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LINKER HISTONE H1 AND ANDROGEN RECEPTOR: TWO DIFFERENT PLAYERS IN THE CHROMATIN ORCHESTRA

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I get knocked down, but I get up again
You're never gonna keep me down
Chumbawamba
ABSTRACT

The linker histone H1 and the androgen receptor are two different players in the chromatin orchestra. The linker histone H1, one of the most abundant proteins in the nucleus, is located at the surface of the nucleosome but despite many important functions reported for this protein it is not as well studied as the core histones. The androgen receptor, AR, is a member of the nuclear receptor family, a conserved family of transcription factors. AR is of utmost importance for many functions in the human body as well as a driving force behind the most common cancer form in Sweden: prostate cancer.

**Paper I:** Here we focus on the linker histone and the question of whether the heterogeneity of the linker histone family has a functional significance. By reconstituting individual H1 subtypes in *Xenopus* oocytes, a model system that lacks somatic linker histone, we have systematically studied their specific binding to chromatin and their effect on the chromatin structure as seen by increase in nucleosomal repeat length, NRL. We have compared linker histones that differ both in terms of origin and expression pattern as well as the ubiquitously expressed human somatic subtypes. We show that the biggest differences in terms of effect on chromatin structure are found between the coexisting human subtypes thus suggesting that H1 subtypes have different roles in the organization and function of the chromatin fiber.

**Paper II:** Previous studies have shown that the binding abilities of H1 are at large determined by the properties of its C-terminal domain while much less attention has been paid to the role of the N-terminal domain. Using the same assay as in Paper I we compared the binding properties of wild type H1.4 and hH1.4 devoid of its N-terminal domain (ΔN-hH1.4). We showed that the lack of N-terminal domain does not have any effect on the hH1.4 induced increase in the NRL; however, the ΔN-hH1.4 displays a drastically lower affinity for chromatin binding as compared to the wt hH1.4 and is more prone to unspecific chromatin binding. We conclude that the N-terminal domain of H1 is an important determinant of affinity and specificity of H1-chromatin interactions.

**Paper III:** Prostate cancer growth is regulated by AR. Antiandrogens (AR antagonists) compete with androgens for binding to AR and are thus used to stall cancer cells. However, invariably patients develop resistance to such therapy and relapse with castration-resistant prostate cancer. This motivates the creation of a second generation of AR antagonists with a more clear-cut anti AR activity. By reconstitution of the hormone regulated mouse mammary tumor virus promoter, MMTV, in *Xenopus* oocytes we previously revealed that the transcription factor FoxA1 is able to convert the glucocorticoid antagonist RU-486 to a partial agonist by presetting of the chromatin structure at the hormone-responsive enhancer. High level of FoxA1 is a negative prognostic factor in prostate cancer and we decided to evaluate the effect of the AR antagonists bicalutamide (BIC) and MDV3100 (MDV) on transcriptional outcome of AR-dependent MMTV promoter in the context of FoxA1. Here we show that both antagonists, upon binding to AR, can translocate the AR-ligand complex to the nucleus, albeit with reduced efficiency for MDV. While in the nucleus both AR-antagonist complexes have the potential to bind sequence specifically to the hormone response elements, HREs, in vivo. The DNA binding is strongly enhanced by co-expression of FoxA1 that makes the HREs more accessible for AR binding. In this context BIC antiandrogenic ability is seriously compromised whereas MDV shows a more persistent antagonistic activity. We believe that these findings may be of clinical relevance.
LIST OF PUBLICATIONS

I. Christine Öberg, Annalisa Izzo, Robert Schneider, Örjan Wrange, Sergey Belikov.

II. Christine Öberg, Sergey Belikov.
    The N-terminal domain determines the affinity and specificity of H1 binding to chromatin
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III. Sergey Belikov, Christine Öberg, Jorma Palvimo, Örjan Wrange.
    FoxA1 strongly corrupts the anti-androgen effect of bicalutamide but has a weak effect on MDV3100 at a hormone regulated enhancer
    *Manuscript*
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LIST OF ABBREVIATIONS

*OH Hydroxylradical
ΔN-hH1.4 hH1.4 devoid of its N-terminal domain
aa Amino acids
AR Androgen receptor
BIC Bicalutamide
CDK-1 Cyclin-dependent kinase 1
CRPC Castration-resistant prostate cancer
DBD DNA-binding domain
DHT 5α-dihydrotestosterone
DHS DNase I hypersensitive sites
MDV MDV 3100
DBD DNA-binding domain
DMS Dimethyl sulfate
FRAP Fluorescent recovery after photobleaching
HRE Hormone response elements
HP1 Heterochromatin protein 1
GFP Green fluorescent protein
GR Glucocorticoid receptor
LDB Ligand binding domain
LTR Long terminal repeat
MMTV Mouse mammary tumor virus
MNase Micrococcal nuclease
NTD N-terminal domain
NR Nuclear receptor
NRL Nucleosomal repeat length
SBMA Spinal and Bulbar Muscular Atrophy
ssDNA Singel stranded DNA
wt Wild type
1 INTRODUCTION

“DNA is just a tape carrying information, and a tape is no good without a player”.
Bryan Turner, PhD, FMedSci

“We are more than the sum of our genes. We are the sum of our gene expression”
Per-Henrik Holmqvist, PhD (slightly modified).

1.1 CHROMATIN –A SHORT INTRODUCTION

Every cell in the human body contains 2 meter of DNA. In order to make it fit into the cell nucleus, that usually has a diameter of 5-10 µm, genomic DNA of all eukaryotes is organized in a complex structure together with proteins called chromatin (Kornberg, 1974). The packaging unit of chromatin is the nucleosome, consisting of 146 bp of DNA wrapped with 1.65 turns around an octamer of so called histone proteins, forming the nucleosome core particle (Luger et al, 1997). Another type of histone protein, the linker histone, often collectively also referred to as histone H1, is located at the surface of the nucleosome core particle contacting the interconnecting linker DNA. The chromosomal DNA in its most expanded form is organized in an 11 nm thick beads-on-a-string structure, with nucleosomes forming the beads and the interconnecting DNA being the strings. However, this structure probably does not exist in vivo, but only in the test tube. In the cell, the nucleosomes are most probably folded into a higher order structure, the 30 nm fiber, a fiber that can be further compacted into 100-400 nm thick structures (Fig 1).

Figure 1 The chromatin structure. The packaging unit of the chromatin structure, the nucleosomes (here displayed without linker histones) are arranged into a 30 nm thick fiber. This can in turn be packed to 100-400 nm thick structures (not shown).
(Picture adapted from Kinner A et al. (2008) Nuc. Acids Res. 36(17)5678-5694)

However, to make the DNA fit into the tiny space of the cell nucleus is not the only function of chromatin. Another just as important function is to control gene expression, i.e. to ensure that a particular gene is expressed in the correct cell, and at the correct time. The human body comprises of some 200 different cell types. Each cell contains the same DNA, i.e. carries the same genetic information. Yet, a liver cell differs profoundly from, for example, a skin cell or a cell in the eye. Likewise, monozygotic
twins carry identical genetic information and still sometimes don’t have identical phenotypes. Hence, what matters is not only if a certain gene is present or not but also its level of expression.

How is the control over gene expression exerted? There are several ways, and to understand them we need to go down to the level of the packaging unit of the chromatin structure—the nucleosome.

1.1.1 The nucleosome

The protein octamer at the center of the nucleosome contains two copies each of the highly conserved histone proteins H2A, H2B, H3 and H4 (Kornberg & Thomas, 1974; McGhee & Felsenfeld, 1980). These proteins are small and they all share a common structure with a globular domain consisting of a long central helix flanked on either side by a loop and a shorter helix and an unstructured tail in the N-terminal domain (reviewed in (Ramakrishnan, 1997)). The crystal structure of nucleosome core particle was solved with high resolution (Luger et al, 1997). The N-terminal tails are protruding from the core of the nucleosome and subjected to covalent modifications such as acetylation, methylation, phosphorylation, etc. These modifications serve as signals for the activation or repression of gene expression, through the recruitment of different factors (reviewed in (Jenuwein & Allis, 2001)). Acetylation of the core histone tails generally leads to more open structures that are more prone to transcriptional activity. On the contrary methylation of certain residues, such as H3K9, leads to repression of transcription whereas methylation of others, such as H3K4 and H3K36, leads to activation.

Thus the expression of information encoded in the DNA is in part regulated by the modifications of the histone tails (Strahl & Allis, 2000).

1.1.2 Epigenetic mechanisms

Epigenetics define the heritable, but reversible, changes in gene expression that are not encoded in the DNA sequence itself. Histone modifications is one example of epigenetic programming; methylation of DNA is another one. The methylation of CpG dinucleotides in the DNA exerts control of gene expression in two ways. First, the presence of a methyl groups can inhibit the binding of transcription factors that recognizes CpG sequences (Watt & Molloy, 1988). Second, the methylated DNA can attract proteins with a so called methyl-binding domain that in turn associate with co-repressor complexes such as histone deacetylases and histone methyltransferases (reviewed in (Deaton & Bird, 2011)).

Epigenetic regulation of gene expression constitutes the basis for the development of different cell types from the same genome. Epigenetic mechanisms are also responsible for the phenotypical differences between monozygotic twins, differences that tend to increase with age (Fraga et al, 2005a).

In recent years, a lot of effort have been made not only to understand how the epigenetic regulation of the organism development but also to elucidate how epigenetic misregulations may cause disease(s) (reviewed in (Ballestar, 2011)). Results from some
studies have also indicated that epigenetic patterns can be inherited through generations (reviewed in (Peaston & Whitelaw, 2006)). For example, the access to food and nutrition in one generation was found to affect the health of the next; when a father experienced a poor availability of food during the period before the onset of puberty, his sons were found to be less prone to cardiovascular death (Kaati et al, 2002). However, the exact mechanisms on the molecular level for this phenomenon remain unclear.

1.1.3 Euchromatin and heterochromatin

Traditionally chromatin has been divided into heterochromatin and euchromatin. These two forms were distinguished cytologically by how intensely they are stained. Euchromatin stains less intense and constitutes more open chromatin structure, while heterochromatin stains intensely and is characterized by a tightly packed chromatin structure, without much gene expression. Heterochromatin can in turn be subdivided into constitutive heterochromatin that remains silent throughout the life span of an organism and facultative heterochromatin which expression is developmentally regulated. Example of constitutive heterochromatin is the tightly packed structures next to the centromeres and in telomeres and examples of facultative chromatin are promoters of genes that are silenced during development (reviewed in (Craig, 2005)).

1.2 THE “FORGOTTEN” COMPONENT OF CHROMATIN: THE LINKER HISTONE

The X-ray crystal structure of the nucleosome core particle was resolved in 1997 (Luger et al, 1997). However, one important piece of information was, and is still, missing; namely the location of the linker histone on the nucleosome. Here it should be pointed out that the linker histone family in mammals is big and heterogenous, consisting of several different isoforms. The linker histone is sometimes referred to as histone H1 and in this thesis the members of the linker histone family will often collectively be referred to as H1, even though some linker histone variants are known by other names.

