DIPLOMARBEIT

Titel der Diplomarbeit
„Control of Elongation of Replication-activated Histone Genes“
Band 1 von 1

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angestrebter akademischer Grad
Magister der Naturwissenschaften (Mag.rer.nat.)

Wien, 2011

Studienkennzahl lt. Studienblatt: A 490
Studienrichtung lt. Studienblatt: Diplomstudium Molekulare Biologie
Betreuer: Dr. Michael Jantsch
ACKNOWLEDGMENT

As I truly appreciate that I got the opportunity to accomplish the practical part of my degree dissertation at Sir William Dunn School of Pathology, University of Oxford, I first of all want to thank Dr. Shona Murphy who not only gave me this chance through warmly welcoming me into her group, but also constantly supported me during my work. Finding a place where colleagues become friends is not a matter of course, and therefore I especially want to thank my friends and supporters Dr. Martin Dienstbier, Dr. Clélia Laitem, Justyna Zaborowska, Hadeel Al-Rawaf, Pilar Vazquez Arango, Dawn O’Reilly and Alice Taylor, all of them members of Dr. Murphy’s group in 2010.

Additionally I want to show my appreciation to the University of Vienna for supporting me with two scholarships for this work and hence eased my stay abroad, and the Sir William Dunn School of Pathology for welcoming me as a visiting scientist and providing its facilities to me.

And last but not least I want to thank my parents for being good ones and for giving me the fundamentals and support to be able to accomplish my academic work, here and elsewhere.
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General Introduction

1.1 THE MEANING OF BIOLOGICAL LIFE
1.2 HOW CHEMISTRY BECOMES MOLECULAR BIOLOGY
1.3 TRANSCRIPTION
1.4 THE CELL CYCLE
1.5 HISTONES
1.6 HISTONE MODIFICATIONS
1.1 The Meaning of Biological Life

To maintain its existence, each biological species, from a protozoan bacterium to the *Balaenoptera musculus*, also known as *blue whale*, has to carry its genetic information save and has to hand it to its progeny as error-free as possible. To recognise how one can fulfil this challenge, one has to take a closer look on the molecules, which are used by most life-forms on earth to carry their genetic information: the Deoxyribonucleic Acid (DNA)

![Illustration of a double stranded DNA-molecule with paired bases](http://www.accessexcellence.org/RC/VL/GG/dna_molecule.php)

### Figure 1

Illustration of a double stranded DNA-molecule with paired bases$^1$: Adenin=Thymin, Guanine=Cytosine

Each DNA strand consists of a periodic chain of a phosphate group linked to a deoxyribose molecule, which again is linked to a phosphate group and so on. Additionally each deoxyribose is connected with one of the 4 bases found in DNA: Adenine, Thymine, Guanine or Cytosine (shown in turquoise). One of these bases together with its associated sugar and a phosphate group is called nucleotide; hence a DNA strand is a periodic succession of 4 possible nucleotides.

The bases themselves can form hydrogen bonds with their associate base (2 bonds between Adenin and Thymin, 3 between Guanine and Cytosine) from another DNA strand, which leads to the known rope-ladder structure of an imagined untwisted DNA Molecule. In fact, the molecule forms a double helix that has a width of 2.37 nm and a length of 3.4 nm per 10 baipairs (bp). As

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the human genome consists of about $3 \times 10^9$ bp, it gives a hypothetical variability of $4^{3000000000}$ possible sequences. But in fact nearly 100% of the DNA sequence is equal in all humans, except of a few but important differences.

Having the 4 bases in different order, the DNA acts as a long molecule coding for the construction and metabolism of each cell, and therefore for each organism. And due to the fact that our genomic information is stored in a double stranded helical molecule, in which each strand is complementary to the other one, the basic mechanism how cells can replicate their DNA to give one set of it to its progeny is quite easy to understand:

![Figure 2: DNA Replication Fork](image)

Each double-stranded DNA molecule becomes untwisted and separated for replication by specific enzymes (not shown) and then a new strand is added by a DNA dependent DNA polymerase (it uses a DNA strand as template, to form a new, complementary DNA strand) always from 5’ to 3’ (see figure 3). Due to that fact and the anti-parallel orientation of the two strands, one daughter strand can be synthesized continuously, but the other one has to be done fragment by fragment as an elongation of 150-200bp long Okazaki fragments that were synthesized before [1, 2]

1.2 **HOW CHEMISTRY BECOMES (MOLECULAR-) BIOLOGY**

Knowing how a double stranded molecule replicates itself is an important information for understanding life, but it does not tell anything about how an organism or even a single cell builds up itself and metabolises. Therefore one has to look in detail on the sequences in DNA and on the mechanism that translates it into the required function.

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For DNA sequences to ‘express’ a function, they must be copied into ribonucleic acids (RNAs) using the DNA as a template. Not all DNA has a clear function. Some have, as far is known, no function at all, some act as regulatory sequences, some code for stable functional RNAs, while others code for messenger (m)RNAs, which are translated into proteins. A region in DNA, which codes for a distinct RNA molecule is called gene. The expression of genes is strictly regulated by different sequences on the DNA and by the involvement of many enzymes during the synthesis of RNA templated by a region of DNA. This process is called transcription. The information within a protein-coding gene is first transcribed to RNA by a DNA dependent RNA-polymerase, an enzyme complex that catalyses this reaction by using DNA as template to produce a complementary RNA. Then the information is translated from RNA to protein by an RNA-protein complex termed a ribosome.

Proteins are the major components of our cells, which can act as structural, enzymatic or signal molecules. They consist of a periodic chain of 21 different amino acids that are assembled during translation where RNA is acting as template. 3 RNA nucleotides (a triplet) code for one amino acid. For example AAA codes for Lysine, AAU for Serine, GAC for Alanine and AAAAAUGACCAU for Lysine-Serine-Alanine-Histidine. A protein can have a length of 50 to 30 000 amino acids, but even much shorter amino acid chains (peptides) can act as signal carrier [1, 2].

**Figure 3** The path from DNA to protein. The DNA is first transcribed to RNA, which is then translated into a protein. During transcription, the same complementary nucleotides are used as during DNA-replication, only Uracil rather than Thymidine is added to the emerging...
RNA strand if there is an Adenine in the template DNA. During translation, 3 nucleotides of RNA code for one amino acid.

1.3 Transcription

The act of transcription is related in all organisms, but there are several differences between prokaryotes and eukaryotes. In prokaryotes, the RNA-polymerase acts together with its detachable subunit $\alpha$-factor that recognizes the site where transcription should start (promoter) [3]. In eukaryotes, many more ancillary factors are required to support the multisubunit RNA polymerases. Furthermore, there are 3 different types of RNA polymerase in eukaryotes [1]:

Polymerase I: transcribes 5.8S-, 18S- and 28S-ribosomal RNAs
Polymerase II: transcribes all protein coding genes and snoRNAs (plus some snRNA genes)
Polymerase III: transcribes tRNA genes, some snRNA genes and 5S-ribosomal RNA

In eukaryotic cells, transcription starts with the recognition of the TATA-Box, a Thymine and Adenine rich sequence 25 base-pairs (bp) upstream from the start of the transcribed region, by the transcription factor (TF) TFIID. This factor contains a TATA-binding protein (TBP), which recognizes the TATA-Box and binds it. Subsequently, other TFs follow and finally recruit the RNA polymerase II (Pol II). The C-Terminal Domain (CTD) of the largest subunit has a tremendous effect on Pol II activity by recruiting and interacting with several transcription and RNA processing factors [4] (see below). It consists of tandem repeats of the heptapeptide sequence Tyr$^1$–Ser$^2$–Pro$^3$–Thr$^4$–Ser$^5$–Pro$^6$–Ser$^7$.

