The Dynamics of CBX2, Subunit of PRC1: Immobilization after Chromatin Release Induced by Heat Shock

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University of Groningen

university of groningen

Author

Iris Lechner

S2470608

i.m.lechner@student.rug.nl

Supervisor

Dr. Steven Bergink

Department of Cell Biology

University Medical Center Groningen

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Author	Iris Lechner
Student number	S2470608
Bachelor thesis in the context of	BSc Liberal Arts and Sciences University College Groningen University of Groningen
Supervisor UMCG	Dr. S. Bergink Department of Cell Biology UMCG
Supervisor UMCG	Dr. Vincent van den Boom Department of Hematology UMCG
Supervisor UCG	Dr. Roland Chiu University College Groningen

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ABSTRACT

Epigenetic machinery influences the activation and inhibition of many cellular processes. Chromatin remodelers can suppress or activate genes, resulting in adequate cellular functioning. Dysregulation of remodelers, however, can induce adverse epigenetic alterations. The epigenetic alterations of chromatin remodelers induced by cellular stress, such as heat shock, are currently unknown. Polycomb Repressive Complex 1 (PRC1) represses various important regulatory genes. Upon heat shock, PRC1 releases from the chromatin and one of the subunits of PRC1, CBX2, localizes in the nucleolus. This study reveals the nuclear dynamics of CBX2 upon heat shock. The advanced microscopy techniques FRAP and FLIP led to the observation of high immobile fractions in GFP-CBX2 fusion cells, in the nucleus and nucleolus. In the nucleus this immobile fraction is likely the result of depletion of the mobile fraction, which has migrated towards the nucleolus. The remaining fraction is tightly bound to structures in the nucleus. The immobile fraction in the nucleolus is possibly a result of an altered nucleolar structure, which might form a gel-like structure upon heat shock.

ACKNOWLEDGEMENTS

I would like to personally thank both of my supervisors at the UMCG: Steven Bergink and Vincent van den Boom. Both of you tremendously helped me through the process of doing research in a laboratory and stimulated me to grow as a researcher. I also would like to thank Roland Chiu for introducing me to molecular biology and helping me find a laboratory in the first place. Moreover, I would like to thank the people in my office at the UMCG for the good conversations, helping with (so many) of my experiments, and all scientific talks. I would also like to thank my parents and sister for listening to me rattle on about my thesis, of which I hope they learned something more than ‘she does something with green cells’. I also want to thank my roommates for their tolerance, since they had to endure my writing sessions in our living room. Lastly, science is never a direct line and this whole process has been a great learning experience, where more questions have been raised than answered.

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List of Acronyms

ALS	Amyotrophic lateral sclerosis
AML	Acute myeloid leukaemia
ChIP	Chromatin immunoprecipitation
ESC	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FLIP	Fluorescence loss in photobleaching
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
HS	Heat shock
HSC	Hematopoietic stem cell
HSPs	Heat shock proteins
NUC	Nucleus
NUO	Nucleolus
PRC	Polycomb repressive complex
RFI	Relative fluorescence intensity
RFP	Red fluorescent protein

1 INTRODUCTION

Chromatin organization plays a crucial role in gene expression. Differences in chromatin organisation between cells, within the same organism, can be explained by understanding the underlying epigenetic mechanisms. DNA is organized in chromatin by being wrapped around histones, and the amino-terminal histone tails extending from the nucleosomes create unique, yet dynamic, properties. Transcription factors can only bind to specific sites on the chromatin, which can be temporarily unwrapped by the epigenetic machinery, and consequently induce histone modifications, allowing the transcription factors to encode only specific genes. Any changes in the organization of the chromatin structure can therefore have an impact on gene expression. Such changes can be made on purpose, for example, by specific histone modifications, ATP dependent chromatin remodeling complexes, transcription factors or other sequence specific DNA-binding proteins. Or they can be induced by various kinds of stress. In both cases the chromatin will be remodeled. In the first case, however, the purpose is to open up or compact the chromatin, and thereby regulating accessibility to the DNA. This induces gene transcription or gene silencing, respectively. In the second case, the remodeling can have more dramatic effects, and it is suggested that upon deregulation of the epigenetic machinery, the genes for cell senescence can be activated for example[1].

Moreover, on a larger scale, epigenetic changes may induce various forms of cancer, and have even been linked to depression and ageing[2-4]. Therefore, it is important to understand the mechanisms by which these deregulations take place. The underlying mechanisms of such unfavourable epigenetic changes are not entirely clear. Cellular stress, including DNA damage, are likely to be linked with such changes. However, little is known on chromatin remodeling induced by cellular stress. Moreover, the different types of stress inducing such epigenetic alterations have not been investigated. Recent findings have shown that stress induced by heat shock (HS), has a surprising effect (Van den Boom, et al., unpublished data). This effect is attributed to Polycomb Repressive Complex 1 (PRC1). PRC1, which consists of several subunits, is involved in the repression of important regulatory genes by binding to the chromatin and thereby silencing gene transcription in conjunction with a second PRC complex: PRC2[5]. Remarkably, upon HS, PRC1 deconjugates from the

chromatin, and one of the subunits, CBX2, localizes in the nucleolus. After a recovery phase, the localization reverts back to its original state (Van den Boom et. al. unpublished data).

This thesis aims to confirm these recent findings, and explore the dynamics of PRC1 upon heat shock. In order to investigate this, protein mobility will be measured using advanced microscopy techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP). Using these methods, this study aims to reveal the dynamics of CBX2, subunit of PRC1, in the nucleus and sub-nuclear compartments upon heat shock.

1.1 CHROMATIN

Structure and Function

Chromatin is the structure which encompasses DNA and other proteins, together this forms an organized construction. Within the nucleus of eukaryotic cells, chromatin usually appears as a neatly folded structure. DNA is tightly packaged around histones, and further leveled by covalent modifications and non-histone chromosomal proteins. The four histones (H2A, H2B, H3, H4) interact in pairs to form an octamer. The DNA wraps twice around the octamer to form a nucleosome. In turn, nucleosomes bind to each other via linker histones (histone H1), and become even more compressed when they are organized in chromatin fiber. The two main types of chromatin in the cell nucleus are heterochromatin and euchromatin. The first is highly compressed, and thus hard to transcribe. This mainly encompasses the inactive genes. The latter has a more open structure, and contains the active genes[6].

There appear to be four main functions of chromatin. First, the compression of DNA; the proteins involved in chromatin folding aid in fitting the DNA inside the nucleus. Second, the dynamic structure of chromatin allows specificity regarding gene expression and DNA replication. Due to histone modifications, selected genes can be activated at the appropriate time in specific cells. Third, the chromatin structure allows reinforcement of DNA macromolecules to favour cell division. Lastly, chromatin plays an important role in preventing permanent DNA damage, by influencing accurate functioning of reparation machineries[7]. Continuously, the organization of chromatin plays an important role in gene expression.