Figure 2 The structure of the metazoan linker histone. The linker histone has a three domain structure; a globular domain of ~60 aa, and two protruding tails that can be subjected to modification. The N-terminal domain is 20-40 aa long, while the C-terminal domain is ~100 aa and rich in positively charged residues.
The metazoans H1 molecule has a three domains structure, with a globular domain, a short N-terminal domain and a long, basic C-terminal domain as shown in Fig. 2 (Allan et al, 1980; Bradbury et al, 1975; Chapman et al, 1976; Hartman et al, 1977). H1 is located close to the nucleosomal dyad and interacts with DNA where it enters and exits the nucleosome (Allan et al, 1980; Goytisolo et al, 1996; Zhou et al, 1998). This brings the segments of the linker DNA together to form a characteristic stem structure (Bednar et al, 1998) (Fig 3) which stabilizes the DNA wrapping and preventing unpeeling of DNA from the histone octamer. The nucleosome core particle protects around 147 bp from digestion with micrococcal nuclease, MNase, an enzyme that cleaves the linker DNA. The presence of a linker histone results in protection of additional ~20 bp from MNase digestion. The nucleosome structure containing one molecule of H1 and 20 additional bp DNA protected from digestion is called the chromatosome. H1 has been shown to be necessary for correct mammalian development (Fan et al, 2003) as well as been implicated in disease development (Duce et al, 2006; Kim et al, 2008; Trojer et al, 2007). However, while discussing factors that influence chromatin structure and gene regulation the role of H1 is often ignored. As can be seen in Fig 1 in some figures of chromatin structure H1 is even omitted. While much effort has been placed on understanding the language of the modifications of the core histones less effort has been placed on elucidating the function of the modifications of H1 (Jenuwein & Allis, 2001). While the role of different core histone subtypes, such as the H2A variant H2A.X or the H3 variant H3.3 have been elucidated, the role of the different H1 subtypes are not as well understood.

Figure 3 Binding of H1 The linker histone is bound at the surface of the nucleosome. In the presence of H1 the two segments of the linker DNA become juxtaposed roughly 8 nm from the nucleosome center, and remain together for 3-5 nm before diverging, forming a ”stem structure” (Bednar et al, 1998). The location of the H1 N-terminal domain is not known, and hence this part is not included in the picture.

Is it correct to call the linker histone a forgotten protein?
Yes and no. YES, because compared to the amount of research that has been done in the core histones field much less attention has been paid to the role of H1. And NO, because to say that H1 is forgotten is of course in one way to belittle the research that has been performed around H1 and to underestimate the knowledge that has been accumulated. The primary research aside, a lot of reviews have also been written, however as Woodcock et al point out in their review (Woodcock et al, 2006) the title of many of the reviews ends with a question mark. And maybe this says something about how limited our current knowledge about H1 and its role in chromatin structure and gene regulation is today. The aim of the studies presented in this thesis was to bring
clarity to some of the questions regarding the role of H1 and its role in chromatin structure.

1.2.1 Linker histone structure

As previously described (Fig. 2) the linker histones of multicellular eukaryotes exhibit a tripartite structure with a globular domain of ~60 aa flanked by a short protruding amino-terminal domain (N-terminal tail) of 20-40 aa and a longer, lysine rich carboxyl terminal tail (C-terminal tail). The structure of the globular domain has been solved by X-ray crystallography and shown to consist of a 3-helix “winged helix” fold containing a helix-turn helix motif (Ramakrishnan et al, 1993). The N-terminal domain consists of two distinct parts; the first part is rich in alanines and prolines while the second part, closest to the globular domain is enriched in lysines (Bohm & Mitchell, 1985). The positively charged domain has been shown to acquire an α-helical structure in trifluoroethanol (Vila et al, 2002), but otherwise its structure is not known. The C-terminal domain comprises almost half of the length of the linker histone and has been implicated to be a major contributor to H1’s binding abilities (Hendzel et al, 2004). The C-terminal domain is an intrinsically disordered domain, that only adopt a structure upon DNA binding (reviewed in (Caterino & Hayes, 2011)) One conserved feature of all linker histone variants is the presence of serine/threonine kinase phosphorylation sites, S/TPXK motifs, distributed throughout the molecule. Several S/TPXK motifs are present in the C-terminal domain of H1 and take part in DNA binding and function in the compaction of DNA during mitosis ((Bharath et al, 2003), reviewed in (Caterino & Hayes, 2011)).

1.2.2 H1 Family

Among the five histone families the H1 family is the fastest evolving and the most diverse (Eirin-Lopez et al, 2005). While the evolutionary origin of the core histones can be traced to archaeas, there is no evidence for the presence of H1 genes (Kasinsky et al, 2001). However in eubacteria several genes coding for basic proteins with similarity to the lysine rich C-terminal domain of metazoan linker histones have been found. Thus it seems like the origin of H1 like proteins can be traced back to eubacteria. The acquisition of the globular domain and the N-terminal tail occurred later in evolution (Kasinsky et al, 2001).

Lower eukaryotes such as fungi and yeast only have one type of H1, whose structure differs from the three domains structure found in metazoans. Tetrahymena Thermophila contains a protein that shares the size, solubility properties and lysine richness of metazoans H1 but lacks the globular domain (Gorovsky et al, 1974; Wolfe et al, 1997; Wu et al, 1994). Saccaromyces cerevisiae contain an H1 homologue Hho1p with two regions that are homologous to the globular domain of higher eukryotes. They are connected with a protein whose amino acid composition and sequence is similar to that of the C-terminal domain of H1 in higher order species (Landsman, 1996; Patterton et al, 1998). However, this non-canonical linker histone is present in very low amount, with 1 H1 molecule per 37 nucleosomes and knock down of Hho1p had no effect on the chromatin structure, viability or growth rate (Freidkin & Katcoff, 2001). In contrast
to this, metazoan cells usually have an H1-to-nucleosome content of 0.5-1 and they are required for proper chromatin function.

Most higher eukaryotes contain several H1 subtypes. One of the exceptions to this rule is *Drosophila melanogaster* (Nagel & Grossbach, 2000), that only contains one H1 subtype. In the African clawed frog *Xenopus laevis* there are six subtypes (Risley & Eckhardt, 1981; Shechter et al, 2009b) and in chicken there are seven (Coles et al, 1987). While some subtypes are present in all analyzed cell types, others are specific for certain cell types and for certain stages in development. In *Xenopus* three subtypes are somatic and they are present in all cells of the body, one is specific for terminally differentiated cells and two are specific for oocytes and sperm cells respectively (Risley & Eckhardt, 1981; Shechter et al, 2009b). In the chicken, six subtypes are somatic whereas one, H5, is present only in the repressed chromatin of erythrocytes (Coles et al, 1987). The chicken erythrocyte is a terminally differentiated cell with very compact and inert chromatin with long NRL. There are indications that the chicken erythrocyte has a total of 1.3 molecules of linker histones per nucleosome; 0.4 chH1 and 0.9 chH5 (Bates & Thomas, 1981; Morris, 1976).

### 1.2.2.1 H1 in humans

Humans have 11 different subtypes of linker histone, each one corresponds to a homologue in mice. There have been difficulties in finding a consensus regarding the nomenclature of the human H1 subtypes. A summary of different nomenclatures is shown in Table 1. In this thesis and our papers we are using the nomenclature proposed by Albig and coworkers (Albig et al, 1993).

<table>
<thead>
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<th>Table 1 Different nomenclatures used in the H1 field</th>
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<tr>
<td><strong>Human</strong></td>
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<tr>
<td>Albig et al (1993)</td>
</tr>
<tr>
<td>Ohe et al (1989)</td>
</tr>
<tr>
<td>Official symbol</td>
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<tr>
<td>Also known as</td>
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<tr>
<td>Parseghian et al (2000)</td>
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<td>Seyedin and Kistler (1979)</td>
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The 11 known human subtypes are H1, H1.1-H1.5, H10, H1oo, H1t, H1T2, H1LS1 and H1x ((Izzo et al, 2008) and references therein). These subtypes can be subgrouped according to their temporal and spatial expression. H10 is expressed throughout the cell cycle, i.e. is replication independent, and is predominantly found in terminally differentiated cell. H1oo is specific for oocytes and the H1t and H1T2 variants are present during mouse spermatogenesis in male germ cells (Drabant et al, 1996; Izzo et
al, 2008; Tanaka et al, 2001). In both human and mouse there are five somatic replication dependent subtypes, hH1.1-hH1.5. While the expression of hH1.1 seem to be restricted to testes, thymus and spleen hH1.2, hH1.3, hH1.4 and hH1.5 has been found in most if not all somatic cell types with H1.2 and H1.4 being the predominant variants in most of the cell types studied (Franke et al, 1998a; Franke et al, 1998b; Izzo et al, 2008; Meergans et al, 1997). hH1.2 and hH1.4 have also been found to exist in two different sequence variants. H1.2 has an Ala17Val substitution in 6.8% of the Swedish population and H1.4 was found to have a Lys173Arg in Raji cells (Sarg et al, 2005). Analysis of H1x distribution showed that it is expressed in all tissues examined (Yamamoto & Horikoshi, 1996), but otherwise very little is known about this subtype.

1.2.3 H1 location in the chromatin fiber

The exact location of the linker histone on the nucleosome has for long been controversial and during the years several different models have been proposed. The original model placed the linker histone symmetrically with the globular domain contacting the DNA at the place where it enters and exits the nucleosome and shielding 10 bp of linker DNA at either side of the nucleosome (Allan et al, 1980). Quantitative DNaseI footprinting experiments supported this view (Staynov & Crane-Robinson, 1988). However, other studies in late 80’s suggested an asymmetrical placement of the H1 molecule. An et al. (An et al, 1998) showed that deposition of H1 on nucleosome core particles resulted in 20 bp protection of the DNA on one side of the core particle and none on the other. Careful analysis performed in the Alan Wolfe lab indicated that H1 binding to nucleosome reconstituted on Xenopus borealis 5S DNA resulted in protection of 5 bp on one side of the nucleosome and 15 on the other (Hayes & Wolfe, 1993). Further studies suggested the linker histone to be located asymmetrically inside the DNA gyres, with the globular domain placed 65 bp away from the dyad axis (Hayes et al, 1994; Pruss et al, 1996). A drawback with these studies is that they were all performed in vitro, with nucleosomes reconstituted on a particular DNA sequence. H1 is known to play a repressor role at the 5S gene (described in further detail below) and thus, what is true for H1 binding at the 5S gene sequence may not be applicable to H1 binding at other sequences.

In a study by Zhou et al. the H5 globular domain was deposited on bulk chromatosomal DNA. It was shown that helix III of the globular domain binds to in the major groove of the first helical turn of the chromatosomal DNA while another binding site at the opposite side of the H5 globular domain contacting DNA near the dyad axis, thereby forming a bridge between the linker DNA and the nucleosomal DNA (Zhou et al, 1998).