After Pol II has formed the preinitiation complex with several TFs (Figure 4), the Ser$^5$ becomes phosphorylated by the cycline dependent kinase 7 (CDK7) subunit of TFIIH near the 5′-end of protein coding genes [5]. This is a signal to recruit the capping enzyme that adds the 5′-7-methylguanosine cap to the newly synthesized RNA [6]. For elongation of the new RNA strand of mRNAs the phosphorylation of Ser$^2$ by the positive transcription elongation factor B (P-TEFb) is an important event [7]. However, this is not the case for transcription of snRNA genes [8]. Finally Ser$^7$ becomes phosphorylated, which appears to be important only for the functional 3′-processing of snRNAs [9].
Figure 4 | Simplified sketch of the initiation of transcription in eukaryotic cells. A) TBP on TFIIID recognizes the TATA-Box on the DNA, binds it and causes a bend in the DNA (not shown). B) Bonded TFIIID enables binding of TFIIA, TFIIB and other TFs including TFIIH. C) Polymerase II (Pol II) binds the TFs to form the preinitiation complex. D) TFIIH opens the double helix and phosphorylates the C-Terminal Domain (CTD) of the polymerase at the position Serine 5. This allows the polymerase to escape the promotier and an RNA is synthesized 5’ to 3’.
Whilst a new mRNA is synthesized during transcription elongation in the nucleus, it is being processed and prepared for transport into the cytoplasm where the translation into a protein takes place. All pol II-transcribed RNAs become 5'-7-methylguanosine capped right after the beginning of transcription. This cap is known to stabilise against exonucleases, promote transcription and splicing and plays a role in RNA 3'-end formation [10]. In mRNAs non-protein-coding sequences of the RNA become spliced out (introns) and the remaining exons are linked together, and the 3’ end becomes poly-adenylated. This final poly-adenine tail (polyA-tail) protects the mature mRNA from degradation, promotes transport into the cytoplasm and plays an important role in translation [11]. Polyadenylation is directed by the polyadenylation signal found at the end of most protein-coding genes.

In contrast, there is a population of mRNAs that lacks intronic regions and the polyA-tail. Some of them are small nuclear RNAs (snRNA) that are part of the spliceosome complex that is responsible for splicing. Those snRNAs do not have a polyA-tail and a gene-specific 3’-box that is located 9-19bp downstream of the coding region of the snRNA promotes correct processing of the 3’-end of the emerging RNA [12].

Figure 5 | Comparison of a common protein-coding mRNA and a replication-activated histone gene mRNA. A) Common mRNAs feature a 5’ 7-methyl-guanosine cap, Introns between their coding sequences that are spliced out during processing, and they are ending in a polyA-tail, which is added by a polyA-polymerase. B) Replication-activated histone gene RNAs are 5’ capped as well, but
they lack Introns in their coding region and instead of a polyA-tail they end in a specific 3’ stem-loop that acts as processing signal.

Another very special subset of mRNAs is the replication-activated histone mRNAs (Figure 5). These mRNAs lack introns and a polyA-tail, and their 3’-end is formed through endonucleolytic cleavage after a conserved ACCCA-sequence upstream of the histone downstream element (HDE). Just upstream of the HDE is a conserved stem-loop. This stem-loop is important for processing and stabilizing the 3’-end of the mRNA of replication-activated histone genes. It interacts with the stem-loop binding protein (SLBP) [13] that recognizes and binds the stem-loop and stabilizes interaction of U7 snRNP with the HDE, which is essential to process the mRNA at its 3’-end [13,14].

1.4 THE CELL CYCLE

In metabolizing eukaryotic cells the cell cycle is divided into two major phases, interphase in which the cell grows, gathers nutrients, metabolizes and replicates its DNA, and the mitotic phase in which the cells divides into two. Interphase itself is divided into 3 phases: G₁, S and G₂

**G₁ phase** (G stands for gap) starts right after the previous cell division. During this phase the synthesis of many metabolites is realized, especially those needed for DNA replication. If there is a lack of some required nutrients, the cell stays arrested in G1 phase. In particular, the cyclins and their partners, the cyclin-depended kinases (CDK) are responsible for continuing the cell cycle. CDKs are only active when bound to a cyclin, and if so, they phosphorylate proteins important for progressing the cell cycle. If a cyclin is missing, which might be a signal for a problem, the cell will not enter the next phase [15]. For example, the cyclin D-cdk4/6 complex phosphorylates the Retinoblastoma-protein (Rb) during early G1. Therefore Rb cannot bind and thus inhibit the transcription factor E2F anymore and several genes activated by E2F can be transcribed [16]. On the other hand, if Rb is in a hypophosphorylated state, it binds E2F and transcription cannot start [17]. In late G₁ cyclin E-cdk2 phosphorylates Rb and maintains the cell cycle [18]. This pathway also plays an important role in the life cycle of some DNA-viruses that depend on the host-cell cycle and therefore try to activate transcription. SV40 virus or human papiloma virus (HPV) express oncoproteins (large T-antigen and E7) that bind Rb and thus activate transcription of genes they need for their own metabolism [19,20].
If all $G_1$ processes are completed correctly, the cell enters **S phase** (S stands for (DNA-) synthesis), in which DNA replication occurs. Here again, several cyclin-cdk interactions are jointly responsible for the progression of the cell cycle. But in S phase another important event takes place, the transcription of replication-dependent histone genes. As new DNA needs new packaging material (see chapter 1.5), right at the beginning of S phase new histones are synthesized [21]. Due to the high demand for histones during S phase, the genes encoding them are clustered and each cluster contains multiple copies of each of the five histones [22]. Hence a large number of copies of mRNAs can be produced within a relatively short time.

When DNA is replicated error-free and chromatin is formed (see chapter 1.5) the cell enters **G2**. Here again several important proteins are synthesized, especially those required for mitosis, like microtubules.

**Mitosis** is usually the shortest of all phases and the DNA that was replicated in S phase is now separated and spilt into two cells. In addition, cell organelles like ribosomes, mitochondria or the Golgi apparatus are partitioned and the nucleus is degraded and reformed in each new cell. Mitosis itself is divided into five phases (see figure 6) and ends with cytokinesis where the cell finally becomes physically divided.

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**Figure 6** | The Cell Cycle and the phases of Mitosis.

During **prophase** the chromosomes, always pairs of two sister-chromatids, are condensing in the nucleus and the spindle apparatus starts forming between the
two centromeres. In prometaphase the nucleus breaks and the chromosomes can attach with the microtubules of the spindle apparatus. Afterwards, in metaphase, the chromosomes align at the equatorial layer of the cell, right between the two centromeres at the two poles of the cell. The chromatids then are separated in anaphase by being pulled in opposite directions to the poles. In telophase, new nuclei are formed around the chromosomes at the poles of the cell. At the same time as telophase starts, the cell is divided by a contractile ring that divides the cytoplasm of the cell in two cells, each with one set of chromosomes. This final process is called cytokinesis [1]

The duration of each phase of the cell cycle can vary, depending on availability of nutrients, environmental conditions, cell type and intracellular issues. There is only one set of chromosomes in G1; in S it is doubled; in G2 there are two sets and during mitosis (M) the cell and the chromosomes are divided into two cells, each with one set of chromosomes.

1.5 Histones
As described before, the information on DNA is transcribed and translated by many different factors. But our genetic material is not just swimming around in the nuclei of our cells and randomly transcribed, it is systematically packed and sorted for several reasons, including saving space and building a structure that ensures that genes are transcribed only when needed. For that reason, DNA is wrapped around basic histone proteins. These protein-octamers consist of 2 sets of 4 different proteins: H2A, H2B, H3 and H4. At first H2A/H2B and H3/H4 form dimers. Then H3/H4 forms a tetramer with another H3/H4 dimer and H2A/H2B does the same with an identical partner. Finally two tetramers combine to an octamer that consists of 2xH2A-, 2xH2B-, 2xH3- and 2xH4-molecules.

The DNA wrapped around the histone-octamers forms nucleosomes. One nucleosome consists of a DNA string with a length of 147bp, which is wrapped 1.65 times around a histone-octamer [23]. The nucleosomes are packed as well in another kind of structure, a fibre, and this again is structured in a higher order. All together, the DNA-Histone- and non-histone-protein complex is called chromatin. This is what our chromosomes are made of.
Between two nucleosomes another type of histone can be placed to shorten the distance between them, histone H1. This is one way histones can significantly influence the accessibility of DNA. They can therefore act as a key player in transcriptional control. Condensed chromatin (heterochromatin) is predominately found in areas on the chromosome that are not transcribed, either never or for a certain time only. For example, the second X-chromosome of women (they carry two X-chromosomes, whereas men have two different sex-chromosomes: X and Y) is silenced for the reason of dosage compensation through a complex mechanism that leads to heterochromatin formation and thus to a near complete stop of transcription on this chromosome [24].