Understanding the dynamics and structure of chromatin is crucial with regards to the activation and silencing of numerous cellular mechanisms. Over the last few decades, a great deal of research has elucidated the structure and mechanisms of chromatin and chromatin remodelers. At the same time, many questions remain unanswered, such as the exact mechanisms by which chromatin remodelers work. This is crucial in the understanding of the epigenetic machinery. Furthermore, elucidating the impact of permanent chromatin alterations, and whether this can have unfavourable pathologic consequences, is of great importance. Therefore, more research on specific mechanisms of chromatin, and chromatin remodelers, is necessary.

Modification of Core Histones

Chromatin is highly dynamic due to the many post-translational modifications made to the histones. Nucleosome interaction is constantly changed by proteins with enzymatic properties. This, in turn, influences DNA transcription. Moreover, the restructuring of chromatin composition also influences other processes, including replication and DNA repair[8]. All four histones have specific amino (N)-terminal tails extending from their structure. These tails, in turn, have an important role in gene regulation because of covalent modifications. A large percentage of the histone tails are methylated, phosphorylated, acetylated and ubiquitinated, amongst other modifications[9]. Of special interest here is the ubiquitination of histone H2A. Ten percent of the H2A is ubiquitinated in mammalian cells[10]. Chromatin remodelling complexes can bind to the modified histones, including ubiquitin or methyl groups, and lead to a structural change from euchromatin to heterochromatin or have the opposite effect.

Polycomb Repressive Complexes

One class of chromatin remodelers are the Polycomb Repressive Complexes (PRCs). There are two distinct PRC complexes: PRC1 and PRC2, which can work in concert, but may also function independently[11]. PRCs epigenetically modify the chromatin, and are of special interest because of their role in silencing important regulatory genes. The most interesting genes PRCs bind to are associated with the determination and establishment of cell fates[12]. Thus, the function, and possibly the composition, of PRCs are different in embryonic stem cells (ESCs), than in fully differentiated cells, where they bind to different genes[13]. PRCs play trivial roles in

ESC development and also in hematopoietic stem cell (HSC) fate[14]. The complexes were first described in *Drosophila melanogaster* where they were found to regulate the transcription of HOX genes, which determine the frontal-dorsal axis in *drosophila* during development[15]. In humans, HOX genes encode for numerous HOX proteins, each associated with specific developmental properties[12]. Mutations in PRC complexes result in derangement of HOX gene expression, which can have detrimental effects on vertebrate development[12]. Polycomb complexes are attributed to be involved in many more processes, including cell cycle regulation, senescence, apoptosis, differentiation, DNA replication checkpoints and DNA damage response[5].

A second important gene that PRC silences are the CDKN2A genes. The INK4A/ARF locus on the CDKN2A genes encodes for the tumour suppressors and cell-cycle regulators $p14^{ARF}$ and $p16^{INK4A}$. INK4A/ARF activation also appears to be involved in cell senescence. In senescent cells, PRCs appear to be lost at these loci[16]. Under normal conditions, p16 is involved in a signalling cascade leading to G1-S cell cycle progression. Both p14 and p16 are involved in signalling cascades which inhibit cell senescence and apoptosis. Upregulation of p16 and p14, which can be induced by several forms of stress, is therefore connected to cell senescence[17]. Because PRC1 and PRC2 have such a crucial regulatory role in gene repression, the complexes should therefore bind to i.e. HOX and INK4A/ARF loci in a stable manner, in order to prevent cell deregulation.

In figure 1, the structure of PRC1 is highlighted. PRC1 is composed of several subunits: PCGF, PHC, CBX, and RING1[18,19]. PCGF2 is also known as MEL18 and PCGF4 is known as BMI1. In the canonical model, PRC1 depends on PRC2 to be recruited to the chromatin. PRC2 is responsible for the trimethylation of histone H3-K27 at Polycomb target genes (H3AK27me3)[20]. As a consequence, CBX targets PRC1 to H3AK27me3. The proximity of PRC1 at H3AK27 induces the mono-ubiquitination at lysine 119 of H2a (H2Ak119ub)[19]. This process is mediated by RING1, which is an E3 ligase. Thus, PRC1 is bound to the chromatin by RING1 at H2AK119ub, and associated with H3AK27me3 by members of the CBX family[14,21].

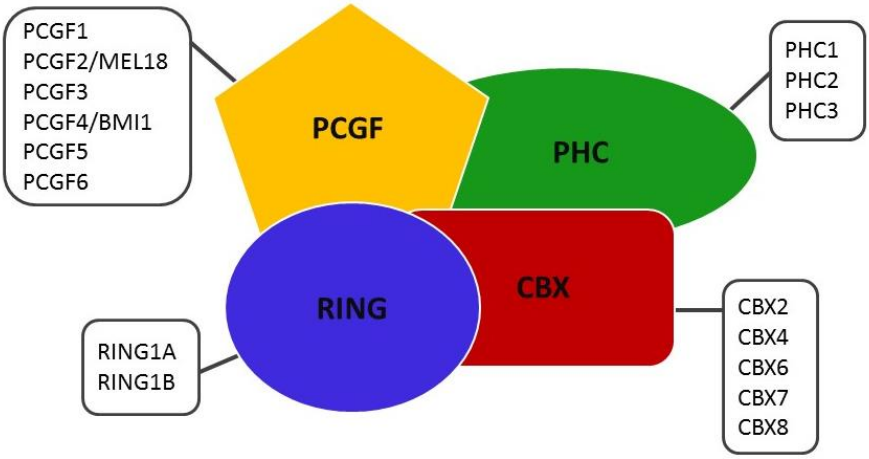


Figure 1: Structure of PRC1. PRC1 consists of PCGF, PHC, RING and CBX, all of which have paralog members (adapted from Connelly & Dykhuizen, 2017)

PRC1 function is altered in various forms of cancer. In acute myeloid leukaemia (AML), for instance, high expression of BMI1 is associated with lower survival rates. Higher expression of BMI1 was also observed in further stages of AML[1]. Furthermore, several models have been provided where dysregulation of PRCs play a role in gain and loss of cell fate, and thereby induce the activation of transcription factors, which can lead to tumour cells[4]. As aforementioned, the functioning of chromatin remodelers under stress conditions is an unexplored field. Different types of stress may evoke different types of responses. These can be reversible and non-damaging, or may have a drastic impact. Thus, understanding the exact mechanisms of PRCs under stress conditions is of importance. This can aid in understanding various types of cancerous cells, the determination of cell fate under stressful conditions, and can provide valuable insights into new therapies.

1.2 HEAT SHOCK

Heat Shock Response

Cells are apt to respond to various kinds of stress via specific molecular pathways. The first and most researched form of stress is the heat shock response, which was first described in 1962[22]. Since then, numerous researchers have tried to elucidate the mechanisms by which cells adapt to stress. The stress responses are intriguing cellular responses, as they are preserved among almost all species, and similar during different kinds of stress[23]. Examples of forms of stress are nutrient

deprivation, DNA damage, unfolded proteins, oxidative stress, cold shock and heat shock.

An important finding was the identification of a class of proteins named Heat Shock Proteins (HSPs), a special set of proteins of which the levels rise during heat shock[24]. HSPs, also known as molecular chaperones, have numerous functions with regards to damaged proteins. The HSP machinery can be seen as the "guard" of the protein homeostasis in cells. Perhaps one of the most important functions is guiding proteins to properly fold, and to refold damaged proteins[23,25]. Other functions of HSPs include ensuring timely degradation, disaggregation, and translocation of proteins across membranes[23]. Continuously, HSPs play a crucial role in the cell, both under normal and stressful conditions.