In a more recent study the linker histone chaperone NAP-1 was used for deposition of H1.5 on the 601 DNA sequence, and the placement of H1.5 was then determined by electron microscopy and hydroxylradical (*OH) footprinting. It was shown that the presence of H1 induced a 10 bp protection from *OH cleavage in the DNA in the center of the nucleosome (Syed et al, 2010), thus supporting a symmetrical positioning. However, since the 601 DNA sequence was developed to provide a high affinity for nucleosome binding and positioning (Lowary & Widom, 1998) and in that sense differs from natural DNA, the conditions for H1 binding in this study may not accurately
reflect the conditions for H1 binding in the cell. Furthermore, another possible explanation for the 10 bp protection is that H1 binds slightly asymmetrically, and with contact points on the DNA on one of the sides of the nucleosome as well as in the middle of the nucleosome. Since H1 in this scenario would alternatively bind to, and thereby protect, either one or another site in different nucleosomes but always contact the same part of the DNA in the middle, the latter segment is the only one to be consistently protected from *OH degradation.

Fluorescence recovery after photobleaching, FRAP, is a widely used method to measure the mobility of a protein in the cell. The protein of interest is tagged with a fluorescent protein, such as GFP. The fluorescence in a certain area in the nucleus is destroyed by intense illumination followed by measurement of the speed of recovery of the signal, i.e. the time it takes before the molecule with destroyed fluorescence is replaced by another molecule. In this way one can measure how long a molecule remains bound to a target site. FRAP studies have shown that while a majority of H1 molecules take part in chromatin interaction at any given time, the interaction with chromatin occurs in “stop-and-go” mode with H1 molecules being continuously exchanged on the target site and with an average time on DNA of 200-400 seconds (Lever et al, 2000; Misteli et al, 2000; Th'ng et al, 2005). This argues for that rather than a certain H1 molecule being continuously bound to a certain nucleosome the nucleosome fiber is surrounded by H1 molecules, that takes turn in binding. By studying how H1’s mobility is changed after point mutations in the globular domain and computer based modelling Brown et al. mapped two binding sites in the globular domain of H1. One site was suggested to interact with the major grove near the dyad axis and the other with the minor groove of the linker DNA (Brown et al, 2006). Interestingly, another study that employed computational docking indicated three binding sites in the globular domain, corresponding to three different binding modes (Fan & Roberts, 2006). One binding mode is consistent with the symmetrical model, where the linker histone contacts the dyad and both strands of linker DNA while the second binding mode is compatible with the model proposed by Zhou et al. (Zhou et al, 1998) where H1 binds to form a bridge between the linker DNA and the nucleosomal DNA. The third binding mode is consistent with the model proposed by Hayes and Wolfe (Hayes et al, 1994) where H1 binds 64 bp away from the dyad axis. The authors propose that different conditions and experimental setups may favour a certain binding mode thus explaining the different results observed in the different studies (Fan & Roberts, 2006).

Using H1 constructs that lack the C-terminal tail and/or with site directed mutagenesis in the binding sites of the globular domain in FRAP experiments H1 has been shown to bind to chromatin with intramolecular cooperativity. This model suggests that the H1 C-terminal domain makes the initial contact with the linker DNA. This interaction positions H1 in a way that makes it possible for either of the binding domains in the globular domain to bind to its target DNA. A conformational change occurs that brings the target for H1’s other binding domain closer allowing both binding domains to bind (Stasevich et al, 2010). The intramolecular cooperativity is negatively affected by acetylation of the core histones (Raghuram et al, 2010).
1.2.4 H1 in the living cell

For many years the well accepted dogma postulated that the H1-to-nucleosome ratio was 1:1. However, more recent studies have indicated that H1-to-nucleosome ratio varies between cell types, and in most cell types the ratio is between 0.5-0.9 (reviewed in Woodcock et al, 2006)). As described earlier, FRAP data has shown that H1 is a highly mobile protein, and that the molecules take turn in binding to the nucleosomes. Eukaryotic cells exhibit a wide range of nucleosome repeat lengths, NRL, that differ between cell types (reviewed in (Woodcock et al, 2006)). The presence of H1 influences NRL. The reason is that more negative charge provided by the DNA phosphate backbone is needed to neutralize the positive charge provided by H1; resulting in an increase in the length of the internucleosomal spacer upon H1 binding to the chromatin ((Blank & Becker, 1995) reviewed in (Woodcock et al, 2006)). Thus, cells with a higher H1-to-nucleosome ratio tend to have a longer NRL, and a reduction in H1 content leads to shorter NRL (Fan et al, 2005; Hashimoto et al, 2010; Woodcock et al, 2006). Shorter NRL is a characteristic of active chromatin domains and rapidly growing cells, such as embryonic stem cells (Berkowitz & Riggs, 1981; Cavalli & Thoma, 1993; Compton et al, 1976; Gottschling et al, 1983). In contrast, mature cells, with more compact chromatin, tend to have longer NRL (Compton et al, 1976; Morris, 1976; Perisic et al, 2010).

The presence of H1 leads to more tightly packed chromatin structures (Routh et al, 2008; Thoma et al, 1979) and thus one of the functions of H1 is to help packing the chromatin and to stabilize higher order chromatin structures. In accord with this, electron microscopy and biochemical studies showed that a 50% reduction in H1 content in the living cell leads to more irregular and open chromatin structures compared to the wild type cells (Fan et al, 2005).

1.2.5 Is there life without H1?

The complete knock down of the non-canonical H1s in *Tetrahymena Thermophilia* (Shen et al, 1995) or *Saccharomyces cerevisiae* had no obvious phenotypic effects (Patterton et al, 1998) and the replacement of the hhoA gene encoding three canonical H1s in *Aspergillus nidulans* gave the same result (Ramon et al, 2000). Knock down of two H1 subtypes in mice resulted in viable mice with no apparent phenotype aside from compensatory upregulation of the genes coding for the other H1 subtypes (Fan et al, 2001). However, when the somatic subtypes H1.2, H1.3 and H1.4 were knocked down simultaneously the embryos died latest at stage E11.5 displaying a wide range of developmental defects (Fan et al, 2003). Hence, a correct amount of linker histone is necessary for mammalian viability. A more recent study showed that knock down of the only H1 gene in Drosophila also results in lethality (Lu et al, 2009b).
The exact role of H1 in transcriptional regulation is obscure. Due to its chromatin compaction abilities and the results from *in vitro* studies H1 was for many years believed to act as a global repressor (O'Neil et al, 1995; Shimamura et al, 1989). Although the 50% reduction of H1 level in mouse embryonic stem cells resulted in global changes in chromatin structure such as decompaction and decrease in NRL, very few changes in gene expression were observed: only 29 genes showed a difference of 2-fold or more, and of them 19 genes showed increased expression and 10 showed decreased expression (Fan et al, 2005). Thus, a reduction of H1 does not result in a global up or down regulation in gene expression but rather affects a subset of genes to different extent. This indicates that the role of H1 is neither that of a global repressor or activator, but rather that of a “lubricant” involved in fine-tuning of gene expression.

### 1.2.6.1 H1 regulated genes
There are though several examples of H1 involvement in the regulation of individual genes. The *Xenopus* somatic subtype xH1A starts to accumulate in the embryo during early gastrula (Dworkin-Rastl et al, 1994). At this time point it is involved in silencing of transcription from the 5S RNA gene (Bouvet et al, 1994; Kandolf, 1994) by positioning the nucleosomes in a way that inhibits transcription factor binding (Sera & Wolffe, 1998). Sometimes H1 exerts its effect on gene expression by the interaction with other factors. For example, the human subtype H1.5 forms a complex with transcription factor Msx1, binds to and induce chromatin repression at the key regulatory element of the gene coding for MyoD, a protein that controls skeletal muscle differentiation (Lee et al, 2004). The human subtype H1.2 was found to be part of a complex that represses p53 mediated transcription by blocking p300-induced acetylation of chromatin at the promoter region. (Kim et al, 2008). H1.2 has also been shown to be recruited by the chromatin remodeling complex CHD8 to suppress the wnt-β-catenining-signalling pathway (Nishiyama et al, 2012).

### 1.2.7 Posttranslational modifications of histone H1
Just like the core histones, H1 is subjected to post-translational modifications. Masspectrometry studies of the modification pattern of H1 showed that the human and mouse H1 subtypes were subjected to phosphorylation, acetylation, methylation and ubiquitination on a number of different residues (Wisniewski et al, 2007). While some modifications were common to all H1 subtypes others were subtype specific. The modification pattern also differed between tissues taken from mice on one hand and MCF7 and HeLa cell on the other. Below the most well studied H1 modifications will be introduced.

#### 1.2.7.1 Phosphorylation
The most extensively studied posttranslational modification of H1 is phosphorylation. Phosphorylation of H1 progressively increases during the cell cycle, reaches a maximal level in mitosis and then decreases rapidly in telophase (reviewed in (Happel & Doenecke, 2009)). A study of interphase phosphorylation pattern in T cells and Jurkat
cells has shown that phosphorylation starts in the second part of G\textsubscript{1} phase and increases in the S phase (Green et al, 2011). Phosphorylation occurs either at the conserved cyclin-dependent kinase 1, CDK 1, S/TPXK motifs or at other sites that are not recognized by CDK1. Examples of the latter are hH1.4S2, hH1.4T4 and H1.4S27 (Garcia et al, 2004). Phosphorylation during interphase was found to occur at serines in a subtype specific manner. H1.2 is phosphorylated on S172, hH1.3 on S188, H1.4 on S171 and S186 and hH1.5 on S17, S172 and S188 (Sarg et al, 2006). During mitosis hH1.5 is phosphorylated also at T137 and T154, and additional phosphorylation of the other subtypes most probably occurs as well (Sarg et al, 2006; Talasz et al, 2009). H1 phosphorylation was originally linked to the mitotic condensation of chromatin (Bradbury et al, 1974), but this view was later changed since chromatin condensation has been shown to occur without H1 in vitro ((Happel & Doenecke, 2009) and references therein). The view that H1 phosphorylation is not involved in condensation was in turn challenged when immunodepletion of H1 from Xenopus laevis egg extracts resulted in 50% elongation of the replicated chromosomes and failed alignment at the metaphase plate (Maresca et al, 2005). Furthermore, inhibition of H1 phosphorylation in FM3A cells resulted in a decline in mitotic cells and decondensation of the metaphase chromosomes (Th'ng et al, 1994). Exactly how phosphorylation of H1 is involved in chromatin condensation is unclear but a model has been proposed that the weakened H1-DNA interactions lead to a positioning of H1 that favors H1-H1 interactions. These interactions may in turn take part in the formation of higher order chromatin structure (reviewed in (Roth & Allis, 1992)).

H1 phosphorylation has also been linked to the decondensation of chromatin (reviewed in (Roth & Allis, 1992)). Local chromatin decondensation during S-phase correlates with the presence of phosphorylated H1, probably in a CDK-2 dependent manner (Alexandrow & Hamlin, 2005; Herrera et al, 1996). Phosphorylation is thought to exert its effect by the provision of negative charge that results in the repulsion of the H1 tails from the DNA thus weakening association with DNA (reviewed in (Happel & Doenecke, 2009) and in (Roth & Allis, 1992)). Due to loss of the positive charge the negatively charged DNA strands will repel each other thus forming a less condensed chromatin structure. This view is supported by the FRAP analyses of H1 mutated to mimic dephosphorylated or phosphorylated H1 and FRAP studies performed in cells treated with kinase inhibitors. The results from these studies show that phosphorylated H1 exhibits increased mobility compared to the non-modified counterpart and argues for that phosphorylation is likely to affect H1 chromatin binding in a variant-specific fashion (Hendzel et al, 2004; Lever et al, 2000).