Another important factor in transcription is the amount and type of post-translational modification of (in most cases) the N-terminal ends of histone proteins.

1.6 Histone Modifications

We know that when our genetic material is handed down to a progeny, the sequence of DNA tells a lot about the prospective properties the offspring will gain. But there are biological characteristics that are not coded in the sequence of DNA. So where do they come from? There are two basic mechanisms: modifications of the DNA and modifications of the histones. The first ones do not change the sequence of the DNA (that would be a mutation), but the DNA is modified. The best-studied modification is DNA-methylation. Here a simple methyl group is added by a DNA-methyltransferase to the 5\textsuperscript{th} position of a cytosine and makes it to a 5-methyl-cytosine. DNA methylation is involved in many processes. Usually 5’ regulatory sequences of genes are not methylated, which helps keep the gene active. On the other hand, methylated regions may recruit proteins that bind 5-methyl-cytosine to promote gene silencing. This modification also plays roles in host-defence as most eukaryotic cell have...
methylated DNA and many parasites do not, meaning they can be recognized and degraded [25].

There is a wider range of modifications of histone than DNA. They can be modified in several different ways and on many different positions and each has a particular meaning.

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**Figure 8** Different kinds of histone modification on different positions. An activating mark for transcription is for example, the methylation on Lysine36 on histone H3 (H3K36me). Repressive marks would be H3K9meth or H4K20meth.

Acetylation of lysine residues is normally a typical marker for active genes. The acetyl group neutralizes the positive charged histones, hence the negatively charged DNA (the phosphate group in its backbone is negative) is bound less strong so that for example TFs can reach it. The acetyl group is added by a histone acetyltransferase (HAT), and removed by histone deacetylase (HDAC). Mis-regulation of acetylation/deacetylation can lead to severe diseases like Rett-syndrome, where the TF MeCP2 is mutated and thus cannot bind methylated DNA to recruit a HDAC like normal [26]. People with Rett-syndrome have amongst other symptoms a high-grade cognitive disability and lose the ability of speech.

Methylation of lysine residues for example can be a mark for active or repressed transcription, depending on the position of the lysine. Whereas a tri-methylation on lysine 36 on histone H3 (H3K36me³) is a well-known mark for a transcribed region, the same modification on lysine 9 (H3K9me) means the opposite [27]. But also the same modification on the same site may act in a different way...
under different conditions. Thus the H3K36me has a negative effect on transcription when it is near a promotor region of DNA [28].

Ubiquitylation is a well-studied modification of proteins and it is best known for triggering degradation of them via the 26S proteosome when four or more entities are bond [29]. Ubiquitylation of histones on the other hand is a less well-studied modification compared to others. It is known that histone H2B can be ubiquitinated at lysine 120 in humans by RNF20/RNF40 and UbcH6 and in yeast by Rad6/Bre1, which enhances H3K4 and H3K79 di- and trimethylation [30, 31, 32]. In humans it is known that H2BK120ub is a mark for active transcription and elongation as well as for DNA repair [32, 33].
Project Description

2.1 Generation of a Cell Cycle Profile
2.2 Control of Elongation of Replication-Activated Histone Genes
2.3 Histone H2B-Ubiquitylation CHIP-sequencing
2.1 Generation of a Cell Cycle Profile

To investigate the regulation of replication-activated histone genes one has to consider many transcription and RNA processing factors, several locations within the cell and all these aspects are time-dependents.

Replication-activated histone mRNAs are mainly produced during S phase of the cell cycle, as this is the time where they are required for forming new chromatin with newly synthesised DNA. Two of the key-players in the genesis of a functional replication-activated histone mRNA are stem-loop binding protein SLBP and U7 snRNP. U7 snRNAs are the shortest known Pol II transcripts and are located in cajal-bodies, small sub-organelles in the nucleus of the cell, which are arranged near the histone-gene clusters [34]. U7 snRNP binds with its complementary RNA-part to the 3’ end of the mRNA, the HDE sequence, and functions as processing factor. SLBP on the other hand binds the stem-loop structure just upstream of the HDE [34]. Both factors are part of the processing machinery, but not both of them are constantly available. SLBP is only produced at the end of G1 phase and rapidly degraded at the end of S phase, along with the bound mRNA [22, 35]. Thus, it was possible that a cell cycle dependent checkpoint is co-responsible for the termination of transcription elongation of those genes. This presumption is supported by previous research data that indicates that TF-dependent checkpoints can pause Pol II during elongation for different reasons [43]. To detect a checkpoint that regulates weather Pol II reads beyond the 3’ processing signal to produce poly(A) transcripts outside S phase, one needs cell populations that are in different phases of the cell cycle to compare the Pol II and histone occupancy of this genes.

My method of choice to synchronise the cells was a double-thymidine block [36], a potent method to pause the cell cycle at the boarder between G1 and S phase due to a reversible inhibition of Pol II with thymidine. When mainly thymidine is available for the polymerase it does not start replication and the cell cycle is stopped at this point until the redundant thymidine is removed. As the cell cycle of each cell strain can differ, it was essential to first establish the cell cycle timing of the cells I was using.
2.2 Control of Elongation of Replication-activated Histone Genes

As mentioned before, the expression of human replication-activated histone genes and some of their processing factors is tightly regulated through the cell cycle and is highest in S phase when newly-synthesized histones are required to form nucleosomes on newly-replicated DNA [22]. The transcripts are short and are not spliced. Furthermore, the 3’-end of the mRNAs for the replication-activated histones is non-polyadenylated and ends instead in a 3’ stem-loop (see figure 9) whose formation is directed by a gene-type specific processing element. A significant amount of regulation is implemented at the level of 3’-processing and post-transcriptionally [22], in particular through factors like SLBP, snRNP, NELF and CBC [22, 36]. Processing occurs after U7 snRNP has bond to the histone downstream element HDE and SLBP to the 3’ stem-loop. The snRNP contains 5 Sm- and 2 Sm-like proteins that contact the zinc-finger protein ZFP100, which again contacts SLBP. In addition, a cleavage-complex is recruited that contains the cleavage and polyadenylation specificity factor subunit 73 (CPSF73), which is responsible for endonucleolytic cleavage after the ACCCA-sequence upstream of the HDE [22].

However, all human replication-activated histone genes have a canonical polyadenylation signal located downstream from the S-phase-regulated processing signal, suggesting that polyadenylated message is made from the same genes outside S phase and that the switch to non-polyadenylated message may involve control of elongation of transcription. In support of this, the positive elongation factor, P-TEFb, is not needed for expression of these genes [8], while the negative elongation factor, NELF, has been implicated in their S phase regulation as it is knocked-down, the 3’-processing signal is not recognized anymore and polyadenylated mRNA is produced [37].

The aim of the project was to investigate whether there is a cell cycle dependent check-point on the level of transcription elongation of the human replication-activated histone genes, using synchronized cells, chromatin immunoprecipitation (ChIP) and reverse transcription PCR (see materials and
methods). The amount of polymerase II at different sites of several histone-
and some reference-genes (e.g. U2 or β-Actin) was investigated. In addition,
the occupancy of histones on those genes was determined. Nucleosome
occupancy is tightly linked to transcriptional activity and therefore a good co-
marker for a checkpoint. Furthermore, previous findings suggest a link between
nucleosome occupancy and the transcription elongation properties of Pol II on
the U2 snRNA and β-actin genes [43]. It was therefore interesting to investigate
histone occupancy during the cell cycle as changes could indicate changes in
transcription properties. I also tested the histone occupancy on the β-actin gene
(ACTB) as preliminary experiments showed differences from previously-
published data [43]. The hypothesis that the amount of antibody used for the
ChIP affects the results was tested.

