 in HeLa chromatin and successfully removes it (Kepert et al., 2005). Similarly, it was reported that ACF can assemble nucleosomes containing histone H1 together with NAP1 when incubated with purified histones (Lusser et al., 2005). Very recently it was reported that NAP1 acts as a histone chaperone for the *Xenopus* early embryo specific histone variant H1M/B4 (Shintomi et al., 2005).

When neutralizing NAP1 in *Xenopus* egg extract there is a decrease of incorporation of H1M/B4 into mitotic chromosomes. Moreover, those chromosomes appear less condensed than normally assembled chromatin (Miller et al., 2015). However, our results obtained in chromatin reconstitution experiments in *Drosophila* preblastodermic extract point to the opposite direction. When neutralizing NAP1 by adding specific antibody we observe an increase of dBigH1 incorporation in the assembled chromatin. Likewise, the nucleosome repeat length appears to increase.

If NAP1 was the chaperone for dBigH1 in *Drosophila*, it could be that when neutralizing it, dBigH1 starts incorporating without control and hence occupies more sites in the chromatin than under normal conditions. This would suggest a

mechanism where NAP1 binds dBigH1 and fine-tunes the incorporation of it into the chromatin.

It is known that transcriptionally repressive chromatin shows a longer NRL than transcriptionally active chromatin (Thomas et al., 1977; Villeponteau et al., 1992). Additionally, it is known that dBigH1 is the responsible factor for the repression of the zygotic genome during early embryo development (Pérez-Montero et al., 2013). These two findings go in line with our observation of more dBigH1 incorporation and the resulting increase in the NRL when depleting NAP1. Taken together, this would support the theory of NAP1 playing a role in transcriptional regulation by removing the linker histone and thus relaxing the chromatin (Kepert et al., 2005).

On the other hand, the chromatin remodeler ACF1 in *Drosophila* has a very similar expression pattern as dBigH1, it is high during embryogenesis and stays expressed in germal stem cells of the adult fly (Chioda et al., 2010). However, ACF1 is also expressed in the neuroblasts while dBigH1 is not. The physiological role of ACF1 in *Drosophila* is still poorly understood. Flies lacking ACF1 assemble nucleosomes less efficiently and therefore show defects in heterochromatin formation. Those effects can be rescued when adding back recombinant ACF1, suggesting a major role in chromatin assembly for ACF1 in *Drosophila* (Fyodorov et al., 2004). In accordance with this observation, flies lacking ACF1 have a shorter S phase, meaning that replication happens faster but is also more prone to errors, most likely due to the lack of heterochromatic regions (Fyodorov et al., 2004). In mice, where the ACF1 homolog BAZ1 is expressed in testis in the adult animal, it was shown that BAZ1 deficiency leads to a massive up-regulation of genes in spermatocytes and spermatids, possibly due to failures in heterochromatin formation (Dowdle et al., 2013). Interestingly, dBigH1 mutant flies show less repressive chromatin and an up-regulation in zygotic genes (Pérez-Montero et al., 2013). This fact, together with the similar expression pattern, could point to a possible interaction between dBigH1 and ACF1.

Neutralizing ACF1, we see more dBigH1 incorporation into the chromatin. However, for the NAP1 neutralized condition the increase of dBigH1

incorporation is higher, and the difference in the nucleosome spacing is more evident. This suggests a possible interaction of ACF1 via NAP1 to regulate dBigH1 deposition. Our results point to a higher NRL when neutralizing NAP1 and a more diffuse, less regular nucleosome spacing when ACF1 is neutralized. Interestingly**,** it was published that loss of ACF1 results in a shorter NRL in bulk chromatin of *Drosophila* embryos as well as in a decrease of the nucleosome array periodicity (Fyodorov et al., 2004). In contrast, we observed a slight increase in the NRL in the ACF1 neutralized condition. Our results do however make sense in the light of the observation that when reducing H1 content, chromatin of mouse embryonic stem cells shows a reduced NRL (Fan et al., 2005). Less ACF1 and more dBigH1 incorporation could therefore lead to an increase in the NRL.

As mentioned, ACF1 was shown to use NAP1 as a histone chaperone during nucleosome assembly with purified components and was also able to incorporate somatic H1 (Lusser et al., 2005). Possibly, when neutralizing ACF1, NAP1 is still able to act upon dBigH1 to a certain level. Therefore, the effect on dBigH1 incorporation and nucleosome spacing is less than when neutralizing NAP1. On the other hand, when neutralizing NAP1, it seems that the direct interactor of dBigH1 is missing, and thus stronger effects on dBigH1 deposition and nucleosome spacing are observed.