When the chaperone machinery does not function properly, however, this can have adverse consequences. For instance, toxic aggregates can be formed, and such aggregates are present in neurodegenerative diseases, including Alzheimer's and Huntington's[25]. Thus, understanding the molecular pathways by which the HSPs function under normal and stressful conditions is essential in preventing toxic cellular conditions.

The Nucleolus and Heat Shock

Next to the HSP machinery, one of the central hubs for coordinating stress responses is the nucleolus. While the exact interplay between the HSP machinery and nucleolus during stress responses has not been elucidated, both have a special role in stress responses. Structurally, the nucleolus is an interesting compartment: it is a liquid body located in the nucleus. A liquid body is a cellular compartment which is not separated from other compartments by a membrane, but rather by a phase separation[26]. The nucleolus consists of several compartments; the fibrillar centres, dense fibrillar component, and a granular component[27]. Under normal conditions the nucleolus has a function in the storage and transcription of ribosomal DNA, and participates in ribosomal biogenesis. Other functions include the assembly of multiple signal recognition particles and modifying transfer RNA[28].

Under stress conditions, however, the nucleolus appears to sense stress, and therefore may have a distinct role under stressful conditions[29]. The structure seems to disintegrate under HS conditions[30]. Several protein complexes depart from the nucleolus to i.e. prevent DNA synthesis[31,32]. It is suggested that the nucleolus can be

seen as a “storage room” for proteins that are released upon stress conditions[23]. Other proteins, however, are suggested to move into the nucleolus under the same conditions. This appears to be a coordinated process guided by molecular chaperones[29]. In the past, HSP70 has been shown to guide misfolded proteins to the nucleolus upon heat shock[33]. This implies that under stress, molecular chaperones may play an important role with regards to the nucleolus.

PRC1 and Heat Shock

Recent findings suggest that canonical PRC1 releases from the chromatin upon heat shock. As is depicted in Figure 2, PRC1 releases from the chromatin binding sites and CBX proteins (CBX2, CBX4, CBX6, CBX7 and CBX8) deconjugate from the complex and localize in the nucleolus (Van den Boom, et al., unpublished data). Furthermore, H2AK119ub also appears to be released. The site to which PRC2 binds, at H3K27me3, does not appear to be lost. Upon recovery, e.g. after heat shock for thirty minutes and a recovery time of three hours, the nucleolar localization dissolves.

Much remains unanswered regarding the dynamics of PRC1 upon heat shock. For instance, the mobility of PRC1 upon heat shock in the nucleus and nucleolus is unknown. Also, it is not certain whether PRC1 binds to the same ubiquitin site upon recovery. Other questions, such as which chaperones and co-chaperones are possibly involved in transporting CBX towards or away from the nucleolus need to be elucidated. Lastly, the large-scale consequences of this possibly adverse epigenetic alteration are unknown. However, to answer the first step of this unexplored field, this research will try to identify the dynamics of canonical PRC1, and in particular subunit CBX2, in the nucleus and sub-nucleolar compartments upon heat shock using advanced microscopy techniques.

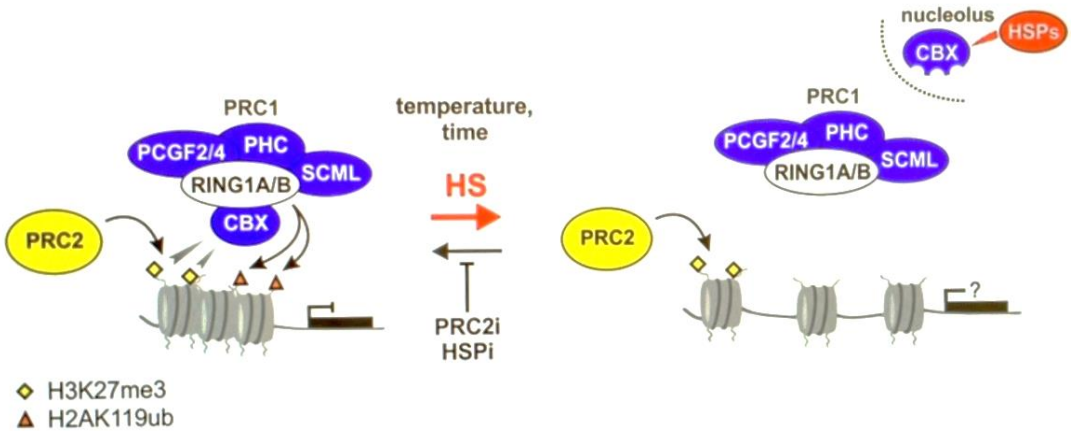


Figure 2: The effects of heat shock upon canonical PRC1 (Van den Boom, et. al., unpublished data)

2 MATERIAL AND METHODS

2.1 CELL LINE CREATION

Stable HeLa cell lines were generated expressing green fluorescent protein (GFP) fusion of canonical PRC1 subunits. Using lentiviral transduction, GFP-CBX2, BMI1-GFP, MEL18-GFP, RING1B-GFP and GFP cell lines were made in HeLa. A lentiviral vector was used because it creates stable cell lines, without high overexpression of the selected proteins. Selection of successful GFP emitting cells was done using fluorescence-activated cell sorting (FACS).

2.2 HEAT SHOCK

Heat shock was administered using a water bath at 44ºC for 30 minutes. Measurements were done immediately after HS; with a 0 minute delay; 30 minute delay; 1,5 hour delay and 3 hour delay. Earlier research (Van den Boom, et al., unpublished data) showed recovery of PRC1 subunits from 44 ºC HS after 3 hours.

2.3 SEEDING AND TRANSFECTIONS

Cells were seeded 48 hours before visualization under the microscope, in glass bottom dishes. Transfections were done 24 hours before visualization, with 0,2 µg of plasmid DNA and 0,8 µg of empty vector, and 6 µl of PEI. Medium was changed 4 hours after transfection. The used plasmids were mRuby-fibrillarin, red fluorescent protein (RFP) and histone H2B-GFP.

2.4 MICROSCOPY TECHNIQUES

In this research, a Zeiss LSM 780 confocal microscope, with a 63x emersion oil lens was used. The used software was ZEN Black. The microscope was brought to a temperature of 37ºC, and 5% CO2 circulated through the microscope to follow the live cells under normal conditions. All treated cells were placed under the microscope immediately after removal from the water bath. FRAP analysis was performed over the span of 10 seconds. All conditions were repeated three times, and measurements were performed on 10 different cells per condition. FRAP regions were small strips, which was chosen to optimize the laser efficiency. FLIP regions were half a nucleus,

with a nucleolus present inside the bleached regions, and outside the bleached region. Analysis was done in the nucleolus and in the nucleoplasm of the bleached region and in the nucleolus and the nucleoplasm of the non-bleached region. FLIP was repeated in ten treated GFP-CBX2 cells. The span of the FLIP analysis lasted 60 seconds.