2.3 Histone H2B-Ubiquitylation ChIP-sequencing
Histone H2B-ubiquitylation is known to be a mark for transcribed regions in the
human genome [33] and for being involved in methylation of histone H3 [38].
This mark and other known Histone modification marks that are involved in the
regulation of transcriptional elongation like H3K36me3 were investigated on
several genes. In addition, genome-wide H2Bub ChIP-sequencing was carried
out. These studies were prompted by the finding of my group-member Hadeel
Al-Rawaf that U2 snRNA genes, which have several similarities to replication-
activated histone genes including the lack of introns and poly(A)-tail, lack H2B-
monoubiquitilation.
Results

3.1 Cell Cycle Synchronization
3.2 ChIP-qRT-PCR and RT-PCR
3.3 H2B-Ubiquitylation ChIP-sequencing
3.1 Cell Cycle Synchronization

The assumption of a cell cycle dependent checkpoint on the level of transcription elongation lead to the need of synchronised cells to investigate differences between the phases, particularly between S and G1 phase. The double thymidine block method of cell synchronisation was optimised for our cells and a timetable was established to enable the cells to be collected at the desired time after that for experiments.

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Figure 10 | A) Cell cycle synchronisation. Cells were arrested at the beginning of S phase by using a double thymidine block and cell synchrony was monitored by flow cytometry of propidium iodide stained cells. B) Ratio of cells in different phases after synchronization. Ratios were analyzed and calculated with FlowJo software. C) Sketch of a cell-cycle-timeline to show how long the used cells have to be incubated after they were blocked on the borderline between S / G1 to pick them in the phase of interest.

The FACS analysis of the cell populations taken at different time points after the synchronisation confirms that up to 86% of the cells are synchronized and gives
a good overview of the cell cycle of the used cells. A signal at 200 in the FACS analysis means n=1, hence 400 means n=2. Between 200-400 shows a population in S phase. Right after the cells were synchronized and released into a new S phase, every 2-3 hours a sample was taken and analyzed. The times denoted in figure 10A show the time after releasing the cells into S phase. The results differs from earlier synchronization experiments with this type of cells that were synchronized with a double thymidine block [39] as they show a longer S and G2 phase, but a shorter G1 phase what leads to different starting points of these phases. Hence it seems that there are small but important differences in the cell cycle depending on on the cell conditions used. There are several reasons for these differences including differences in the media, incubation conditions, nutrition or the cell-splitting protocol. Thus, the results suggest that it is necessary to analyse the duration of the single phases for each cell type and protocol before using them for cell cycle experiments. Due to the successful outcome, the following Pol II ChIP and RT-PCR experiments could be performed with synchronised cells and the established protocol and timetable for this cells are now available for synchronisation experiments for the group of Dr. Murphy at Oxford University.
3.2 ChIP-qRT-PCR and RT-PCR

3.2.1 HIST1H2BD seems not to be controlled on the Level of transcription elongation

For ChIP experiments, the histone H2BD gene (HIST1H2BD) was selected as it had already been used by the group [9] and it was used in a previous paper investigating a similar problem [40]. Furthermore, it gave the highest and most reproducible signal for all tested genes.

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Figure 11 | Top: ChIP with Pol II (left diagram) and H3 (right diagram) on HIST1H2BD with cells in S- or G1 phase. Bottom: RT PCR to compare the amount of mRNA in S and G1. Black bars show the signal with random primers (all mRNA) and the grey bars show the signal for polyadenylated mRNAs as only oligo(dT) primers were used. Below the positions of the probes on the gene can be located. The signals from multiple experiments were normalised relative to probe prom in S phase for Pol II-ChIP (average signal of that probe was 0.44% ± 0.13% of the total input control (TIC)) and to probe 3 in S phase for H3-ChIP (average signal of that probe was 2.1% ± 0.66% of the TIC). Future experiments would include a control polyadenylated RNA to compare the different approaches. Total mRNA in S Phase from probe 1 was twice as much as in G1.

Neither Pol II- nor H3-ChIP on HIST1H2BD (histone H2BD gene) show evidence for a cell cycle dependent checkpoint, but it confirms a higher amount of Pol II
on the gene in S- than in G1 phase. The Pol II ChIP indicates that, contrary to the starting hypothesis, Pol II is still detectable after the 3’ processing signal (between probe 1 and 2) in S phase, whilst in G1 one sees a drop of Pol II after that signal. A checkpoint was expected near probe 2, but the signals were too near background at this point to come to any conclusion.

The profile of the H3-ChIP shows histone H3 occupancy all over the gene except on the promotor region, but on probes 3 and 5 during S phase H3 occupancy is significantly increased. This is an interesting finding and indicates cell cycle-specific regulation of H3 levels in these regions. It would be worth investigating the role of this difference between S and G1 phase.

Due to low signals in the Pol II-ChIP downstream from the promoter it might be difficult to detect cell cycle-specific differences in transcription termination. For this reason, reverse transcriptase PCR (RT PCR) experiments were carried out to compare the H2BD mRNAs in S and G1 phase (Figure 11). The results are not easy to interpret as they show more polyadenylated mRNA than total mRNA on probe 5. The oligo(dT) primers might work more efficiently than the random primers, making the results difficult to interpret. However, the results suggest that there is more polyadenylated mRNA in G1, what supports the starting hypothesis.

Investigation of expression of the histone H2BE gene proved to be impossible, as the gene does not seem to be transcribed at all since Pol II is only detectable at the promoter region of the gene (Figure 12).

![Figure 12](image.png)

**Figure 12** | ChIP with Pol II on histone H2BE gene. The results indicate that this gene is not transcribed in S- or G1 phase.

Signals were normalised to probe 1 in S phase (the average signal of that probe was 0.5% ± 0.17% of the TIC). Probe 1 is on the promoter region, 2 within the
transcribed region, the probe at polyA corresponds to a predicted poly(A) signal right at the end of the transcribed region and 2\textsuperscript{nd} polyA is 500bp downstream. Probes 3 and 4 are located 1 and 2kb downstream of the transcribed region.

![Poll II on U2-gene](image)

**Figure 13** | ChIP with Pol II on the U2 snRNA gene, whose expression is not expected to change between S and G1 phases. The results confirm the comparable efficiency of the ChIP between the two samples (S and G1)

To confirm that the different Pol II signals on the replication-activated histone genes in different phases do not derive from differences in the samples, the pol II levels on the U2 snRNA gene were determined since expression of this gene is not expected to be affected by the progression of the cell cycle. As seen in Figure 13, no significant difference is noted.

### 3.2.2 The amount of anti-body used does not effect the histone profile of ChIP on ACTB

The ChIP profile of H2B on ACTB and the results of qRT-PCR analysis revealed a different profile from that previously published [43]. The major difference was that the promotor proximal side is not cleared of histones in my experiments, whereas it was in the previous experiments. In particular, I found much higher H2B signals on probes A and B and a low but significant signal on probe 1 (see Figure 14). No reason could be found for this difference as the same protocol was followed. It is possible that some differences in media or cell culture conditions accounts for the differences.
Another possibility is that different concentrations of antibody were used for the ChIP. To test this, I titrated the antibody. However, no significant difference could be found in the profile that would explain the different results from published data after testing a broad range of antibody concentrations [41] (Figure 15). The ratio of probes “upB” and “int5” differs, but not because of the higher amount of anti-body but because of the difference between the experiments. Within one experiment the profiles did not change significantly.

**Figure 14** | ChIP of histone H2B on ACTB.

Probe ex3 in the diagram is equivalent to probe 2, and int5 to probe 3. Probe “up” functions as a control and lies upstream of the promotor region.

**Figure 15** | In two approaches, increasing amounts of H2B anti-body were used for ChIP. In the first experiment 0.25µg, 1µg and 3µg whereas in the second one 3µg, 3µg in a sample diluted with more dilution buffer (see materials and methods) and 9µg.
3.3 H2B-ubiquitylation CHIP-sequencing

3.3.1 The way of blocking Staph A cells does not effect the histone profile of ChIP on U2 gene

In contrast to the protein-coding ACTB gene, no H2B-monoubiquitination (H2Bub) can be detected on the U2 snRNA gene [42]. I confirmed this independently and also analysed the profile of H2Bub on the replication-activated histone gene HIST1H2DB. The replication-activated histone genes have several similarities to snRNA genes, including the lack of introns and poly(A)-tail. It was therefore of interest to test whether the lack of H2Bub is also shared.