A few studies found a potential link between H1 dephosphorylation and apoptosis (Guo et al, 2000; Kratzmeier et al, 2000). However later it was shown that dephosphorylation is rather caused by cell cycle arrest (Happel et al, 2005) and yet another study argued that H1 dephosphorylation is dependent on the method used to induce apoptosis and, thus, is no general hallmark of early apoptosis (Green et al, 2008).

There are data indicating that H1 phosphorylation can also be increased at the sites of active transcription and site-specific interphase H1 phosphorylation facilitates transcription by RNA polymerases I and II (Zheng et al, 2010).
Studies of the mouse mammary tumor virus, MMTV, promoter have shown that phosphorylation of H1 is required but not sufficient for glucocorticoid-dependent transcription from this promoter (Bhattacharjee et al, 2001; Lee & Archer, 1998). Binding of the progesterone receptor, that activates the MMMV promoter, increases the level of phosphorylated H1 present at MMTV minichromosomes reconstituted in HeLa cell extracts. (Koop et al, 2003).

1.2.7.2 Methylation
The human linker histone subtype hH1.4 is methylated on lysine 26 by the Polycomb repressor complex 2 (Kuzmichev et al, 2004) and methyltransferase G9a (Trojer et al, 2009). This methylation site is recognized and bound by heterochromatin protein 1, indicating a role of this modification in the formation of heterochromatin. Interestingly, phosphorylation of the neighboring hH1.4S27 prevents HP1 from binding (Daujat et al, 2005). The phosphorylation of S27 is cell cycle dependent and peaks in metaphase (Hergeth et al, 2011).

The methyltransferase G9a also methylates lysines in the C-terminal domains of H1.2, H1.3, H1.5 and H1o. H1.2 is methylated on K187, a modification that is specific to H1.2. It is cell cycle independent and is not associated with HP1 (Weiss et al, 2010).

1.2.7.3 Acetylation
The human subtype hH1.4 may also be acetylated on residues K26 and K34 (Kamieniarz et al, 2012). The K34 modification is enriched at transcription start sites and has been proposed to facilitate transcription by the “opening up” of chromatin and by assisting in the recruitment of transcription factors. The acetylation of K34 also increases the mobility of H1.4 as monitored by FRAP. Aside from the H1.4K34ac modification, acetylation of H1 is not well studied.

1.2.7.4 Ubiquitination
Ubiquitination has been found in H1.2, H1.3 and H1.4 at K46 from HeLa cells. It was, however, missing in the same H1 subtypes from MCF7 cells. Ubiquitination was also found in H1.1 at K116 from mouse tissue (Wisniewski et al, 2007). The functional significance of these modifications in H1 has yet to be elucidated, however, ubiquitinylation of core histones is most often associated with increased gene expression.

1.2.8 Other roles for H1
This thesis, as well as most other studies made on H1, is focused on the effect of H1 subtypes on chromatin structure and gene regulation. However, another reason for having several H1 subtypes could be that they have different functions during mitosis and/or as signaling molecules.

1.2.8.1 Role in mitosis
A study of the distribution of the human subtypes H1.2, H1.3 and H1.5 showed different localization pattern during mitosis (Green et al, 2010). While hH1.3 was detected on chromatin during all cell cycle phases, with only small quantities present in the cytoplasm during late anaphase, hH1.2 was found interacting with chromatin during
interphase and prophase but located exclusively in cytoplasm during prometaphase/metaphase. In late anaphase/early telophase hH1.2 was relocated to chromatin. hH1.5 largely followed the pattern of hH1.2 but was found both in cytoplasm and on chromatin during prometaphase/metaphase (Green et al, 2010). These differences in distribution indicate different roles of the H1 subtypes in mitosis. The dissociation of hH1.2 and hH1.5 from chromatin during mitosis can be explained by mitosis specific phosphorylation that decreases affinity for chromatin binding. However, both hH1.2 and hH1.3 contain four phosphorylation sites, of which three are in the C-terminal domain while hH1.5 contains five phosphorylation sites of which four are located in the C-terminal domain. hH1.2, hH1.3 and hH1.5 all contain two TPKK phosphorylation sites in the C-terminal domain. Hence, in terms of total number of phosphorylation sites H1.2 is similar to H1.3 while it behaves more like H1.5. Thus, one can speculate if these differences in behaviour are, at least partly, due differences between the H1.2 and H1.5 that are not coupled to modification status, such as inherent differences in chromatin binding affinity.

1.2.8.2 Signalling molecule
As was mentioned above, H1.2 is part of a complex that represses p53 mediated transcription (Kim et al, 2008). It was shown that hH1.2 is involved in apoptosis signalling induced by DNA double strand breaks. After exposure to X-ray irradiation, all H1 isoforms are released into the cytoplasm in a p53 dependent manner but only H1.2 could induce cytochrome c release from mitochondria and thereby trigger apoptosis (Konishi et al, 2003), thus, indicating that hH1.2 plays a role of a signalling molecule.

1.2.8.3 H1 involvement in the functioning of the immune system
A few reports suggest that H1 can be involved in the functioning of the immune system. The H1 subtype HIS-24 in C.Elegans has been shown to be a regulator of genes involved in antimicrobial defense; the level of monomethylated HIS-24K14 in the cell rises after bacterial infection. This modification is bound by the HP1-like protein HPL-1 on promoters involved in antimicrobial response (Studencka et al, 2012).

The forkhead transcription factor FoxP3 controls the development and is necessary for the function of the cells required for the maintenance of self-tolerance, the regulatory T-cells. FoxP3 exerts its function by binding to and mediating repression of the IL-2 promoter. Linker histone subtype hH1.5 was shown to interact with FoxP3 in human T cells and co-expression of FoxP3 and hH1.5 lead to synergistic repression of the IL-2 promoter. Knock down of hH1.5 inhibited the ability of FoxP3 to suppress IL-2 expression, indicating a role for at least hH1.5 in the human immune system (Mackey-Cushman et al, 2011). Subtypes H1.1-H1.5 exhibit antimicrobial activity against S. typhimurium when extracted from terminal ileal mucosal cells. When immunohistochemical analysis was performed on tissue sections with a monoclonal antibody to H1 immunoreactive linker histone was shown to be present in the nuclei of epithelial cells as well as in the cytoplasm of some villus epithelial cell. Collectively these findings may indicate a role for H1 in the human innate immune system (Rose et al, 1998).
1.2.9 Potential role in disease development

1.2.9.1 Cancer
In recent years it has become clear that changes in the pattern of covalent core histone modifications are not only associated with certain types of cancer but also predictive of outcome (Fraga et al, 2005b; Schneider et al, 2011; Seligson et al, 2005). The role of linker histone H1 and its modifications, though, tends to be ignored. Even so, some results suggest that H1 not only plays important roles in the development of a healthy organism but also in the development of diseases.

As discussed above the human linker histone subtype hH1.4 can be methylated on lysine 26 by the methyltransferase G9a (Trojer et al, 2009). This modification has been shown to interact with both the malignant brain tumor protein L3MBTL1 (Trojer et al, 2007) and with heterochromatin protein 1. Mutations of the l3mbt gene in Drosophila causes brain tumors, and the L3MBTL1 protein in human is a transcriptional repressor (Boccuni et al, 2003). Loss of HP1 causes chromosome segregation defects, HP1 has also been shown to play a prominent role in the progression of many cancer types (reviewed in (Dialynas et al, 2008)).

1.2.9.2 Alzheimer’s disease
Alzheimer disease is one of the most common forms of neurogenerative diseases. One of the characteristics of the disease is the accumulation of senile plaques in the brain (Tiraboschi et al, 2004). The major component of senile plaques is the amyloid-β-protein (Aβ), a protein that under normal conditions is soluble but in the diseased brains aggregates into insoluble so called amyloid-like structures (Makin & Serpell, 2005).

Duce et al. showed that the H1 subtypes H1.1-H1.5 are present in the amyloid plaques in mice model for Alzheimer disease and that H1 shows increased binding to the amyloid-like forms of the Aβ-peptide in these mice (Duce et al, 2006). Disruption of this interaction in vitro required the presence of NaCl concentrations higher than 1M and thus cannot be explained just by electrostatic interactions (Duce et al, 2006). No differences were shown in the total H1 expression level in the brain of affected and control mice, however, in affected mice H1 tended to be localized to the cytoplasm of both neurons and astrocytic-like cells. It was speculated that both the release of H1 and its translocation into the cytoplasm as well as H1’s interaction with the amyloid-like motifs might trigger disease development.

Aberrant interaction between H1 and amyloid-like fibrils may also be related to other neurodegenerative diseases such as Parkinson’s and Creutzfeldt-Jakob disease.

1.2.10 The H1 family: Why so many different subtypes?
Linker histones serve very important functions in chromatin structure and development. However, there are still a lot of unanswered questions and uncertainties around this protein. One such question is whether the heterogeneity of the H1 family is functionally important.

The different subtypes of H1 are far more conserved between species than within them. Fig. 4 shows an alignment of the somatic human subtypes as well as the differentiation
specific subtype H1\textsuperscript{0}. The amino acid sequence of the globular domain is relatively conserved both between different subtypes from the same species, and also between species. The N-and C-terminal domains are more divergent between subtypes, however there is a high degree of sequence conservations between subtypes from different species. The mouse somatic subtypes H1.2, H1.3 and H1.4 are aligned to the human homologues in Fig 4B, and as can be seen they differ at around 25 residues. Since the C-terminal comprises about half of the protein, much of the diversity of the H1 family can be attributed to that part.

Figure 4 (A) Alignment of the somatic human H1 subtypes and the differentiation specific H1\textsuperscript{0}. (B) Human H1.2, H1.3 and H1.4 aligned to their homologue in mouse.
(Sirotkin et al, 1995). The bulk H1/nucleosome ratio remained the same since the loss of H1\textsuperscript{0} was compensated for by elevated expression of the somatic subtypes H1.2, H1.3 and H1.4. A knock down of either one of H1.2, H1.3 and H1.4 alone or in combination with H1\textsuperscript{0} did essentially show the same result – i.e. no visible phenotypic effects except upregulation of the remaining H1 subtypes (Fan et al, 2001). These results show that the somatic subtypes are able to replace each other to a certain extent and may argue for that the heterogeneity of the H1 family is not functionally important and rather is an evolutionary reminiscence.

However, other studies led to different results. For example, a more careful analysis of the knock down mice showed that knock down of different H1 subtypes had different effects on the age dependent silencing of a transgene (Alami et al, 2003), different subtypes were shown to bind to chromatin with different affinity and localize to different parts of the chromatins structure in the living cell (Th'ng et al, 2005), the level of different subtypes has been shown to differ before and after T-cell activation (Green et al, 2011) and there are indications that different subtypes associate with different regulatory proteins (Kim et al, 2008; Lee et al, 2004).

Thus, the question of whether this heterogeneity has any function to fill remain unanswered. And if yes, what is then the role of each different subtypes?