2.5 Data Analysis

Intensity rates after FRAP analysis were converted to Relative Fluorescence Intensity (RFI) rates, to compare different cells and different conditions. Immobile fractions were calculated based on the average of the recovery RFI rates (from 7s-10s). FRAP analysis of GFP was used as the baseline, and was set at 0%.

3 RESULTS

In order to study the dynamic behaviour of PRC1 under normal and stress conditions, GFP-fusion cell lines were created, of the aforementioned PRC subunits (GFP-CBX2, MEL18-GFP, RING1B-GFP, BMI1-GFP and GFP), using lentiviral transduction. FACS analysis showed efficient transduction rates. Upon visualization, HeLa GFP-CBX2 only showed fluorescence in the nucleus, as expected, and also showed an equal fluorescent distribution. To confirm previous results (Van den Boom, et al., unpublished data) HS was administered to GFP-CBX2 cells. After HS with various delay times, visualization of fixated cells showed localization of CBX2 in sub-nucleolar compartments inside the nucleus (figure 3A). Immediately after HS (e.g. a 0 minute delay), 30 minute and 1,5 hour delay, sub-nucleolar localizations were present. Three hours after HS, localization of GFP-CBX2 reverted to the normal pattern (figure 3A). In order to confirm localization inside the nucleolus, mRuby-fibrillarin was expressed in GFP-CBX2 cells by means of transient transfection. mRuby-fibrillarin is a known fluorescent nucleolar marker[34], and confirmed the presence of GFP-CBX2 inside the nucleolus upon HS (Figure 3B).

To follow the dynamics of PRC1 upon heat shock over time, microscopy techniques are highly valuable. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are best suited to follow the dynamics of PRC1. In this research, FRAP is used to show that at a specific location an immobile fraction is present[35]. Whereas FLIP can also show protein transportation and protein aggregation[36]. The advantage of using advanced microscopy techniques is being able to follow the dynamics of the PRC1 complex in the entire nucleus, and, in live cells.

Surprisingly, FRAP analysis of GFP-CBX2 cells after HS showed a lower recovery in nuclear compartments of treated cells than in untreated cells. Moreover, after HS, a lower recovery rate was present in the nucleolus than in the nucleus (Figure 3E). Based on above results, immobile fractions were calculated. Analysis of GFP-CBX2 after HS in the nucleolus showed the highest immobile fraction of 13,4%, followed by GFP-CBX2 after HS in the nucleus, with 8,6%. Untreated GFP-CBX2 cells, on the other hand, showed an immobile fraction of only 3,4% (Figure 4D).

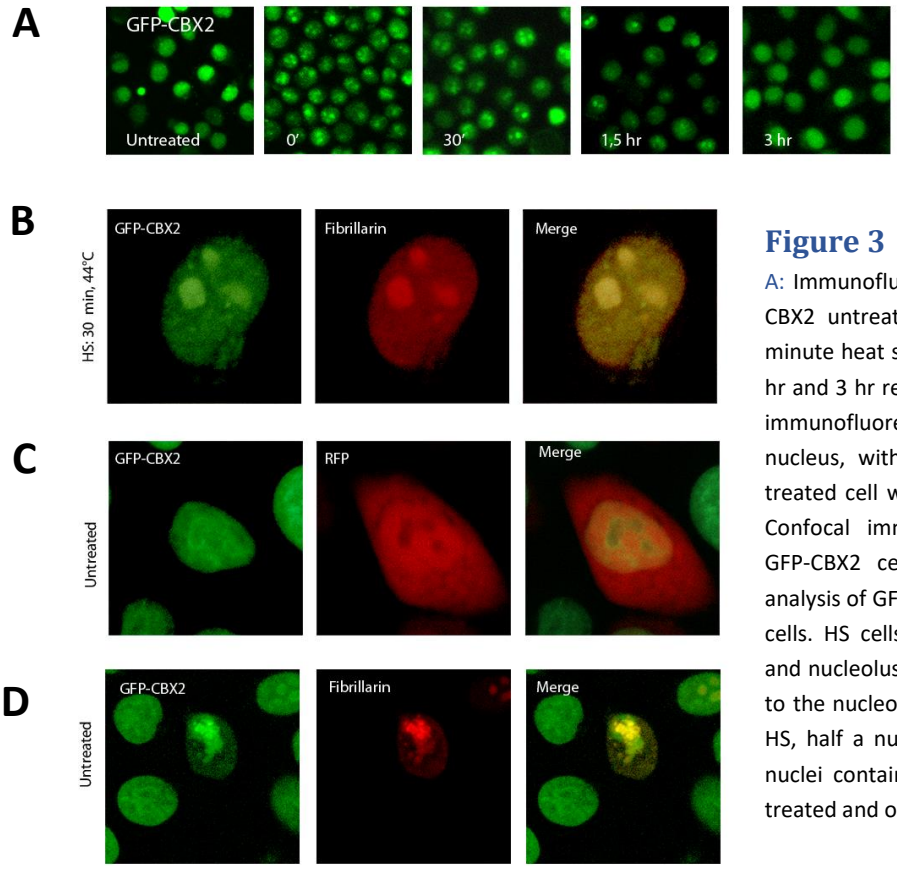
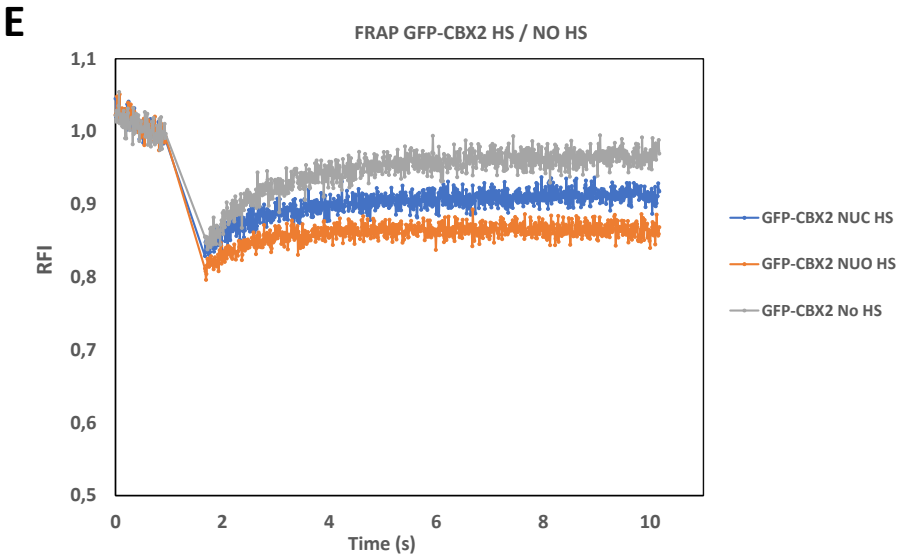
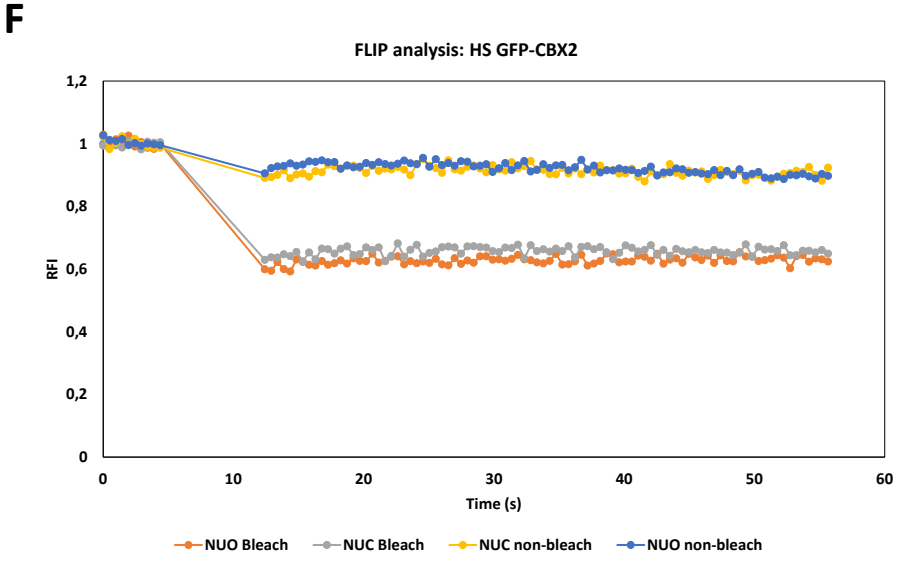