As seen in Figure 17A, H2Bub is found on the HIST1H2BD gene. The promoter is cleared, but immediately downstream this histone mark peaks and then decreases slowly with the distance from the TSS.

I thereafter decided to carry out genome-wide ChIP-sequencing for the H2Bub mark to be able to analyse this mark on all human genes as a foundation for further analysis.

As the Staph A cells for the ChIP-sequencing cannot be blocked with herring sperm DNA as usual as it would be sequenced as well, the consequences of blocking with heparin or without blocking were investigated (Figure 16). The result does not show any major differences between the profiles except for an elevated signal of Pol II on probe ctf for the sample that was not blocked. The Staph A cells used for the sample for H2Bub ChIP-seq were therefore not blocked at all.

![Figure 16](image)

**Figure 16** | H2B and Pol II profiles on the U2 snRNA gene with different blocking conditions. Staph A cells for ChIP were blocked with herring sperm DNA (left), heparin (middle) or not blocked at all (right).
3.3.2 H2Bub is cleared near the TSS and reaches its maximum about 1.5kb downstream

Before the genome-wide ChIP-sequencing could be accomplished, the procedure had to be optimised concerning purity and amount of DNA material submitted for sequencing. Finally, two high-grade ChIP samples were pooled together and submitted. Previous results [33] suggest a constant increase of this histone modification from -1kb till +2kb (according to the TSS). However, the authors normalised the H2Bub mark to H3 levels and transcriptional activity. The results of my genome-wide ChIP-seq show a bi-modal distribution with the minimum signal at the TSS, a small maximum at -1kb and a larger one at +1.5kb, followed by a gradual decrease downstream (Figure 19). Figures 17 and 18 compare the results of the genome-wide H2Bub ChIP-seq with results of H2Bub ChIP - qRT-PCR analysis of HIST1H2BD and ACTB. These results confirm each other and show that the experiment worked successfully.
Figure 17 | A) H2Bub ChIP with unsynchronised cells on H2BD gene analysed with qRT-PCR. Sample of probe 1 got lost and was not analysable B) Frame of the genome-wide H2Bub ChIP-seq to compare the results with “normal” ChIP above. Screenshot shows the region including probes prom, 1 and 2. C) As part B, but this frame shows the region including probes 4 and 5.
Figure 18  

A) H2B-ubiquitylation vs. H2B on β-Actin gene. Left diagram shows total ubiquitinated H2B, right it is divided by total H2B to give a relative result.  

B) Frame of the genome-wide H2Bub ChIP-seq to compare the results with “normal” ChIP above.  

C) Position of the probes, whereas probe “2” is on the same position as probe “ex3”, and probe “3” as “int5”. Probes 2 and 3 seem to match other regions in the genome as well, and these may be pseudogenes.
**Figure 19** | Genome-wide distribution of mono-ubiquitinated H2B on significantly enriched genes in three different resolutions. X-axis shows the position relative to the gene (0=TSS), Y-axis shows the gained signal, hence the amount of H2Bub on a certain position. Analysis was accomplished by Dr. Martin Dienstbier, Oxford University.
Discussion and Summary

4.1 DISCUSSION
4.2 SUMMARY
4.3 ZUSAMMENFASSUNG
4.1 DISCUSSION

The cell cycle data presented here differs in some aspects from that published by Whitfield et al. 2002 [39]. Specifically, the duration of G2 differs, which alters the start time of Mitosis and G1 phase in the cells I used. My results indicate that my cells have a longer S and G2 phase, but a shorter G1 phase, keeping the total period of one cell cycle nearly the same. The double thymidine block method worked very well with our cells compared to other cell strains [43]. The technique is now available to the group of Dr. Murphy and will be a powerful tool to investigate cell cycle-dependent mechanisms affecting gene expression.

Narita et al. published in 2007 [37] that the negative elongation factor, NELF and the cap binding complex, CBC are essential for proper 3’ processing in replication-activated histone genes. They showed an aberrant production of polyadenylated histone mRNAs if one of the two factors was absent and concluded that NELF, CBC and SLBP play major roles in 3’ processing. Our hypothesis was that in addition a cell cycle-dependent checkpoint at the level of transcription elongation regulates whether a polyadenylated or the canonical mRNA ending with a 3’ stem loop is produced.

Our experimental method was Pol II and histone H3/H2B ChIP. Unfortunately, the average transcription rate based on the presence of polymerase II is significantly lower on replication-activated histone genes than the reference U2 snRNA and ACTB genes, making analysis difficult. This method therefore seems not to be sensitive enough to accurately measure small differences of Pol II on genes that are transcribed at low levels like HIST1H2BD or HIST1H2BE. These genes are transcribed at quite low levels in G1 phase in particular and testing the hypothesis further would require a different approach. For this reason, it was not possible to fully test the starting hypothesis. However, the results obtained suggest that there is no control at the level of transcription elongation.

The results of RT-PCR on the H2BD gene do indicate that there is a difference in the amount of polyadenylated mRNA and non-polyadenylated mRNA at the different phases of the cell cycle. The RT-PCR results of RNA from S phase and G1 phase (Figure 11) indicate that the signal of the poly(dT) primers is higher than the signal or random primers on probe 5, which is most likely due to
different efficiency of these primers, which could tested using purified polyadenylated RNA.

Unfortunately, the results of analysis of the histone H1 gene by ChIP were not reproducible and no conclusions could be drawn (see appendix). For this reason, I decided to concentrate on histone H2B genes. As the H2BE gene did not seem to be transcribed at all (Figure 12), I focused on the H2BD gene HIST1H2BD.

The ChIP data of HIST1H2BD suggests that regulation occurs within the intronic region after the 3′ processing signal of that gene. This might indicate the presence of another transcription unit at that position. But more tests would have to be done to prove that hypothesis.

An interesting outcome was that the amount of antibody for ChIP does not significantly affect result. It was expected that low amounts of antibody (-> all antibodies are bound to proteins but not all proteins are occupied) would predominantly lead to more differences between weak and strong signals, and that high amounts of antibody (-> not all anti-bodies are bound to proteins, but all proteins are occupied) would equalize that but lead to unspecific binding and thus to different profiles. However, the profiles were the same even though the ratio was 1:36 concerning the amount of antibody that was tested.

The genome-wide ChIP-sequencing for H2Bub worked well after the parameters of DNA-blocking of Staph A cells and used amount of cells were optimized. Blocking could be important to prevent non-specific binding of DNA that is not of interest. However, the blocking method did not significantly affect the ChIP profiles. Whereas there is an elevation on one probe as seen in Figure 16, the other approaches do not show any difference. Finally, a sample with unblocked Staph A cells (only with protein) was submitted for sequencing. The ChIP-sequencing generated a large amount of data that is still being mined in Dr Murphy’s laboratory. Initial analysis indicates that H2Bub is associated with the middle of genes and is lower at the beginning and end and may be a mark associated with elongating Pol II. Unlike the snRNA genes, which have no H2Bub, the replication-activated histone genes do have this mark, suggesting that elongation is controlled differently in these two gene types.

Unfortunately no reason could be discovered why the H2B signal on ACTB was different from the one published by Egloff et al. 2009 [43], particularly since
the probes and procedures used were the same. As the experiment was repeated several times and my results were reproducible, I assume that the cells or their condition was different.
4.2 Summary

At the beginning of my work was the assumption that the transcription of replication-activated histone genes is not only regulated by various factors such as negative elongation factor NELF or cap binding complex CBC, but that there is a cell cycle dependent check-point at the level of transcription elongation that regulates whether polymerase II goes beyond the 3’ processing signal of these genes. Contrary to other protein coding genes, replication-activated histone genes end in a 3’ stem-loop instead of being polyadenylated. My ChIP/RT-PCR results suggest that our hypothesis is not the case. However, more sensitive methods would be required to be conclusive. Unfortunately, the total transcription rate of the replication-activated histone genes was quite low compared to reference genes like the U2 snRNA gene or the ACTB gene, and it was hard to get evidence for significant differences of Pol II profiles on these genes depending on the cell cycle. In addition, other transcription factors and histone modification marks as H3K36me3 (see appendix) or H2Bub were investigated and gave interesting and novel results. Other genes might lie within that region studied and the histone occupancy and modifications marks may relate to these. The synchronisation of the cells by double thymidine blocking worked very well, providing novel data concerning the duration of the single cell cycle phases of the HeLa cells used and turned out to be a powerful tool for investigating cell cycle dependent factors.