1.3 NUCLEAR HORMONE RECEPTORS

The members of the nuclear receptor (NR) family are examples of another type of players in the chromatin orchestra. Nuclear receptors are a conserved family of transcription factors with more than 300 recognized members that are known to play crucial roles in reproduction, development, metabolism, homeostasis and other processes (reviewed in (Whitfield et al, 1999)). Nuclear receptors are subdivided into different types (reviewed in (Tsai & O'Malley, 1994)). The type I receptors are also called the steroid receptors, since they are dependent on the binding of steroid hormones for their activation. Examples of this type of receptor are androgen receptor, estrogen receptor, glucocorticoid receptor, progesterone receptor and mineralcorticoid receptor. In the absence of ligand the steroid receptors resides in the cytoplasm where they are bound to heat shock proteins. However, upon ligand binding steroid receptors undergo conformational changes and translocate to the nucleus where they recognize and bind to response elements, so called hormone response elements, HREs, at the promoter of their target genes. In the case of type I NR, the HREs are made up of inverted repeats of DNA separated by a few bases. The second class of NRs, type II, includes the thyroid hormone receptor, vitamin D, retinoic acid and 9-cis retinoic acid receptors. This type of nuclear receptor binds DNA even in the absence of ligand and their HREs are made up of direct repeats. Type III nuclear receptors are also called orphan receptors, due to the fact that their ligand is not yet known or a ligand binding pocket may be missing. This is the biggest class of nuclear receptors (Kumar & Thompson, 1999; McKenna et al, 1999; Tsai & O'Malley, 1994).
1.3.1 Structure of nuclear receptors

Nuclear receptors share a common structure with four main regions; an N-terminal trans-activating domain (NTD), a DNA-binding domain (DBD), a ligand binding domain (LBD) and a hinge region that connects the DNA-binding domain with the ligand-binding domain, see Fig. 5.

![Common structure of nuclear receptors](image)

**Figure 5 The common structure for nuclear receptors**: An N-terminal domain (NTD), a DNA-binding domain (DBD), a ligand binding domain (LBD) and a hinge region (Hinge) that connects the DNA-binding domain with the ligand binding domain.

The least conserved part of NRs is the N-terminal domain containing a transcriptional activating domain called activation function 1 (AF-1) that can act in a ligand independent manner when separated from the receptor (Germain et al, 2006; Kumar & Thompson, 1999; Warnmark et al, 2003). The DNA-binding domain (DBD) is the most conserved domain. It consists of two zinc finger motifs that each contains four cysteine residues and forms a tertiary structure that interacts with the DNA at the HREs (Kumar & Thompson, 1999). The ligand domain is less conserved than the DBD in terms of sequence, however among the LBDs that have been solved by different means a common structure has been identified (Germain et al, 2006; Kumar & Thompson, 1999; Warnmark et al, 2003).

Steroid hormone or receptor agonist is a compound that binds the receptor and activates a biological response. A nuclear receptor antagonist is a substrate that can bind to the receptor but does not provoke the normal biological response and, thus, acts as a competitive inhibitor of receptor function. One example of a widely used glucocorticoid and progesterone receptor antagonist is RU486 that binds to and blocks the action of the progesterone as well as the glucocorticoid receptor. Progesterone is necessary to sustain a pregnancy, and RU486 is therefore used as an abortion pill (Fiala & Gemzel-Danielsson, 2006).

1.4 ANDROGEN RECEPTOR

Unlike the linker histone, the steroid receptor androgen receptor (AR), is far from being forgotten or underestimated. While a search at Pubmed for “linker histone H1” yielded ~1000 hits, a search for “androgen receptor” gave ~18250 hits (May 2012).

Just like linker histones, the androgen receptor is necessary for correct mammalian development and, as can be understood from its name, it is especially important for male sexual development. During the eighth to ninth weeks of gestation of a normal genetic male, the so called Leydig cells appear in the observable testis (Chemes, 2001). The Leydig cells secret testosterone that is converted by the enzyme 5α-reductase to 5α-dihydrotestosterone, DHT - an even more potent androgen. Both testosterone and
DHT bind to and activate the androgen receptor which is necessary for the development of the male reproductive organs; prostate, testis and growth of the penis during embryo stages as well for the development of secondary sex characteristics later in life; muscle mass, hair and beard growth (reviewed in (Haendler & Cleve, 2012; Hughes et al, 2001)).

AR also contributes to several aspects of development of the central nervous system and to maintenance of its proper function in adulthood (reviewed in (Li & Al-Azzawi, 2009)). In aging men, the levels of testosterone in the brain decreases, something that is thought to give consequences for the brain function and has been implicated to be a risk factor for Alzheimers disease (Rosario et al, 2004). Androgens also affect bone formation. The AR is expressed in osteoblasts (cells that are responsible for bone formation), osteocytes (cells found in the mature bone) and osteoclasts (cells that control the amount of bone tissue). Androgens stimulate the longitudinal growth in boys during puberty and after the cessation of bone growth, both the AR and the estrogen receptors are responsible for the maintenance of skeletal integrity (reviewed in (Carnevale et al, 2010)). Androgens directly stimulate the proliferation of osteoblast precursor via the Wnt signaling pathway (Liu et al, 2007), but also indirectly by increasing vitamin D synthesis that enhance intestinal calcium absorption (reviewed in (Foradori et al, 2008; Hagenfeldt et al, 1989).

Except for its role as transcription factor, the AR has been shown to take part in non-genomic signaling. It has been shown to rapidly modulate ion channels and increase the intracellular level of Ca\(^{2+}\) in a variety of cell types by a variety of non-genomic mechanisms. The AR has also been shown to activate other second messenger pathways, for example, it has been shown to stimulate Raf-1 and ERK-2, members of the MAPK signaling pathway as well as activate cAMP and protein kinase A, PKA and it also directly interacts with and stimulates signaling cascades (reviewed in (Bennett et al, 2010; Foradori et al, 2008)).

### 1.4.1 AR gene

The gene encoding the AR is located on the X chromosome (q11-12) and encodes a cDNA of roughly 2760 nucleotides. The human AR protein consists of approximately 920 aa; residues 1-558 constitute the N-terminal domain, residues 559-624 constitute the DBD and 625-676 constitute the Hinge-domain and 677-920 constitute the LBD (Bennett et al, 2010). However, the exact length of the protein may vary, due to variations in length of a poly-glutamine (poly-Q) and a poly-glycine (poly-G) tract in the N-terminal domain.

The normal poly-Q repeat length is usually said to be some 10-35 residues (Bennett et al, 2010; Itkonen & Mills, 2011). One study found the mean repeat length among 75 healthy patients to be 21±2, with a range of 17 to 26 residues (La Spada et al, 1991). Expansion of the repeats over 35 residues is associated with a decrease in AR transactivating function (Chamberlain et al, 1994). An extension of the repeat to 40-62 residues is the cause behind the neuromuscular degenerative disease spinal and bulbar muscular atrophy (SBMA), or Kennedy’s disease (La Spada et al, 1991). This extension is thought to cause AR to aggregate inside motor neurons and induce neuronal cell
apoptosis, leading to gradual development of neurodegenerative disorders (Finsterer, 2009). The longer the repeat length the earlier the onset and severance of the disease (Igarashi et al, 1992). However, also shorter repeat lengths have been associated with disease development and problems. For example ≤ 19 repeats has been associated with benign enlargement of the prostate defective, ≥ 28 repeats spermatogenesis and ≤ 18 repeats with prostate cancer (reviewed in (Bennett et al, 2010)).

Dissociation from the heat shock proteins and subsequent translocation to the nucleus is triggered by ligand binding, but also by phosphorylation (Lalevee et al, 2010). The androgen receptor is phosphorylated in several residues (reviewed in (Itkonen & Mills, 2011; Kuiper & Brinkmann, 1995)). The binding of a ligand has also been shown to change the phosphorylation pattern of the AR (Wong et al, 2004).

1.4.2 Androgen receptor and prostate cancer

Prostate cancer is the most common form of cancer in Sweden (Cancerfonden) and the second leading cause of male cancer deaths in many Western countries (Cancer.org; Prostate.org.au). Since abnormal AR signalling is a driving force behind the development of prostate cancer the treatment often involves androgen deprivation therapy. The first line of treatment often involves ligand deprivation, while treatment of metastatic prostate cancer involves the use of AR antagonists ((Huggins & Hodges, 1972) reviewed in (Balk & Knudsen, 2008; Barmoshe & Zlotta, 2006; Heinlein & Chang, 2004)). In most patients androgen deprivation therapy is a very effective and well-tolerated method, however the challenge is that the disease will resurface after 18-24 months leading to the development of castration-resistant prostate cancer (CRPC). The development of CRPC coincides with the increased levels of AR in the cells, and a study using prostate cancer xenograft models showed that this increase in AR mRNA production and AR protein level was both necessary and sufficient to convert prostate cancer to CRPC (Chen et al, 2004). The antiandrogens that are available today for treatment of metastatic prostate cancer, such as Bicalutamide (BIC), have a mixed agonist/antagonist activity in certain cellular context (reviewed in (Barmoshe & Zlotta, 2006) and, thus, are not effective against CRPC. The recent development of AR ligands with a more clear-cut antiandrogenic activity may improve the prospects of anti-AR therapy in CRPC.

1.5 CHROMATIN PRESETTING

As was discussed earlier higher eukaryotes such as humans constitute some 200 different cell types and each cell type is defined by its specific combination of expressed genes. The expression patterns for the different cell types are established by a cell-type specific combination of different transcription factors that bind the enhancer DNA and activate transcription. Genome wide studies have made it clear that the HREs are generally a part of a composite element, comprised of multiple factor binding sites (So et al, 2007). Thus, the promoters/enhancers containing HREs are not only bound by hormone receptors,
but also by different regulatory factors. The components of these composite elements tend to be strongly conserved for the same or similar cell type in different species (So et al, 2007).

The same steroid receptor regulates a different set of genes in different cell types. But how is this different transcriptional response achieved? The answer is that the different combinations of transcription factors available in different cell types are responsible for orchestrating the cell-type specific binding of a steroid receptor. Genome wide studies of the chromatin structure in different cell types have revealed that DNase hypersensitive sites (DHS) in the vicinity of steroid receptor binding sites often are established before hormone activation (John et al, 2011). This is due to the binding of other transcription factors to the composite enhancer elements that remodels the chromatin to a more open configuration. In this way the binding of a hormone receptor, or basically any other signalling protein, to a certain enhancer is facilitated. For example, members of the C/EBP transcription factor family were shown to bind to and remodel the chromatin for PPARγ-binding during adipogenesis (Siersbaek et al, 2011). In the case of TGF-β signalling Smad3 has been shown to bind together with Oct4 in embryonic stem cells and Myod1 in myotubes, and hence activate a different set of genes in the different cell types (Mullen et al, 2011).

Thus, the fact that the same steroid receptor can regulate a distinct set of genes in different cell types can be explained by the binding of different sets of cell-specific transcription factors. They act as pioneer factors interacting with chromatin in vicinity of HREs before receptor binding and, thus, programming, or preparing, the chromatin for further binding of steroid receptors. We refer to this process as chromatin presetting. In our system, the MMTV enhancer, we have shown that chromatin presetting can be achieved by various transcription factors (Astrand et al, 2009; Belikov et al, 2004). This will be discussed in detail below.