Figure 3

A: Immunofluorescence visualization of fixed GFP-CBX2 untreated cells, and treated cells with 30 minute heat shock at 44°C, with 0 min, 30 min, 1,5 hr and 3 hr recovery time, respectively B: Confocal immunofluorescence image of a GFP-CBX2 nucleus, with mRuby-fibrillarin transfection of a treated cell with 30 minute heat shock at 44°C C: Confocal immunofluorescence of an untreated GFP-CBX2 cell, with RFP transfection D: FRAP analysis of GFP-CBX2 of treated cells and untreated cells. HS cells were analysed in the nucleoplasm and nucleolus. NUC refers to the nucleus and NUO to the nucleolus E: FLIP analysis of GFP-CBX2 after HS, half a nucleus underwent photobleaching, all nuclei contained two nucleoli, of which one was treated and one untreated





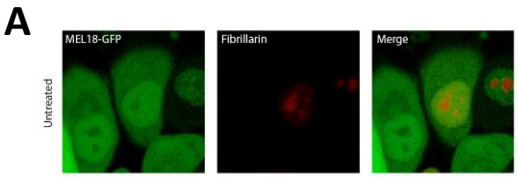
CBX2 in treated cells display very different behaviour in treated cells than in untreated cells. FLIP analysis after HS of GFP-CBX2 cells provided further confirmation of the immobile fractions. After photo bleaching of half the nucleus, with recovery of 1 minute, the immobile fraction of GFP-CBX2 in both the nucleus and nucleolus firmly sustained. This indicates that there is no movement between compartments and inside compartments (figure 3F).

A curious finding was encountered during visualization of mRuby-fibrillarin transfection of GFP-CBX2 untreated cells. Whereas in other untreated cells GFP-CBX2 was equally distributed, mRuby-fibrillarin transfection evoked a response in untreated cells, leading to migration of the protein towards the nucleolus (Figure 3D). This puzzling finding cannot be entirely explained, and needs future research.

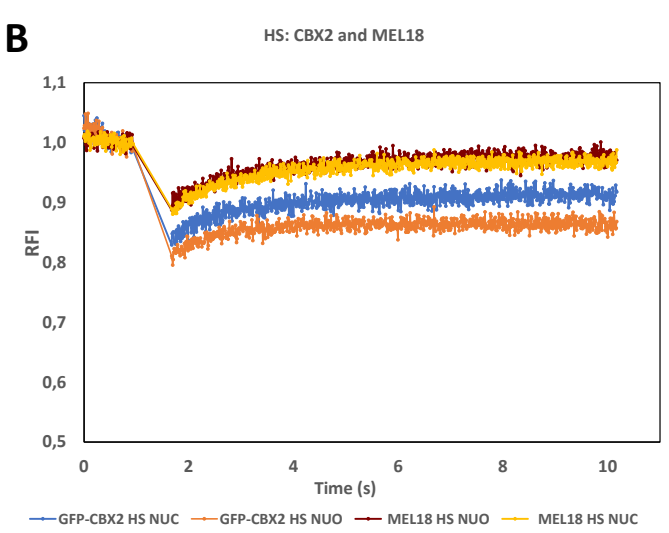
To verify similar behaviour of GFP-CBX2 in untreated cells in the nucleolus and nucleus, visualization of the nucleolus was necessary, since under normal circumstances the nucleoli are not visible in untreated GFP-CBX2 nuclei. Therefore, RFP transfection was performed. Opposite of mRuby-fibrillarin, RFP does not bind to structures for visualization, nor does it localize in the nucleolus, and thus interferes minimally with cellular processes. RFP transient transfection of GFP-CBX2 cells resulted in clear visualization of the nucleoli inside the nucleus, and allowed FRAP analysis of the nucleus and the nucleolus in untreated cells (Figure 3C). Furthermore, GFP-CBX2 appeared to be minimally influenced by RFP transfection. Contradictory to the difference in dynamic behaviour of GFP-CBX2 after HS, GFP-CBX2 RFP transfected cells showed equal behaviour, and displayed similar recovery rates and immobile fractions, of the protein in the nucleolus and the nucleus (Appendix 1).

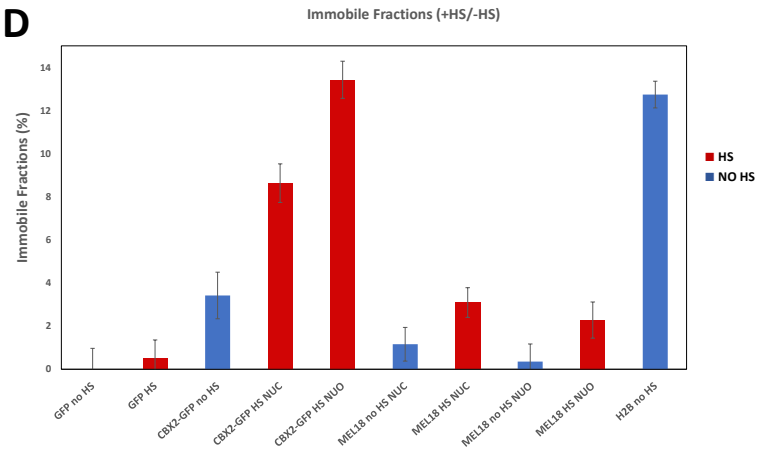
To compare the results of GFP-CBX2 to other PRC1 subunits, MEL18-GFP was analysed. MEL18-GFP is a known PRC1 subunit which does not localize in the nucleolus upon HS. It does, however, also release from the chromatin (van den Boom et al., unpublished data). Moreover, MEL18-GFP is the most suitable control, because both untreated and treated cells allowed for clear visualization of the nucleolus. MEL18-GFP is present both in the cytoplasm and nucleoplasm, but appears not to be localized in the nucleoli (Figure 4A). mRuby-fibrillarin transfection also showed this. In sharp contrast to CBX2, MEL18 did not show a large increase in the immobile fraction after HS. In untreated cells, CBX2 showed an immobile fraction of 3,4%. In MEL18 these fractions were substantially lower: at 1,2% and 0,4%, in the nucleus and nucleolus, respectively. Remarkably, when treated, the immobile