The second project was a genome-wide ChIP-sequencing of H2Bub, which is known to be positively linked with transcriptional activity. It worked very well after parameters for the ChIP were optimized. The result is promising as it shows a bi-modal distribution with its minimum at the transcription start side. However, further analysis is beyond the scope of this thesis concerning data and time.
4.3 ZUSAMMENFASSUNG


Materials and Methods

5.1 Cell Culture
5.2 Cell Cycle Synchronization
5.3 FACS-Analysis
5.4 ChIP
5.5 Quantitative RT-PCR
5.6 RNA Isolation and RT-PCR
5.1 CELL CULTURE
If not mentioned differently, MEM and DMEM media supplemented with 10% fetal bovine serum (FBS), 1x penicillin/streptomycin and 1x L-glutamine (all by PAA) were used for cell culturing. To the PBS used for maintaining (washing/splitting) the cells 1mM EDTA was added. The cells that were used were HeLa cells [44] received from Dr. Clélia Laitem, Sir William Dunn School of Pathology, who was a postdoctoral fellow at the Lab of Dr. Shona Murphy during the time I accomplished my work.

HeLa cells that had been grown to about 80% confluence in six 75cm² flasks in MEM media were transferred to a 15ml falcon tube, centrifuged with 1000rpm for 10min, resuspended in 6ml mix composed of 90% FBS and 10% DMSO and then divided into 6 cryovials (1ml per vial). After that the cells were cooled on ice for about 2 hours and finally stored at -80°C. Approximately once a moth (maximum after 5 weeks) fresh cells from a vial were taken for cell culture.

Cells were thawed at 37°C and then transferred into a 10cm flask with 10ml DMEM media and incubated over night at 37°C. On the following day cells were grown to about 100 percent confluence and split. Therefore the media was aspirated and cells were washed 3 times with PBS, then 2ml PBS+EDTA were added and incubated at 37°C for about 10min. After that, 8ml media were added and the cells divided into new flasks. The amount of cells that were transferred depended on the use, for maintaining cells were split about 1:10, for experiments for next day 1:5 thus they were about 60-80 percent confluent when harvested.

5.2 G1/S PHASE CELL SYNCHRONIZATION USING DOUBLE THYMIDINE BLOCK
This protocol [39] is designed to synchronize HeLa cells at the G1/S border. It will not work in cell lines with intact p53 apoptotic response.

HeLa cells were cultured in DMEM media to about 40% confluence (2.5ml per well in a 6-well plate, 10ml in a 10cm dish). Thymidine that has been resuspended in PBS to a final concentration of 2mM in the media (37.5µl per well, 150µl per plate of 130mM thymidine) was added, hence the DNA replication cannot start as there is an overspill of thymidine, which inhibits the polymerase from incorporating other deoxynucleoside triphosphates (dNTPs). Then the culture was incubated at 37°C for 19hrs. Afterwards DMEM media was
removed and cells washed 3x with PBS (without EDTA!). Then fresh media without thymidine was added and the cells were incubated for 9hrs at 37°C. After that thymidine was added to the cell culture again to a final concentration of 2mM and incubated for another 16hrs.

Finally cells were washed 3x with PBS and fresh media was added. At this point nearly all cells were at the borderline from G1/S phase and were “released” to progress through the cell cycle over the following ~15hrs. The cells should be uniform for about 1-2 cell divisions and then regain their asynchronous state.

5.3 FACS Analysis
Fixing cells for FACS analysis
Media was aspirated and cells were washed twice with PBS, then 10ml of PBS were added and incubated for 5min at 37°C. Afterwards the cells were carefully scraped off with a cell scraper or by slightly shaking the flask/dish and collected in a 15ml falcon tube. There the cells were centrifuged at 700g for 5min at 4°C. After discarding the supernatant 2ml of 79% EtOH were added and left for 30min at 4°C. Then the cells were centrifuged again at 700g for 5min at 4°C. Supernatant was removed and cells were washed with 5ml cold PBS (without EDTA) and then centrifuged again as before. After supernatant was discarded, cell pellet was resuspended in 300µl PI-solution (0.1mg/ml Propidium iodide in PBS + 0.2mg/ml RNase A) and incubated for 35min at 37°C in the dark. Afterwards cells were centrifuged at 700g for 5min at room temperature (RT) and then resuspended in 300µl PBS and stored for a maximum of 18hr at 4°C in the dark.

Propidium iodide intercalates with nucleic acid (DNA/RNA) not sequence specific and increases its fluorescence, hence it is distinguishable if a cell contains one or two sets of chromosomes (→ G1 or G2 phase) since doubled DNA/RNA means doubled fluorescence signal.

Fluorescence activated cell sorting (FACS) analysis
5.4 CHROMATIN IMMUNOPRECIPITATION (ChIP)

Solutions for ChIP:
- Cell lysis buffer: 5mM PIPES pH 8.0, 85mM KCl, 0.5% NP40
- Nuclei lysis buffer: 50mM Tris-Cl pH 8.1, 10mM EDTA, 1% SDS
- IP dilution buffer: 0.01% SDS, 1.1% Triton X100, 1.2mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167mM NaCl
- 1x dialysis buffer: 2mM DTA, 50mM Tris-Cl pH 8.0, 0.2% Sarkosyl
- 1x dialysis buffer (for monoclonal anti-bodies): 2mM DTA, 50mM Tris-Cl pH 8.0
- IP wash buffer: 100mM Tris-Cl pH8.0, 500mM LiCl, 1% NP40, 1% deoxycholic acid
- IP wash buffer (for monoclonal anti-bodies): 100mM Tris-Cl pH9.0, 500mM LiCl, 1% NP40, 1% deoxycholic acid
- Elution buffer: 50mM NaHCO₃, 1% SDS
- 5x PK buffer: 50mM Tris-Cl pH 7.5, 25mM EDTA, 1.25% SDS

all in ddH₂O

Preparation of Staph A cells for ChIP:
1 gram of lyophilized Staph A cells (Pansorbin® cells, stored at 4°C) were resuspended in 10ml dialysis buffer for monoclonal antibodies and centrifuged at 4000rpm for 30min at 4°C (in 15ml falcon). This step was repeated once and then cells were resuspended in a mixture of 4ml PBS + 1.2ml of SDS + 400µl β-mercapto ethanol and boiled for 30min. Afterwards cells were centrifuged at 10000rpm for 5min (in Eppendorf tubes) and the pellet washed with 500µl 1x dialysis buffer for monoclonal antibodies. This centrifuging/washing step was repeated once. Finally the cell pellets were resuspended in 400µl of dialysis buffer for monoclonal antibodies and divided into 90µl aliquots and stored at -80°C.

5.4.1 ChIP protocol:
Staph A cells were prepared (for each plate one needs 50µl: 10µl to pre-clear the chromatin and 40µl for Immunoprecipitation [10µl for each sample]). 1 tube (90µl) of cells was thawed for approximately 10^8 cells that one begins with. 10µl of herring sperm DNA (10mg/ml) and 10µl of BSA (10mg/ml) were added
to each tube of Staph A cells. Following Staph A cells have to be incubated on a rotating platform at 4°C for at least 3 hours, over night is fine as well.

Then HeLa cells were taken from 37°C and 270µl (for 10ml of media) of formaldehyde solution (37%) were added directly to tissue culture media (to a final concentration of 1%) for cross-linking the DNA with proteins like TFs, histones or polymerase. Afterwards adherent cells were incubated on shaking platform for 10 min at RT.