### 1.6 FOXA1

One example of a transcription factor that has been found to be a critical mediator of nuclear receptor signalling is the forkhead box protein A1, FoxA1 (Belikov et al, 2009; Lupien et al, 2008). *In vitro* chromatin studies indicated that FoxA1 can stably bind within nucleosomes (Cirillo et al, 2002). FoxaA1 belongs to a large family of forkhead transcription factors, with over 40 members in mammals. FoxA1 has been shown to play a vital role in development of pancreas and lung, in kidney function as well as in metabolism and glucose homeostasis (reviewed in (Friedman & Kaestner, 2006)).

FoxA1 is expressed during prostate development and it regulates a number of prostate specific genes (Friedman & Kaestner, 2006; Gao et al, 2003). A genome wide study showed that FoxA1 binding sites often located near the AR binding sites (Wang et al, 2007) and FoxA1 has been shown to mediate both androgen, estrogen as well as glucocorticoid receptor signalling by presetting of the chromatin structure (reviewed in (Augello et al, 2011)). For example, FoxA1 has been shown to promote the expression of AR target genes in prostate development and estrogen target genes in ductal morphogenesis (reviewed in (Augello et al, 2011)).

Overexpression of FoxA1 is a good prognostic marker for breast cancer (Wolf et al, 2007), however, it is a bad prognostic factor for prostate cancer (Gerhardt et al, 2012). Overexpression of FoxA1 was observed 19% of primary and 89% of metastatic tumors
and FoxA1 expression positively correlates with tumor size (Jain et al, 2011). Furthermore, knock down of FoxA1 leads to a reduction of cell proliferation and inhibition of cell migration (Gerhardt et al, 2012).

As mentioned above, the treatment of advanced metastatic prostate cancer includes the use of AR antagonists that block the activity of the androgen receptor. Unfortunately, despite initially good response prostate cancer patients develop resistance to antiandrogen treatment and develop CRPC (Chen et al, 2004). Earlier in our lab it was shown that presetting of chromatin structure by FoxA1 can convert the glucocortiocoid receptor, GR, antagonist RU486 into a partial agonist (Belikov et al, 2009). Since FoxA1 is a bad prognostic marker for prostate cancer and since AR antagonists are not working after a certain time of treatment one may ask: Are these two phenomena related? Could the overexpression of FoxA1 in prostate cancer cells have the same effect on AR antagonists as on RU486?
2 EXPERIMENTAL SYSTEM

2.1 XENOPUS OOCYTE SYSTEM

In this thesis the *Xenopus* oocyte has been used as a model system, or rather an *in vivo* test tube, to study chromatin structure and DNA-protein interactions involved in gene regulation. The oocyte is a giant cell with high capacity to translate introduced mRNA into protein and to assemble injected DNA into chromatin, and has been used since late 1950 to study a variety of cellular processes (reviewed in (Brown, 2004)). The idea behind our experiments is to reconstitute a hormone dependent signal transduction event via expression of protein(s) of interest in the oocyte by cytosolic injection of the corresponding mRNA(s) and intranuclear injection of reporter DNA, and thereafter study how the different transcription factors affect gene regulation and chromatin structure.

2.1.1 Inside the oocyte

![Diagram of Xenopus oocyte with mRNA and ssDNA injection](image)

**Figure 6** Schematic drawing of a Xenopus oocytes, and injection of mRNA and ssDNA respectively (Courtesy of Dr. C. Åstrand)

The stage VI *Xenopus* oocyte has a diameter of 1.3 mm. This can be compared to the size of a human egg that is 100 µm in diameter. The total volume of the *Xenopus* oocyte is about 1 µl. A schematic drawing of this oocyte, and of oocyte injection is presented in Fig. 6. The lower, white part (lighter gray in the picture) of the oocyte is called the vegetal pool, and in a fertilized egg the cells stemming from that part will form the inner tissues such as the gut. The brown pool (darker grey in the figure) is called the animal pool and gives rise to the outer tissues. The remarkably large nucleus of the oocyte, also called the germinal vesicle, is located inside of the animal pool. The diameter of the nucleus is one third of that of the oocyte, i.e roughly 0.4 mm. For comparison: the nucleus of a somatic cell has a diameter of 6 µm. The *Xenopus* oocyte harbors 12 pg of chromosomal DNA and 4000 pg of mitochondrial DNA (Hausen & Riebesell, 1991).

During the maturation of the oocyte the cell accumulates a huge amount of histones and other proteins including basal transcription factors and other components of transcriptional/remodelling machinery as well as tRNAs and rRNA in the cell.
(Adamson & Woodland, 1974). This forms a reserve for later development; the oocyte contains enough material to support the somatic cell division up to mid-blastula transition, equal to about 12 cell divisions. This means that the *Xenopus* oocyte has exceptionally high capacity of translating mRNA introduced in the oocyte into protein. The total mass of histone proteins in the stage VI is 140 ng (Hausen & Riebesell, 1991) indicating that the oocyte contains a huge excess of histones compared to chromosomal DNA. These core histones are often coupled to chaperones, including nucleoplasmin for later deposition on chromatin (Kleinschmidt et al, 1990). An analysis of the core histone content in the oocyte has been carried out showing that except for the canonical core histones the *Xenopus* oocytes contain a high amount of an embryo specific form of H2A.X (Shechter et al, 2009a; Shechter et al, 2009b). It should be noted that the sequence conservation between the human core histones and the *Xenopus* core histones is very high; they differ at only a few amino acids residues.

Collectively, this means that the *Xenopus* oocyte is a useful model system for studying different aspects of chromatin structure, especially for linker histone studies, since they lack somatic H1 (Hock et al, 1993). This makes it possible to introduce different H1 subtypes one by one in the cell, and study their effect on chromatin structure and transcription in a living system.

### 2.1.2 Experimental setup

The schedule for a typical experiment is shown in Fig. 7. The oocytes are injected in the cytoplasm with mRNA(s) coding for a protein(s) of interest. The exact amount of mRNA can vary, but is usually between 0.5-5 ng in an injection volume of 23 nl. After 3-6 h incubation reporter ssDNA is injected into the nucleus. In the studies of H1 effect on chromatin ssM13 filamentous phage DNA was used as template. After injection ssDNA rapidly undergoes second strand synthesis concomitantly with chromatin assembly.

![Figure 7 Experimental setup](image)

*Figure 7 Experimental setup* The oocyte are isolated from the ovaries of the frog and treated with enzyme, liberase, to remove follicular cell layers from the oocytes. The next day mRNA is injected in the morning and ssDNA in the afternoon. One the second day the analysis, MNase digestion, DMS treatment, determination of protein expression, or other kinds of analysis, is performed.

Typically 3-7 ng of ssDNA is injected in a volume of 18 nl. The recovery rate is around 90%, meaning that, for example, injection of 5 ng of ssDNA results in formation of 9 ng of dsDNA. Thus the injected reporter DNA is the dominant one in the nucleus as
compared to the 12 pg of frog DNA. Detection of the chromatin pattern can be made in either by indirect means, such as Southern blotting or directly by the co-injection of $\alpha$-dCTP$^{33}$ that upon incorporation into the DNA during second strand synthesis uniformly label the DNA.

The level of protein expression is monitored either by Western blot or by incorporation of $^{14}$C-lysine during protein synthesis. In the latter case, the proteins are separated with SDS-PAGE and dried gels exposed to a phosphorimager screen. The relative intensity of the bands is then determined with the Image Gauge V4.1 Software.

2.2 MICROCOCCAL NUCLEASE DIGESTION AND DETERMINATION OF NRL

In Paper I we studied the effect of different H1 subtypes on the chromatin structure, using their effect on nucleosome repeat length, NRL, as a readout (Fig. 8). The NRL is determined by measuring the length of DNA that is protected from digestion with micrococcal nuclease, MNase that cleaves the linker DNA between the nucleosomes. Digestion of chromatin with MNase results in a characteristic cleavage pattern, known as nucleosomal ladder. As was discussed above the binding of H1 to chromatin induces an increase in the NRL. This in turns increases the length of the DNA protected from MNase digestion, and thus decreases the electrophoretic mobility of the corresponding DNA fragments on an agarose gel. This can be seen as an upward shift in the nucleosome ladder (see Fig 8B).

To detect the MNase digestion pattern the single stranded DNA was co-injected with $\alpha$-dCTP$^{33}$ and the DNA fragments resulting from MNase digestion of chromatin were separated on an agarose gel. The dried gel was analyzed using Fuji Bio-Imaging FLA-7000 analyzer and the Image Gauge V4.1 Software. To determine the NRL the pattern of each lane was digitalized and presented as a profile. The upwards shift in the nucleosome ladder induced by the H1 protection of the linker DNA can be seen as shift in the location of the peaks corresponding to the polynucleosomes (Fig. 8C). By determining how the maximum of each peak is related to the known DNA marker the NRL can be determined.

However, objective determination of the location of each maximum could be a problem. In addition, we sometimes dealt with very small differences. Therefore I developed a method where one makes use of a computer based curve-fitting algorithm in the program PeakFit® (Seasolve Software) that smoothens each peak and assigns it a value based on its electrophoretic mobility. This was done both for the profiles of the DNA markers and the profiles of the MNase digestion patterns. The known marker DNA lengths were then plotted as a function of their electrophoretic mobility. By inserting the value obtained for the threenucleosomal band into thus obtained equation for the closest ladder, the size of that band could be determined and, hence, the NRL calculated.
Figure 8 Determination of H1 induced increase in NRL (A) The nucleosome repeat length (B) The MNase digestion pattern in the absence and presence of H1 (C) Profile of lanes 1 and 4 in Fig 8B

The profiles of the MNase patterns were smoothened using the Loess algorithm while the DNA markers were smoothened using FFT filtering. Different algorithms were used for the profiles of the MNase pattern and for the peaks of the ladder since the FFT filtering algorithm is more suitable for sharp peaks.

Another possible difficulty when determining NRL is bending of or “smiling” of the gel that results in shifting of the MNase pattern in relation to the ladder. When performing MNase digestion the oocyte homogenate was split in three equal portions that were digested individually. In this way we ended up with three independently analyzed samples from each H1 mRNA concentration that was used. To further minimize the influence of the bending of the gel the triplicates were loaded next to each other on the gel, in between two ladders so that each lane could be compared to the marker loaded in the next or second next lane. The NRL in the two lanes at the edges were determined using the equations of the marker next to it. The NRL of the lane in the middle was calculated with the equations of the markers on either side, and then a mean value was used.

It should also be noted that due to technical problems occurring during gel drying the peaks are not totally smooth and this unevenness may play a role in where the program places the maximum. To minimize these problems gels were dried on positively charged membranes, to make sure that the DNA was not sucked through the nylon support during the drying process. As described above, each group was run in triplicates. If the pattern was very uneven, and the peak(s) clearly misplaced the sample was disregarded.
2.3 THE MMTV ENHANCER

As a model system for chromatin studies we have utilized reporter DNA containing the long terminal repeat, LTR, of the mouse mammary tumor virus, MMTV fused to the herpes simplex virus thymidine kinase gene and cloned in an M13 vector (Belikov et al, 2000). The mouse MMTV is a retrovirus that causes tumors in the mammary tissue of mice. It is activated by pregnancy hormones and transmitted through the milk of the infected mothers to their offspring (reviewed in (Gunzburg & Salmons, 1992)). The 1.2 kb MMTV LTR harbors four steroid hormone binding elements (Figure 9) and it is one of the most widely used model system for glucocorticoid regulation (Gunzburg & Salmons, 1992; Ringold, 1979) but it can also be activated by progestins and androgens via binding of progesterone and androgen receptor, respectively. In addition to the steroid hormone receptors response elements, the MMTV LTR also harbors binding sites for nuclear factor 1 (NF1) (Nowock et al, 1985) and octamer binding factor 1 (Oct1) (Bruggemeier et al, 1991). Furthermore, binding sites for the forkhead transcription factor FoxA1 were found at -225 (single site) and at -51/-39 (double site) as well as -360/-332 (double site) relative the transcription start site (Belikov et al, 2009).