Taken together, our results from these experiments imply an involvement of the chromatin remodeler ACF1 and histone chaperone NAP1 in the regulation of deposition of dBigH1 into the chromatin. Putting them into relation with other studies mentioned above, it seems reasonable that ACF1 plays a crucial role in dBigH1 deposition, likely through the help of the histone chaperone NAP1. The fact that dBigH1 gets more incorporated when neutralizing one of the two proteins suggests a fine-tuned mechanism by which ACF1 and NAP1 prevent an excessive incorporation of dBigH1.

5.5. The interplay between dBigH1 and ACF1 in spermatogenesis

We have analyzed the possible interplay between dBigH1 and ACF1 during *Drosophila* spermatogenesis. dBigH1 is present in the GSCs and GBs, is absent is spermatogonia and is strongly expressed in spermatocytes.

This pattern of dBigH1 expression seems interesting when we think about transcription during spermatogenesis. Most of the transcription happens in the early primary spermatocytes and ends in late primary spermatocytes. The untranslated transcripts are stored until when they are needed at later stages of spermatogenesis. In mammals, post-meiotic transcription is known to happen during spermatogenesis until the stage of chromatin compaction (reviewed by Schäfer et al., 1995). Recently it has been shown that there is also post-meiotic transcription going on in elongating spermatids, just before histone-to-protamine chromatin remodeling. Yet, in *Drosophila* this seems to affect only 24 genes (Barreau et al., 2008).

We know that dBigH1 is the repressive factor during early embryogenesis (Pérez-Montero et al., 2013), and from our experiments it seems evident that dBigH1 represses transcription in vitro. Surprisingly, in spermatogenesis dBigH1 is present at a time when transcription is robust in spermatocytes (Olivieri and Olivieri, 1965; Vibranovski et al., 2010; White-Cooper et al., 1998).

As mentioned before, during early embryogenesis when dBigH1 is present, it is distributed rather uniformly along the genome (Pérez-Montero et al., 2013). During early embryogenesis, this pattern makes sense, as the zygotic genome is silenced in early embryos, whereas this is not the case during spermatogenesis. In spermatocytes, dBigH1 is not uniformly distributed along the genome. Rather it seems that dBigH1 accumulates around the transcriptional start site (TSS) of repressed genes and is underrepresented at genes that are important for proper spermatogenesis (Carbonell et al., 2017).

On the other hand, we show that the chromatin remodeler ACF1 colocalizes with dBigH1 in GSCs and GBs. However, contrary to dBigH1, ACF1 stays expressed in spermatogonia and is down-regulated in spermatocytes.

Our first experiments with the ACF1 mutants and RNAi against ACF1, showed an effect on expression of dBigH1 in the GSCs. In both cases, dBigH1 expression was diminished in GSCs but not at the spermatocyte stage. Interestingly, it seems that the loss of ACF1 did not influence the spermatogonia region or its differentiation into spermatocytes. It looks like ACF1 is responsible for proper dBigH1 deposition in GSCs, as its loss diminishes their dBigH1 content. However, loss of ACF1 in the spermatogonia is not sufficient for dBigH1 to be expressed, meaning that other factors are responsible for its absence at that stage.

ACF1 overexpression showed increased dBigH1 staining in the testis tip, where GSCs are resident, which is consistent with the reduced dBigH1 content observed in the absence of ACF1. Not only is the signal stronger but also the region is larger, suggesting increased proliferation of GSCs/GBs, which was confirmed using and escargot-LacZ reporter that labels GSCs and GBs. Altogether these results suggest that ACF1 regulates dBigH1 deposition and proliferation in GSCs/GBs.

As discussed previously, when neutralizing ACF1, we see more dBigH1 incorporation into chromatin in vitro. This observation contradicts the results we obtained in vivo, probably reflecting the involvement of additional factors in the GSCs. On the other hand, ACF1 does not seem to have an influence on the levels of dBigH1 in spermatogonia since dBigH1 is not being expressed at this stage.

6. Conclusions

1. dBigH1 incorporates into chromatin assembled in vitro using DREX.
2. dBigH1 depletion does not impair chromatin assembly in DREX.
3. Chromatin assembled in dBigH1-depleted DREX shows a larger nucleosomal spacing than chromatin assembled in complete DREX.
4. dBigH1 inhibits transcription of in vitro assembled chromatin in a dose-dependent manner.
5. dBigH1 inhibits transcription more effectively than somatic histone H1.
6. Increased transcription inhibition of dBigH1 depends on its acidic N-terminal domain region.
7. Neutralization of ACF1 or NAP1 leads to increased dBigH1 incorporation into in vitro assembled chromatin.
8. ACF1 colocalizes with dBigH1 in male germ stem cells (GSCs) and gonialblast cells (GBs).
9. ACF1 mutant flies show reduced dBigH1 expression in GSCs.
10. ACF1 overexpression leads to an increase of GSCs/GBs in the testis tip.

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