![Cross-link reaction of DNA and protein induced by formaldehyde.](image)

Cross linking reaction was stopped by adding glycine to a final concentration of 125mM (625µl of 2M stock solution were added), then continued to shake at RT for 5 min. Afterwards the media was poured off and plates rinsed twice with cold 1x PBS. To scrape off the cells of the dishes, 1ml PBS was added and cells were transferred into an Eppendorf tube. Following they were centrifuged at 1000 rpm for 5 min at 4°C and washed once with 1x PBS (plus PMSF - 10µl per ml). The cell pellet was resuspended in 200µl (per plate) of Cell Lysis Buffer plus 4µl (per plate) of 50x stock protease inhibitor (one tablet in 1ml dH2O=50x) and incubated on ice for 20 min. After that the sample was centrifuged at 5000rpm for 5min at 4°C to pellet the nuclei. The nuclei were resuspended in 200µl (per plate) Nuclei Lysis Buffer plus same protease inhibitor as used before and incubate for 10 min on ice. To cut the DNA, chromatin was sonicated to an average length of 300bp while samples were kept on ice (Sonicator settings: amplitude 12 microns – 10 times for 10 sec – separated by 40 sec). Following the samples were centrifuged at 14000 rpm for 10 min at 4°C and supernatant taken and transferred to a new tube.

Chromatin was pre-cleared by adding blocked Staph A cells. 10µl of pre-blocked Staph A cells for every 10^7 cells were used (10µl for each plate) and samples
were incubated on rotating platform for 15min at 4C and following centrifuged at 14000rpm for 5 min. Supernatant was transferred to a new tube and divided equally among the samples. Final volume of each sample was adjusted with IP dilution buffer plus protease inhibitor and phosphatase inhibitor if necessary. Samples volumes were between 250µl. After that 1µg of anti-body was added to each sample.

Anti-Bodies:

- anti-Pol II: rabbit N-20, lot# A1207
- mock: normal rabbit IgG, sc-2027, lot# J2009, Santa Cruz
- anti-ubiquitinated H2B: mouse monoclonal cat.: MM0029, lot# 299040927, MEDIMABS
- anti-H2B: α-1423
- anti-ctcf: milipore® anti-ctcf, rabbit, lot# DAM 1682158
- anti-H3K36meth³: rabbit polyclonal, lot# 65835
- anti-H3: rabbit, lot# 65835, Abcam
- anti-cdk9: rabbit, L-19 sc 7331, lot# c1704, goat IgG, Santa Cruz

Samples were incubated with respective anti-bodies over night on rotating platform at 4°C.

When a monoclonal anti-body was used, 1 µg of an appropriate secondary antibody was added and incubated for another 60min. Then 10µl of blocked Staph A cells were added to each sample and incubated on rotating platform at 4C for 15 min. Then the samples were centrifuged for 3 min at 13000rpm and supernatant from the “mock” sample was saved as TIC to subsequently have a reference for evaluation of the amount of DNA with qRT-PCR. Afterwards the pellet was washed twice with 1.4ml of 1x dialysis buffer (for samples with monoclonal anti-body appropriate buffer was used) and four times with 1.4ml of IP Buffer (for samples with monoclonal anti-body appropriate buffer was used). For each wash, pellet was dissolved in 200µl of buffer and additional 200µl of buffer were taken to wash the pipette tip. Then an additional 1ml of buffer was added. After each wash, sample was centrifuged at 14000rpm for 3min at RT. After the last wash the sample was centrifuged once more and the last traces of buffer were removed. To elute AB/protein/DNA complexes, 150µl of IP elution buffer were added and (NOT ON ICE FROM NOW) shaken on a vortex for at least 15 min at setting “vortex 3”. After that, sample was centrifuged at
14000rpm for 3min and the supernatant transferred into a new tube. The elution was repeated once and both elutions combined in the same tube. The sample then was centrifuged at 14000rpm for 5 min to remove any traces of Staph A cells, and supernatant was transferred to a clean tube. After that 1µl RNase A (10mg/ml) and 5M NaCl were added to a final conc. of 0.3M. 50µl of TIC were taken and 250µl IP elution buffer plus 1µl of RNase A (10mg/ml) and 5M NaCl to 0.3M) added. The sample then was incubated at 67ºC for 4-5 hours to reverse formaldehyde cross-links. To precipitate the DNA, 2 ½ volume of EtOH was added and left over night at -20ºC. The following day, the sample was centrifuged at 14000rpm for 20 min at 4ºC. Afterwards ethanol was removed and the pellet air-dried completely. After that each pellet was resuspended in 100µl of TE + 25µl of 5x PK buffer and 1.5µl PK. Finally the sample was incubated at 45ºC for 1-2 hours.

To purify the DNA, the QIAquick PCR purification KIT 250 (QIAGEN) was used after protocol and finally the DNA was eluted with 50µl ddH2O. For analysis with qRT-PCR one 1µl of DNA was used.

**Figure 21** | Schematic sketch of the functionality of chromatin immunoprecipitation (ChIP) with quantitative real time PCR (qRT-PCR) analysis. [A] After DNA has been cross-linked to associated proteins, it is sonicated to small fragments of about 600bp length but still linked to the proteins [B]. Afterwards specific anti-bodies (AB) against the specific protein-domains are added and bind the DNA-Protein complex [C]. Afterwards Staph A cells are added which have the protein A on their surface that binds the Fc-region of the anti-bodies and hence separate the DNA-protein complex from other cell-components [D]. Then the
DNA-protein complex is eluted [F] and the cross-linking reversed [G], thus the DNA can be purified from other components [H] and be used for qRT-PCR analysis [I].

To prove the length of the sonicated DNA fragments, the “total input control” (TIC, see next page) was loaded on a 1% Agarose Gel:

![Figure 22](image)

**Figure 22** | Control of sonication of ChIP DNA. TIC 1 and TIC 2 are total input controls of two different ChIPs. Both show an average length of 300bp as requested.

### 5.4.2 Measuring of DNA/RNA concentration and purity with NanoDrop

For confirming the purity of RNA (see 5.6) or DNA, a NanoDrop ND-1000 with ND-1000 software (v 3.1.0) was used. Especially for the H2Bub ChIP-sequencing sample the purity was of special interest. The 40μl ChIP-sequencing sample that was sent in had a concentration of 3.25ng/μl, hence 130ng of DNA were used. The 260/280 ratio, which gives a value for Protein or Phenol contamination, was 1.75, hence there was nearly no contamination of Protein (1.8 would be “pure”). The 260/230 ratio was 0.9 (ideal would be 1.8), which might be due to some EDTA residues.

### 5.5 Quantitative real-time PCR (qRT-PCR)

Samples, each consisting of 5μl SYBR-green, 3μl ddH2O, 1μl primer-mix (5μM each) and 1μl sample DNA, were heated up to 95°C for 15min, and then the following cycle was conducted:

- 94°C for 15sec
- 58°C for 20sec  x 42
- 72°C for 25sec

Finally the temperature was heated up to 99°C degree by degree to observe the melting temperature of the DNA fragments. The analysis was accomplished with
*Rotor-Gene 6* software. The qRT-PCR device that was used was a Corbett Research RG-300.

**Primers used for qRT-PCR amplification:**

<table>
<thead>
<tr>
<th>HIST1H1C gene</th>
<th>Name of primer</th>
<th>Sequence 5' - 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 far upstream F</td>
<td>TCAGGTGATCCGCCGCCGAG</td>
<td></td>
</tr>
<tr>
<td>H1 far upstream R</td>
<td>GGCTATTCTCAGAGTGACAGGGT</td>
<td></td>
</tr>
<tr>
<td>H1 @ poly A F</td>
<td>GTCCTCGCATGACTACGGTTGC</td>
<td></td>
</tr>
<tr>
<td>H1 @ poly A R</td>
<td>CAGAGGGAACCATTACGAAGGCC</td>
<td></td>
</tr>
<tr>
<td>H1 0 F</td>
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**HIST1H2BE gene**