![Figure 9 The MMTV reporter construct with indicated transcription factor binding sites](image)

In tissue culture cells, the MMTV LTR is organized on six nucleosomes, A-F, where the transcription start site is located at nucleosome A and the steroid response elements are located at nucleosome B. Hormone induced transcriptional activation does not cause any major changes to the nucleosome positioning, however the region corresponding to the B nucleosome, -60 to -250, becomes hypersensitive to DNaseI due to chromatin remodeling induced by the binding of steroid hormone receptors (Fragoso et al, 1995; Richard-Foy & Hager, 1987).

2.3.1 MMTV enhancer studies in Xenopus oocytes

In contrast to the translational positioning of the nucleosomes in the MMTV LTR in tissue culture cells, no preferred nucleosomal positioning along the DNA is observed in the inactive MMTV promoter upon reconstitution in Xenopus oocytes (Belikov et al, 2000). However, hormone induction results in nucleosome arrangement similar to that in tissue culture cells, i.e a distinct translational positioning of six nucleosomes covering the MMTV LTR (Belikov et al, 2000). This rearrangement of nucleosomes is independent of transcriptional activity, since it occurs also when transcription is
inhibited; the presence of one high affinity steroid hormone response element is sufficient for induction of nucleosome positioning after addition of hormone in the presence of glucocorticoid receptor, GR (Belikov et al, 2000). This nucleosome positioning is reversible since addition of glucocorticoid antagonists results in transcription secession and reverts it to the random arrangement of nucleosomes as observed in the absence of hormone (Belikov et al, 2001). Addition of NF1 and Oct1 preset the MMTV chromatin in *Xenopus* oocytes for hormone induction; i.e arrange nucleosomes in a positioning intermediate between the non-induced state and the transcriptional arrangement, which leads to a faster binding of hormone receptors and a stronger hormone response (Belikov et al, 2004). Also FoxA1 has the capacity to open the chromatin structure for a more active response of the GR and enhanced hormone induced transcription (Belikov et al, 2009; Belikov et al, 2012).
3 RESULTS AND DISCUSSION

3.1 PAPER I

One unsolved question regarding the role of H1 is whether its presence in several similar variants in higher metazoans has a functional role. The Xenopus oocyte has no somatic linker histones but do contain an oocyte-specific maternal linker histone, B4, which, however, does not compete with somatic H1 for chromatin binding (Belikov et al, 2007). The lack of somatic types of H1 in the Xenopus oocyte thus offered the possibility to introduce and study H1 variants in the living cell individually. As readout for changes in chromatin we used the H1 induced increase in NRL. While the positive correlation between high H1 content and longer NRL is well established (Woodcock et al, 2006), the effect of individual subtypes is not previously known. We focused on comparing the human ubiquitously expressed somatic subtypes, H1.2-H1.5 as well as the Xenopus somatic subtype H1A, the Xenopus differentiation specific subtype H10 and the chicken differentiation specific subtype chH5.

We decided to use the human hH1.4 subtype as a reference, and thus studied how different subtypes behave in comparison to hH1.4. Expression of increasing amounts of hH1.4 by cytoplasmic injection of hH1.4 mRNA results in a gradual increase in the NRL (Fig 8B and 8C). The increase in NRL reaches a plateau corresponding to ~1 H1 bound per nucleosome (Belikov et al, 2007) thus arguing for that H1 binding is a saturable and, hence, specific.

We started by comparing hH1.4 to the linker histones that differed from hH1.4 in terms of origin and/or expression pattern. Surprisingly no significant differences were observed between chH5, xH10, xH1A and hH1.4 in terms on their effect on NRL, however, we note that xH1A displays a lower affinity for chromatin binding. When comparing hH1.4 to hH1.2 or hH1.3 we saw significant differences in the effect on chromatin structure: hH1.4 typically results in an increase of 16.5±3.5 bp, while both hH1.2 and hH1.3 give rise to an increase of roughly 6 bp (See summary in Fig 10).

A comparison of hH1.4 and hH1.5 revealed that at the same level of expressed protein, hH1.4 elicits a much stronger effect on the NRL in comparison to hH1.5 and not even when hH1.5 is vastly overexpressed in the oocyte does the saturation curve show any signs of approaching a plateau. Thus, H1.5 expressed in Xenopus oocytes is not able to bind specifically to the chromatin target.

We conclude that the ubiquitously expressed somatic human H1 variants have distinct effects on chromatin structure in vivo as well as different affinities for chromatin binding. hH1.4 has the strongest effect on NRL and hH1.2 and hH1.3 have the weakest. hH1.5 does not appear to bind to a specific chromatin site, since saturation is not reached, and has the lowest affinity for chromatin binding. These results argue for that the biggest differences neither take place between H1 subtypes from different species nor H1 subtypes differing in terms of expression pattern; on the contrary the biggest differences are found between the somatic human subtypes, the subtypes that in mice
The increase in NRL induced by the presence of the different H1 subtypes have been shown to be able to replace each other to a certain extent and that are present in one and the same cell. Thus, our data supports the idea that different H1 subtypes play different roles in chromatin structure.

In our hands hH1.4 resulted in the same increase in NRL as chH5, xH1\(^0\), and xH1A - H1 subtypes known to participate in formation of heterochromatic chromatin and gene silencing. xH1A is involved in the silencing of the 5S gene (Bouvet et al, 1994; Kandolf, 1994). chH5 is present in chicken erythrocyte which is a terminally differentiated cell with very compact and inert chromatin with long NRL. The chicken erythrocyte is indicated to have a total of 1.3 molecules of linker histones per nucleosome; 0.4 chH1 and 0.9 chH5 (Bates & Thomas, 1981; Morris, 1976). During the erythropoiesis the NRL increases from 190 bp to 212 bp. This increase is correlated with the rising concentration of H5, but not H1, in these cells (Weintraub, 1978). During the maturation of Xenopus erythrocytes, the content of xH1\(^0\), namely xH1\(^0\)-2 the subtype used in the study, increases threefold (Koutzamani et al, 2002; Rutledge et al, 1984). This argues for that the xH1\(^0\)-2 function in Xenopus erythrocytes may be analogous to that of chH5 in chicken erythrocytes.

Given the similarities in effect on chromatin structure between hH1.4 and chH5, xH1\(^0\) and xH1A we suggest that hH1.4 mainly participates in the formation and maintenance of heterochromatic structure and gene silencing. This would agree with a study by Th’ng et al. (Th’ng et al, 2005) who by using GFP-tagged H1 subtypes demonstrated that H1.4 is preferably localized in heterochromatic regions and H1.2 and H1.3 in euchromatic regions. The overexpression of mouse H1\(^0\) and mouse H1.2 in cultured mouse fibroblasts showed that while both variants resulted in an increase in NRL the H1\(^0\) overexpression resulted in significantly more dense chromatin resistant to MNase.

**Figure 10 Summary of result from Paper I.** The increase in NRL induced by the presence of the different H1 subtypes.
digestion (Gunjan et al, 1999). This is in line with our data, that shows that H1.2 and H1.3 yielded a short increase in NRL. Yet another study classified hH1.4 (as well as hH1') as a strong chromatin condenser, hH1.3 as an intermediate and hH1.2 (and hH1.1) as a weak condenser (Clausell et al, 2009). Thus our data are in agreement with the results from others and suggest a primary role in formation and/or maintenance of more dense and/or inactive chromatin structures for hH1.4 and a role in formation and/or maintenance of more active chromatin structures for hH1.2 and hH1.3.

Regrettably we did not have enough time to elucidate the effects of the different H1 subtypes on gene expression. We performed initial studies aiming at elucidation of the effect of hH1.4 and hH1.2 on transcription factor, GR, binding and the MMTV transcription. Both hH1.4 and hH1.2 were found to stimulate GR binding, however the effect of hH1.2 was more pronounced. hH1.2 also give slightly more stimulation on MMTV transcription level as compared to hH1.4. These differences were, however, not significant. Our results are in agreement with studies by others where the roles of H1 subtypes in functioning of eukaryotic genome have been elucidated. Overexpression of either murine H1'0 or murine H1.2 in 3T3 cells showed that H1'0 inhibited transcription of more genes than H1.2 (Bhan et al, 2008; Brown et al, 1996). When H1'0 and H1.2-H1.5 were knocked down one by one in human breast cancer cell the ratio of down-to-upregulated genes after knock down of hH1.2 was of 2.7, while the same ratio for knock down of hH1.4 and hH1.5 was 1.4 and 1 respectively. This strengthens the notion of hH1.4 and hH1.5 having more repressive role compared to hH1.2. Interestingly, the ratio of down-to-up regulated genes in hH1'0 knock down cells was 1.9, i.e. higher than for hH1.4 and hH1.5 (Sancho et al, 2008). As described earlier knock down of either H1.2, H1.3 or H1.4 had no visible effect on the phenotype of the affected mice (Fan et al, 2001). A more detailed analysis revealed that deletion of H1.3 and H1.4, or H1.4 in combination with H1'0 attenuated the age-dependent silencing of the human β-globin transgene, while the deletion of H1.2 or H1'0 alone did not affect the rate of silencing (Alami et al, 2003). ChIP based studies have shown that hH1.3 and hH1.4 are depleted from active chromatin, as opposed to hH1.5. The same study also showed that H1.2, unlike the other subtypes, remained present at the HSP90 promoter after heat shock induction (Parseghian et al, 2001; Parseghian et al, 2000). Hence, this study supports the notion of H1.4 as being a part of repressive chromatin structure and H1.2 to be enriched in active chromatin. It should be noted that the observed depletion of H1.3 from active chromatin and the presence of H1.5 in the same study disagree with results by others (Sancho et al, 2008) as well as our expectations based on its short increase in NRL. Here it should be pointed out that there have been major problems in the making of antibodies that are specific for a certain H1 subtype and hence, one cannot exclude that the data from this study might be compromised depending on the quality of the antibodies.

Taken together, these results indicate that the chromatin structure induced by H1.2 binding is more open and facilitates transcription while the chromatin structure formed by H1.4, H1'0 and H1.5 is more repressive. When it comes to H1.3 the results differ, and we suggest that this one might be more of an intermediate subtype. However,
neither of the H1 subtypes is fully either euchromatic or heterochromatic. For example, H1.2 and can also act as repressor of certain genes, as when it is part of the complex that represses p53 mediated transcription (as described above) (Kim et al, 2008).