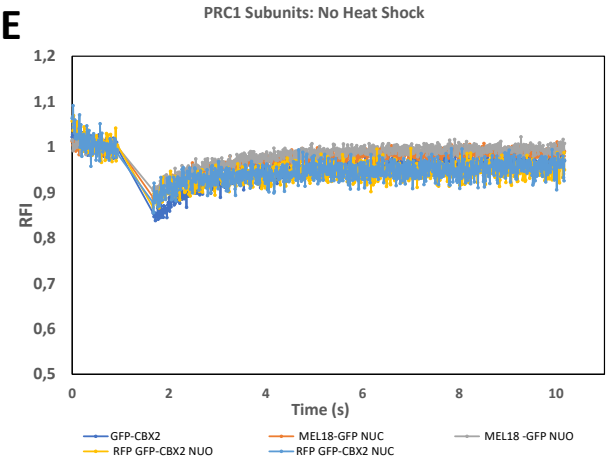
Figure 4

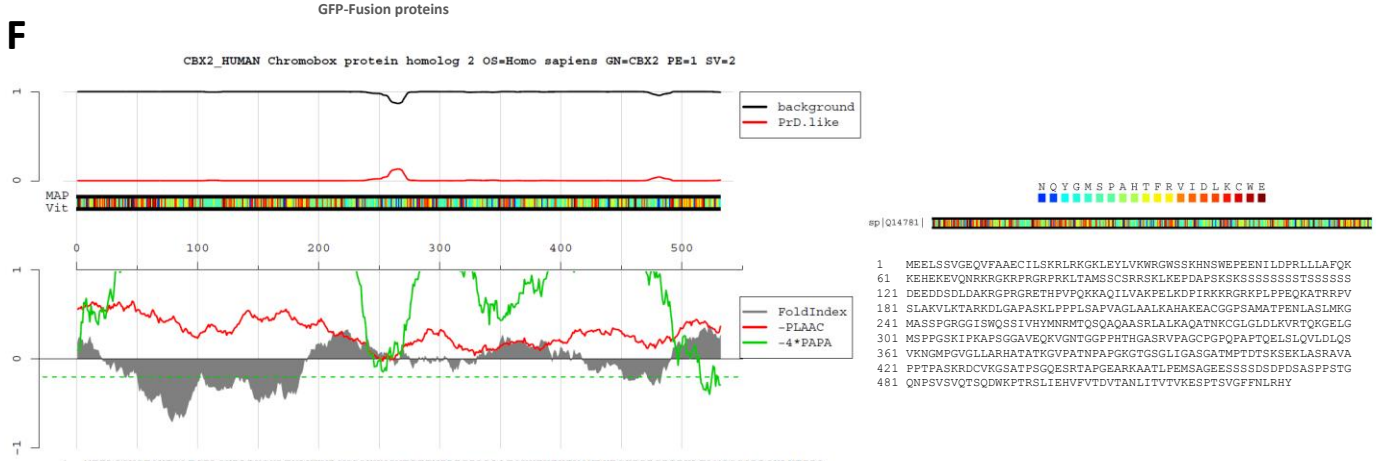


A: Confocal immunofluorescence of untreated MEL18-GFP cells with mRuby-fibrillarin transfection B: FRAP analysis of treated GFP-CBX2 compared to MEL18-GFP cells. Cells were analysed in the nucleoplasm (NUC) and the nucleolus (NUO) C: FRAP analysis of GFP-CBX2 compared to GFP cells and in comparison to H2B-GFP transfected cells. HS GFP-CBX2 cells were analysed in the nucleolus (NUO) and nucleoplasm (NUC), GFP cells and H2B cells in the nucleus









D: Immobile fractions of selected GFP-fusion proteins, in relation to GFP, which is set at 0% immobile. Selected proteins were untreated or treated with HS. NUC refers to the nucleus and NUO to the nucleolus E: FRAP analysis of untreated selected cells. NUC refers to the nucleus and NUO to the nucleolus F: HMM prediction of CBX2. The PrD. like (prion domain like) versus background shows the prediction of the probability of a prion like domain. The amino acid composition is highlighted in red if a prion like domain is present

fractions of MEL18 only rose slightly: to 3,1% and 2,3% (Figure 4D). The recovery rates of MEL18 did not also show a substantial change (Figure 4B). The comparison of MEL18 to CBX2 puts the results of CBX2 in context.

Furthermore, FRAP analysis of GFP HeLa cells and H2B-GFP transiently transfected HeLa cells was performed to appraise the extremes of the level of mobility, with GFP HeLa cells having the highest expected mobility and H2B-GFP transfected HeLa cells the lowest expected mobility. GFP cells, both treated and untreated, had a high recovery, and virtually no immobile fraction (Figure 4C). FRAP analysis of H2B transfected cells resulted in a low recovery rate and a high immobile fraction; at 12,7%. However, the immobile fraction of H2B was lower than the fraction of GFP-CBX2. Plausibly the transient expression of H2B-GFP caused an overexpression of histone H2B, resulting in a pool of mobile histones.

Analysis of untreated cells of PRC1 subunits (MEL18 and GFP-CBX2) displayed similar recovery rates (Figure 4E). When comparing treated GFP-CBX2 to other proteins, e.g. GFP, MEL18-GFP and H2B; GFP-CBX2 had a small mobile fraction, especially relative to H2B-GFP (Figure 4D).

To investigate one of the possibilities of the striking behaviour of CBX2, an amino acid composition analysis was performed. The online software ‘PLAAC’ uses a computational algorithm based on the hidden Markov model (HMM) to analyse any amino acid composition, and can result in evidence for a prion-like domain[37]. In the case of CBX2, a prion-like domain could point towards a natural high affinity for a phase separated compartment such as the nucleolus. Previous studies have shown that proteins with prion-like domains form liquid droplets. If such localizations sustain for a prolonged period of time, toxic aggregates are formed, which is the case in proteins associated with amyotrophic lateral sclerosis (ALS)[38]. In the case of CBX2, a prion-like domain could possibly explain the localization in the nucleolus. HMM prediction of CBX2 did, however, not result in enough evidence for a prion-like domain (Figure 4F). Thus, it is unlikely that the construct of CBX2 alone results in the localization of the protein in the nucleolus.

4 CONCLUSION

In conclusion, heat shock induces immobilization of GFP-CBX2. The highest immobile fraction and lowest recovery rate is present in the nucleolus, and a lower immobilization and recovery rate in the nucleus. Compared to untreated cells, treated GFP-CBX2 cells show a large increase in immobilization. Untreated cells of PRC1 subunits show very similar behaviour in the nucleolus and nucleus, both in GFP-CBX2 cells, and in MEL18-GFP cells. Upon heat shock, however, the behaviour of GFP-CBX2 changes drastically, and results in an immobile fraction, which was first observed by FRAP analysis and further substantiated with FLIP analysis.