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**ACTB gene**

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5.6 RNA ISOLATION

For homogenization, cells were lysed direct in the culture dish by adding 1ml of TRIZOL® Reagent to a 3.5cm diameter dish and passing the cell lysate several times through a pipette. Then the homogenized samples were incubated for 5min at 25°C to permit the complete dissociation of nucleoprotein complexes and 0.2ml of chloroform per 1ml of TRIZOL Reagent were added. Afterwards tubes with samples were shaken vigorously by hand for 15 sec. and incubated for another 2-3min at 25°C. After that time, samples were centrifuged at 11500 x g for 15min at 4°C. The mixture was separated in a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase. For precipitation of the RNA, the aqueous phase was transferred into a fresh tube and mixed with 0.5ml isopropyl alcohol and incubated at 25°C for 10min. Afterwards samples were centrifuged at 11500 x g for 10min at 2-8°C. Then supernatant was removed and the pellet washed once with 75% EtOH, at least 1ml of EtOH per 1ml TRIZOL Reagent was used. The Sample then was mixed by vortexed and centrifuged at 7 000 x g for 5min at 4°C. At the end of the procedure, RNA pellet was briefly air dried, but not completely, and dissolved in RNase-free H₂O by passing the solution several times through a pipette and incubating for 10min at 55-60°C. As still some DNA might have been in the samples, 1 x DNase buffer and 50 units of DNase were added and incubated for 1hr at 37°C. Second extraction was performed by adding 100µl Phenol/Chloroform and 3M 10µl NaOAc before vortexing the sample. Afterwards the sample was centrifuged for 5min at 7 000 x g. The RNA remained again in the aqueous phase, which was transferred into a fresh tube and 250µl (2.5 x of sample volume) of 100% EtOH were added to precipitate it. After that, the sample was centrifuged for 5min at 7 000 x g at 4°C. Finally the pellet was air-dried (not completely) and resuspended it in 20µl RNase-free H₂O.

Reagents:

TRIZOL® by Invitrogen: contains Phenol and guanidine thiocyanate.

DNase I, RNase-free by Roche; 10 776 785 001
5.6.1 Reverse transcriptase PCR

Using SuperScript™ III by invitrogen™

First-Strand cDNA Synthesis:
For the first-strand cDNA synthesis, following components were added to a nuclease-free microcentrifugate tube: 1µl of oligo(dT)$_{20}$ (50µM) or 50-250ng of random primers, depending on approach, 10pg-5µg total RNA (500ng), 1µl 10mM dNTP Mix (10mM each), sterile and distilled water to 13µl. That mixture was heated to 65°C for 5min and then incubated on ice for at least 1 min. Contents of the tube were collected by brief centrifugation and then in each sample was added: 4µl 5x First-Strand buffer, 1µl 0.1 M DTT, 1µl RNaseOUT™ Recombinant RNase Inhibitor (Cat. No. 107777-019, 40units/µl) and 1µl of SuperScript™ III RT (200units/µl). Then the sample was mixed by pipetting gently up and down. Samples with random primers were incubated at 25°C for 5min, afterwards all samples were incubate at 55°C for 45min. Finally increasing the temperature to 70°C for 15min stopped the reaction, thus cDNA could be used as a template for amplification in PCR.

PCR Reaction:
5µl SYBR-green, 3µl ddH$_2$O, 1µl primer-mix (5µM each) and 1µl cDNA were mixed. Afterwards the mixture was heated to 94°C for 2min to denature, followed by

- 94°C for 15sec
- 58°C for 20sec $\times 42$
- 72°C for 25sec

Finally the temperature was heated up to 99°C degree by degree to observe the melting temperature of the DNA fragments. The analysis was accomplished with Rotor-Gene 6 software. The qRT-PCR device that was used was a Corbett Research RG-300.
**APPENDIX**

**Figure 14** | Pol II ChIP on histone H1 gene. The three different experiments are shown separately as they show such different results. The signal is given as percentage of the TIC.

On histone H1 gene several ChIPs with Pol II anti-bodies were accomplished, but the results were not reproducible and differed each time. Even more approaches than the ones shown above were accomplished with different PCR programs and new probes. Due to no explanation could be found no further experiments were accomplished with that gene and finally HIST1H2BD was selected.

**Figure 15** | Cyclin dependent kinase cdk9-ChIP on HIST1H2BD, β-Actin gene and U2 gene. The signal was too low in all experiments to present any results.

A protein involved in transcription that was tried to look at was P-TEFp subunit cdk9. Unfortunately the signal was too weak to believe that the used anti-body actually worked. Also here it was not possible to repeat the experiment with the same result. But even if, the signals were too weak to trust them.
Figure 16 | ChIP-qRT-PCR result of H3K36me\(^3\) and ctf on HIST1H2BD. Probes ctf1 and ctf2 were assumed ctf binding-sides, probe 3 was used as negative control.

As tri-methylation of Lysine on position 36 on histone H3 is linked with positive transcription [45], it was interesting to see if this histone mark supports our thesis, but only far after the 3’processing signal the tri-methylation was detectable at low signal.

Another protein of interest to ChIP was ctf, which functions as an insulator protein that binds DNA and blocks transcriptional enhancers [46, 47]. Therefore 2 possible sites on the gene that could act as recruiting signal for ctf where searched and found and probe *ctf1* indeed showed presence of the protein, but exclusively in G1 phase and with a very low signal. Due to lack of time and no significant outcome no further experiments were accomplished with this protein.
# CURRICULUM VITAE

## DETAILS

<table>
<thead>
<tr>
<th>Name:</th>
<th>Philipp Velicky</th>
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<tbody>
<tr>
<td>Address:</td>
<td>1070 Vienna, Austria</td>
</tr>
<tr>
<td>Date of birth:</td>
<td>10/03/1985 - Vienna</td>
</tr>
<tr>
<td>Nationality:</td>
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## EDUCATION

Since 10/2005  
Student of *Biology* and *Molecular Biology* at the University of Vienna (degree *Bachelor of Science* in Biology in 11/2010)

09/1998 – 06/2005  
Bundesrealgymnasium Wien XX, Austria

Erich Fried Realgymnasium Wien IX, Austria

## ROTATIONS/DISSERTATION

03/2010 - 09/2010  
**University of Oxford**, Degree Dissertation “*Control of Elongation of Replication Activated Histone Genes*” for reaching the grade *Magister rer. nat.* in Molecular Biology (equivalent *MSc.*)  
Sir William Dunn School of Pathology, Dr. Murphy Group

03.08. – 30.09.2009  
**Medical University of Vienna**, Institute for Cancer Research, with Dr. Maria Sibilia - *Cancer Histology-Immunochemistry*

22.04. – 15.06.2009  
**NYU Langone Medical Center**, New York, Department of Pathology, with Sandra Demaria, M.D. - *Cancer Immunology*

27.10. – 22.11.2008  
**University of Vienna**, Max F. Perutz Laboratories, with Dr. Michael Jantsch – *Protein/RNA Biochemistry*

## FURTHER EDUCATION

13. – 15.01.2009  
*EMBO Workshop*  
‘Regulatory RNAs in Pro- & Eucaryotes’

## SCHOLARSHIPS

2010  
“KWA Stipendium für kurzfristige wissenschaftliche Auslandsaufenthalte”  
*Scholarship for students with a good GPA (grade point average) to conduce scientific projects abroad (University of Vienna)*
2010
“Förderstipendium der Universität Wien”
Scholarship for students with a good GPA to perform research projects that would come with an elevated financial strain (University of Vienna)

EMPLOYMENT

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<td>06/2005 - 09/2008</td>
<td>„Food on Wheels“, Sozial Global</td>
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<tr>
<td>10/2004 - 06/2005</td>
<td>Military Service (Guard Commander)</td>
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<tr>
<td>07/2002</td>
<td>DOMAD Bau (summer job)</td>
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<tr>
<td>07/2001</td>
<td>Architekturbüro Dr. Suske (summer job)</td>
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FURTHER SKILLS

IT knowledge
Good knowledge of MS Word, MS PowerPoint and MS Excel
Average knowledge of Macromedia Fireworks MX (image editing)

Languages
German (mother tongue)
English (very good - spoken and written)
French (basic)
REFERENCES

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11 Bernstein P., Ross J. (1989) Poly(A), poly(A) binding protein and the regulation of mRNA stability
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30 Seunghee Oh, Kiwan Jeong, Hyunhee Kim, Chang Seob Kwon, Daeyoup Lee (2010) A lysine-rich region in Dot1p is crucial for direct interaction with H2B ubiquitylation and high level methylation of H3K79, Biochemical and Biophysical Research Communications 399; 512-517


42 Al-Rawaf H., unpublished results

43 Liska O., Proudfoot N., personal conversation


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