But what is the cause of the different effects on NRL and the different affinity for chromatin binding that we observe? Results from others have revealed that H1 binding for the most part is controlled through cooperation between the globular and C-terminal domains (Brown et al, 2006; Stasevich et al, 2010) while chromatin compaction abilities and affinity for chromatin binding are largely influenced by the C-terminus (Clausell et al, 2009; Th'ng et al, 2005). In Paper I, we do not find any correlation between effect on NRL and the factors that have been proposed to influence H1’s binding abilities i.e. C-terminal amino acid composition length, distribution of positive charge and number of phosphorylation motifs. The biggest differences are found between xH1A, xH10 and chH5 on one hand and the human somatic subtypes on the other. Data from others have suggested that different regions of the C-terminal domain contribute differently to H1’s binding affinity in vitro (Caterino et al, 2011) and that some parts are more crucial than others for H1’s ability to stabilize folded secondary chromatin structures (Lu & Hansen, 2004). Another study shows that amino acid composition in different parts of the H1 C-terminal domain rather than sequence determines H1’s binding abilities (Lu et al, 2009a). However, when aligning the C-terminal domains of the different linker histone subtypes to that of hH1.4 and dividing each tail in four parts in order to compare the distribution of positively charged residues as well as phosphorylation motif distribution between subtypes the biggest similarities were once again seen between the human subtypes, while chH5, xH10 and xH1A differed (Paper I, Supplements). In Paper I we thus conclude that other factors than amino acid composition of the C-terminal domain affects H1’s effect on NRL.

3.2 PAPER II

The N-terminus of H1 has received much less attention compared to the C-terminal domain. In Paper II we used the same experimental strategy as in Paper I to elucidate the behaviour of hH1.4 devoid of its N-terminal domain, ΔN-hH1.4, as compared to hH1.4 in its wild type form when it comes to effect on chromatin structure and its affinity for chromatin binding. We showed that wt hH1.4 and ΔN-hH1.4 results in the same final increase in NRL. However, 3-4 fold more ΔN-hH1.4 compared to wt hH1.4 was needed to reach the same effect on NRL and at higher levels of ΔN-hH1.4 the nucleosome ladder pattern started to deteriorate and become smeary. This kind of pattern can otherwise be seen when H1 is vastly overexpressed in the oocyte, and starts to bind unspecifically (Belikov et al, 2007). In the case of ΔN-hH1.4 the smearing of the pattern was present already at the point where saturation of the DNA was reached. At the same amount of wt hH1.4 no deterioration in the nucleosome ladder was observed. When doubling the amount of ΔN-hH1.4 the smearing of the pattern was even more pronounced. The lack of N-terminus seems to impair the ability of hH1.4 to bind specifically to chromatin, as reflected by the progressive deterioration of the pattern. Thus, a larger fraction of ΔN-hH1.4 takes part in unspecific chromatin interactions and it is not surprising that more protein is needed to reach saturation as
compared to the wild type hH1.4. Our results imply that the N-terminal domain is an important determinant of affinity and specificity of H1-chromatin interactions.

However, since the absence of a N-terminal domain did not affect H1.4’s effect on increase in NRL it seems unlikely that differences in the N-terminal domains are directly responsible for the different effect on NRL we observed in Paper I.

So what is then the explanation for the differences observed? The N-terminal domain together with the globular domain have been shown to be responsible for correct localization pattern of the oocyte specific H1 subtype H1FOO (Becker et al, 2005) and domain swapping revealed that the individual effects on transcription of H1^0 and H1.2 upon overexpression in 3T3 cells (Brown et al, 1996) followed the globular domain (Brown et al, 1997). By the use of point mutations in the globular domain and FRAP the binding domain of H1.2 was shown to be distinct from that of H1^0 (George et al, 2010). This would lead to different binding geometry of these subtypes, and this would most probably lead to different kinds of chromatin structure. At a first glance the differences in globular domains does not provide the explanation for the differences observed between the human subtypes in Paper I since the globular domain of hH1.2 and hH1.3 are identical to that of hH1.4 (Fig 4). However, if one is allowed to speculate; the fact that the amino acid sequences of the globular domains of these linker histones are identical does not necessarily mean that they bind DNA in the same way. In this respect one should not disregard the potential impact of the C-and the N-terminal domains and their influence on the geometry of H1 binding. A recent Paper by Vyas and Brown indicates that swapping the C-terminal domains of H1.2 and H1^0 also resulted in the corresponding change in chromatin interaction surface (Vyas & Brown, 2012). This result indicates that the C-terminal domain is responsible for the correct placement of these H1 subtypes. The different effects of H1 subtypes may thus be explained by a combined effect of the globular and C-terminal domain. In the study by Vyas and Brown, swapping the N-terminal domain of H1.2 for that of H1^0 did not change the interaction surfaces of the globular domain, however, switching the N-terminal domain of H1^0 to that of H1.2 did slightly impair the binding mode of H1^0, although not to the same extent as when the C-terminal domains were exchanged. Thus it seems like the N-terminal domain is of slightly more importance for the binding of H1^0 than for H1.2. The result from this study indicates that the N-terminal domain is of more importance for some subtypes than others. When the C-terminal domains of H1.5 and H1.4 were exchanged for that of H1.1 the resulting chimera protein behaved almost identical to H1.1 but when the C-terminal domain of H1.1 was swapped to that of H1.4 or H1.5 the chimera protein displayed recovery rate after photobleaching that was an intermediate of H1.1 and H1.4/H1.5 (Th'ng et al, 2005). The binding affinity thus follows the C-terminal domain in some cases but not in others, lending further proof to the concept of the domains having different roles in different subtypes.

We conclude that the answer to the question of what decides H1’s binding abilities cannot be found solely in the sequence of the N-terminal, the C-terminal or the globular domain, but most probably involves a combination of all these domains. We also propose that the exact contribution of each domain may differ between subtypes.
As described previously, many antiandrogens that are available today for treatment of prostate cancer, such as bicalutamide (BIC), have mixed agonist/antagonist activity in certain cellular context. The recent development of AR ligands with a more clear-cut antiandrogenic activity may improve the prospects of such therapy. MDV3100 (MDV) or Enzalutamide is an AR antagonist developed by Medivation for the treatment of CRPC and is currently in Phase 3 clinical trials. Since high levels of transcription factor FoxA1 is a negative prognostic factor in prostate cancer and FoxA1 was previously shown to convert the glucocorticoid antagonist RU486 to a partial agonist we found it of interest to investigate the possible effect of FoxA1 on the transcriptional outcome of AR antagonists.

To address the effect of FoxA1 on AR action oocytes were injected with AR mRNA only or with a combination of AR and FoxA1 mRNA and placed in media containing agonist R1881 or either of the antagonists: BIC or MDV. As control we used oocytes injected with DNA only since a drawback of the Xenopus oocyte system in these studies is that the oocytes contain significant amounts of endogenous androgens. Nuclear translocation induced by either of the ligands was studied by analysis of the protein content of manually isolated nuclear and cytoplasm by Western blotting. We were able to show that both antagonists, upon binding to AR, can translocate the AR-ligand complex to the nucleus, albeit with reduced efficiency for MDV. While in the nucleus both AR-antagonist complexes have the potential to bind sequence specifically to the HRE. The DNA binding is strongly enhanced by co-expression of FoxA1 that presets the enhancer chromatin and, hence, makes the HRE more accessible for AR binding. In this context the AR-BIC complex antagonistic activity is considerably compromised as demonstrated by a significant chromatin remodeling and induction of a robust MMTV transcription whereas AR-MDV shows a more persistent antagonistic activity. Since the Xenopus oocyte contains endogenous androgens we are unable to separate the loss of inhibition from the intrinsic agonistic activity that may be exerted by the AR-BIC complex in the FoxA1 context. On the other hand the endogenous androgens present in oocytes may mimic a clinical situation since the tumor tissue may produce androgenic ligands and where high levels of FoxA1 expressed in the cancer cells might compromise the therapeutic effect of BIC. This motivates the search for compounds with a more persistent antiandrogenic activity.

In conclusion our work provides a mechanistic explanation of how prostate cancer cells may become insensitive to BIC and sheds light on how AR-antagonist complex function in the context of FoxA1.
4 PRELIMINARY RESULTS

One factor that makes the study of the linker histone subtypes problematic is difficulties is raising specific antibodies against different H1 subtypes. Thus many studies on the linker histones have been performed with GFP-tagged H1, for example when studying intracellular localization of different subtypes or H1 chromatin binding properties by FRAP (Hendzel et al, 2004; Lever et al, 2000; Misteli et al, 2000). However, the addition of the GFP-protein, i.e. 238 extra amino acids, to the 200 aa H1 protein has the potential to affect its properties, such as affinity for chromatin binding and its effect on chromatin structure. A study by Hendzel et al showed that H1.1 had much lower affinity for chromatin binding when the GFP tag was placed in the C-terminal domain as compared to when it was placed in the N-terminus. In fact, the presence of a GFP-tag in the C-terminal domain had almost the same effect as deleting the whole N-terminus (Hendzel et al, 2004). However, no study has been made where GFP-tagged H1 has been compared to untagged H1. Thus one cannot exclude that the GFP tag also affects the properties of H1 when placed in the N-terminus.

Using the same experimental setup as described in Paper I and Paper II we have compared the wild type hH1.4 to hH1.4 tagged with GFP in either N-or C-terminal domain. Our preliminary results indicate that when placed in the N-terminal domain the GFP-tag has no influence on hH1.4’s affinity for chromatin binding or effect on NRL. However, when fused to the C-terminus, the GFP tag significantly compromises H1 affinity for chromatin binding, but again has no effect on the increase in NRL.

We are planning to continue these studies by addressing how the GFP-tag affects hH1.4’s mobility in the oocyte nucleus by the use of FRAP to be able to compare the two methods.
5 CONCLUSIONS

In this thesis I have shown that:

(I) Ubiquitously expressed H1 subtypes differ in their effect on chromatin structure. This strengthens the idea of H1 subtypes having individual roles in chromatin organization and that the presence of a certain subtype on the chromatin fiber may contribute to the formation of a distinct type of chromatin structure.

We suggest that hH1.4, which results in an increase in NRL comparable to those of subtypes found in repressed chromatin in specialized cells, may contribute to the formation and/or maintenance of repressed chromatin. hH1.2 and hH1.3 both result in about half of the effect on NRL as compared to hH1.4 and in agreement with data from others (Alami et al, 2003; Clausell et al, 2009) we propose an euchromatic role for hH1.2 and an intermediate role for hH1.3.

(II) The N-terminal domain was shown to support H1’s ability to bind specifically to chromatin, but it had no effect on NRL. We suggest that the N-terminus is involved in the correct positioning of H1 along the chromatin fiber.

(III) The chromatin presetting by transcription factor FoxA1 heavily compromises the antagonist properties of the first-generation androgen receptor antagonist BIC and to some extent also of the second-generation antagonist MDV.

We showed that the AR-MDV complex is translocated to the cell nucleus, albeit to a lesser extent than the AR-BIC complex. While in the nucleus, both complexes have the same ability to bind specifically to the HREs. Upon DNA binding agonist-AR complex recruit co-activators needed for chromatin remodeling. This ability is only slightly compromised for BIC-AR complex in the presence of FoxA1 while in the same context AR-MDV fails to recruit coactivators and other components of chromatin remodeling machinery. Since high levels of FoxA1 have been shown to be a bad prognostic factor for prostate tumors (Gerhardt et al, 2012; Jain et al, 2011) these findings may be of clinical relevance.
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