5 DISCUSSION

The high immobilization of CBX2 after HS is a striking result. After HS, PRC1 releases from the chromatin, and CBX2 moves towards the nucleolus where it immobilizes. The pool of CBX2 in the nucleus also shows a heightened immobile fraction after HS. At first sight this is a curious observation; a pool of unbound PRC1 complexes would be expected to have a higher mobility than a pool of bound PRC1 complexes. A likely explanation is that due to the movement of the mobile fraction of CBX2 to the nucleolus, the remaining fraction of CBX2 in the nucleus is tightly bound, and thus causes the presence of a higher immobile fraction in the nucleus. The fact that in untreated cells the immobilization rate is 3,3%, gives rise to the idea that there is a large free pool of CBX2 in the nucleus. If the majority of available PRC1 complexes would be bound to the chromatin, the immobilization rate of untreated cells would have been substantially larger. Chromatin binding complexes, and also PRC complexes, are thought to bind to the chromatin in a dynamic manner[39,40]. Thus, there may be a tightly regulated transient binding of the PRC1 complexes to the chromatin. This can explain the idea that there is such a large pool of CBX2, which is readily available to bind to the chromatin.

Another question remains whether PRC1 is recruited anew to the chromatin after recovery, at the same Polycomb target genes as pre-heat shock. These possible changes start with the immobile fraction in the nucleus after HS. There is a possibility that CBX2 is bound to different loci pre-heat shock and immediately after HS. It may also be that CBX2 is bound to other structures which could explain the immobilization in the nucleus. If compared to MEL18, which also releases from the chromatin but does not immobilize, this could be a possibility. What happens upon recovery of CBX2 is not entirely clear. Chromatin immunoprecipitation (ChIP) analyses previously showed that H2AK119ub is lost upon HS, whereas H3K27me3 does not appear to be lost. It cannot be said with certainty if this allows for recruitment of PRC at the same Polycomb target genes after recovery. However, ChIP analyses did show recovery of GFP-CBX2 at Polycomb target genes four hours after HS, concomitant with increase of H2Ak119ub. The increase of H2AK119ub likely depends on an increase of H3K27me3. Furthermore, the exact mechanisms by which PRCs are recruited to the chromatin are unknown, even though several models have

been suggested[41], making it challenging to elucidate the specific recruitment mechanisms after recovery of HS. Moreover, since CBX2 is deconjugated from PRC1 after HS, reassembly of the complex is necessary. The mechanisms of the reassembly, and, if PRC1 reassembles in the same formation, remains up for questioning. ChIP analysis, however, can give future insights into the presence of PRC1 at Polycomb target genes.

A possible model for the dynamics of CBX2 before and after heat shock is provided here. Untreated nuclei contain both a free pool of CBX2 and a pool that is bound to the chromatin. Upon HS a large fraction of CBX2 disassociates from the chromatin. Both the free pool and the disassociated CBX2 proteins move towards the nucleolus. After HS, a small fraction of CBX2 remains in the nucleus, where it is still bound to the chromatin. Upon recovery, the CBX2 localization in the nucleolus dissolves, and is possibly recruited anew to the chromatin at Polycomb target sites (Figure 5).

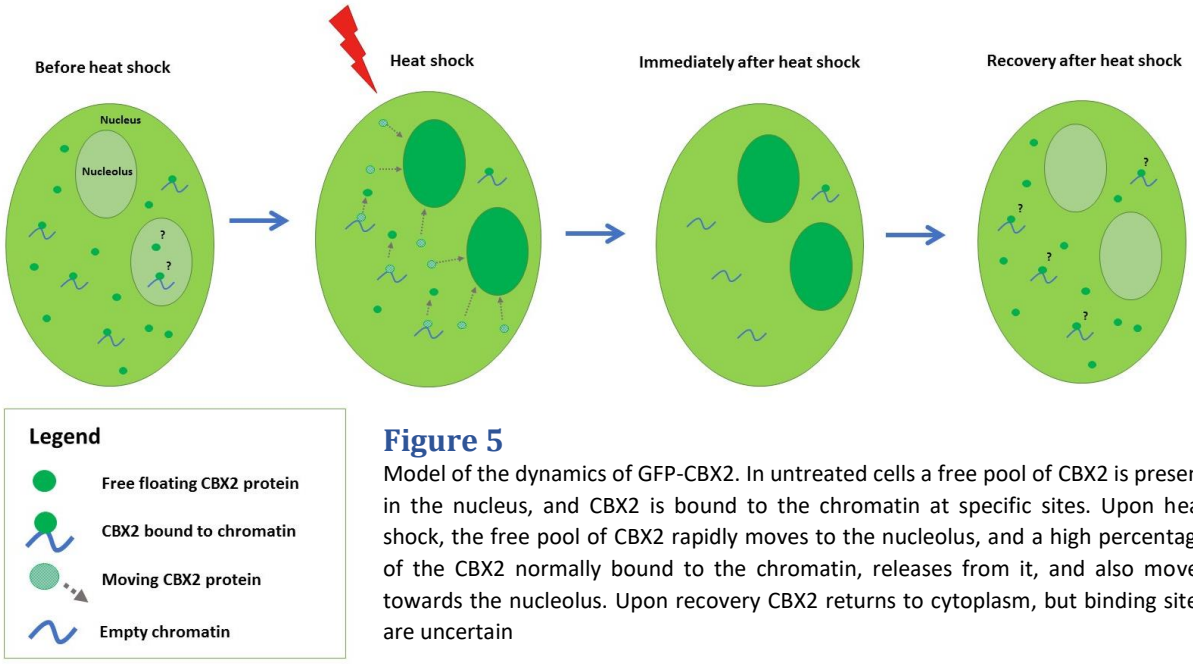


Figure 5
Model of the dynamics of GFP-CBX2. In untreated cells a free pool of CBX2 is present in the nucleus, and CBX2 is bound to the chromatin at specific sites. Upon heat shock, the free pool of CBX2 rapidly moves to the nucleolus, and a high percentage of the CBX2 normally bound to the chromatin, releases from it, and also moves towards the nucleolus. Upon recovery CBX2 returns to cytoplasm, but binding sites are uncertain

The high immobility of CBX2 in the nucleolus after HS could possibly be explained by recent findings. A recent study on liquid droplets induced by stress, which were phase-separated from the plasma, showed interesting results[42]. These findings directly concern this research, since the nucleolus is a phase separated sub-nuclear compartment. The liquid droplets have a gel-like structure, which can possibly

explain the immobilization. FRAP analysis of small regions inside the liquid droplets showed a high immobilization and little recovery after bleaching. However, opposite to misfolded protein aggregates, which remain highly immobile, the gel-like structure of the liquid droplets is proposed to be reversible. While this is all speculative for CBX2 immobilization inside the nucleolus, it can provide an exciting insight into the structure and functioning of the nucleolus under stress.

The curious finding of CBX2 moving to the nucleolus after mRuby-Fibrillarin transfection provokes several questions. Because of fibrillarin expression, the nucleolar structure appears to be altered. Perhaps the movement and immobilization of CBX2 towards the nucleolus is evoked by the altered nucleolar structure, rather than CBX2 being changed. It may be that the altered nucleolar structure evokes the response of CBX2 migration towards the nucleolus, and thus evokes epigenetic alterations. Such alterations have not been observed before. Therefore, ChIP analysis in mRuby-Fibrillarin transfected CBX2-GFP cells could possibly indicate the epigenetic changes that result from this curious observation.

As aforementioned, upon recovery, CBX2 departs from the nucleolus. Incubation of GFP-CBX2 cells with the HSP70 inhibitor VER-155008 was previously shown to induce more accumulation of GFP-CBX2 in the nucleolus after HS and a slower recovery, suggesting a role for HSP70 in exporting CBX2 from the nucleolus. One of the functions of HSP70 is to refold misfolded proteins, however, so far it has not been researched if this is also the case with CBX2 after HS. The role of HSP70 in possibly transporting CBX2 also need to be established.

5.1 Concerns

There are several concerns regarding this study, which might slightly distort the results, but do not change the conclusions.

One possibly worrying feature are the microscopy settings and the laser used for photobleaching. The laser is unable to bleach optimally and deep, and does therefore not give constant measurements. This alters the exact numbers of this research, but does not alter the observed trends. However, because both FLIP and FRAP indicated immobilization of CBX2, there is no doubt about the conclusions. In future research, FRAP settings have to be optimized in order to obtain even more accurate results.

A second concern is whether using FRAP analysis the actual biological pool of CBX2 is measured, or, if the free pool of CBX2 is depleted and the measurements

reveal a relative diminished equilibrium pool of CBX2, which has simply shifted towards the nucleolus. One indication of this is the difference between the immobile fractions of treated GFP-CBX2 in the nucleus and nucleolus after FRAP analysis. Because of the depletion of GFP-CBX2 in the nucleus, less proteins are available to be measured, resulting in a lower immobile fraction than what is actually present. Because of repetition of the FRAP experiments, the immobile fractions relatively to each other show the actual trends.

Another concern is that even though lentiviral transduction stably expresses the GFP fusion proteins, the selected proteins are plausibly overexpressed, and thus do not represent the endogenous function of fusion proteins. One indication that this might be the case is that there appears to be a large free pool of both proteins, which might explain the high recovery rates of the selected proteins. In endogenous cells, the recovery might be much lower, and the immobile fractions after HS much higher. Western blot analysis should verify equal or lower expression of GFP-CBX2 compared to endogenous CBX2. A CRISPR/CAS9 GFP construct specifically targeted to PRC fusion proteins can, in future research, represent the most endogenous pool of fusion proteins.

Lastly, H2B-GFP transiently expressed in HeLa cells plausibly evoked high overexpression of H2B, which resulted in an immobile fraction of 12,7%. In other studies, this number is substantially lower[43]. To provide a good control to appraise the extremes of immobile fraction for PRC subunits, a stable H2B-GFP cell line is required.

5.2 Future Research

Future research regarding PRC1 and HS can take many directions and gives rise to many questions. What causes the immobilization of CBX2 inside the nucleolus and nucleus? What is the effect of an altered nucleolar structure on CBX2 and other proteins? What is the function of the possible free pool of CBX in the nucleus? By which means does CBX2 move towards the nucleolus? Which chaperones and co-chaperones are involved? Is PRC1 recruited by the chromatin at Polycomb target sites upon recovery of the cell? This last question is perhaps the most concerning. If PRC1 is not the recruited by the chromatin, this can have drastic consequences. If, for instance, the epigenetic machinery which normally induces repression of the INK4a/ARF locus is lost, this can activate gene transcription which can consequently

induce cell senescence[16]. Even more concerning, in ESCs and HSCs cell fate can be lost. FRAP analysis might prove a valuable technique in answering several of the aforementioned questions. In combination with other techniques, insights from different perspectives can be gained.

One suggestion for future research is the creation of stable cell lines with a mutation in the chromatin binding domain of H3K27me3. This can be used to compare the endogenous recruitment of PRC to impaired recruitment of PRC after HS, upon recovery[40]. A stable cell line expressing both mRuby-fibrillarin, which localizes in the nucleolus, and GFP-fusion proteins of the PRC complex can provide valuable insights into the endogenous protein mobility in the nucleolus, and can prove an important control for subunits of PRCs which are not easily visible in the nucleolus.

ChIP analysis will also be very valuable for future research. Exposing stem cells to HS could possibly induce changes to cell fate. If this were the case, a strong connection between stress and epigenetic changes could be concluded. Repeated stress on cells could also result in altered Polycomb binding. ChIP analyses and gene expression assays could indicate aforementioned possible changes.

Moreover, it is not certain if subunits of PRC are misfolded after HS. If this were the case, chaperones interacting with CBX2 and other PRC subunits, would likely guide the refolding process.

Lastly, the nucleolus appears a much more diverse and complex structure than previously thought. It may well be that the nucleolus prevents the degradation of CBX2 after HS, and only releases the protein under stable conditions, which can be a process guided by HSPs. It firmly stands that CBX2 is immobilized after heat shock. This research has provided valuable insights into the immobilization of CBX2 in the nucleus nucleolus upon heat shock, but many questions remain unanswered, and new questions have been raised. While the mechanisms are not yet understood, this research provides a possible model for the nuclear dynamics of CBX2.

6 References

1. Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*. 1999;397(6715):164-168.

2. Benayoun BA, Pollina EA, Brunet A. Epigenetic regulation of ageing: Linking environmental inputs to genomic stability. *Nature Reviews Molecular Cell Biology*. 2015;16(10):593-610.

3. Sananbenesi F, Fischer A. Remodeling the susceptibility to stress-induced depression. *Nat Med*. 2015;21(10):1125.

4. Bracken AP, Helin K. Polycomb group proteins: Navigators of lineage pathways led astray in cancer. *Nature Reviews Cancer*. 2009;9(11):773-784.

5. Sauvageau M, Sauvageau G. Polycomb group proteins: Multi-faceted regulators of somatic stem cells and cancer. *Cell stem cell*. 2010;7(3):299-313.

6. Dawson MA, Kouzarides T. Cancer epigenetics: From mechanism to therapy. *Cell*. 2012;150(1):12-27.

7. Lukas J, Lukas C, Bartek J. More than just a focus: The chromatin response to DNA damage and its role in genome integrity maintenance. *Nat Cell Biol*. 2011;13(10):1161-1169.

8. Bannister AJ, Kouzarides T. Regulation of chromatin by histone modifications. *Cell Res*. 2011;21(3):381-395.

9. Kouzarides T. Chromatin modifications and their function. *Cell*. 2007;128(4):693-705.

10. Carlson N, Rechsteiner M. Microinjection of ubiquitin: Intracellular distribution and metabolism in HeLa cells maintained under normal physiological conditions. *J Cell Biol*. 1987;104(3):537-546.

11. Kahn TG, Dorafshan E, Schultheis D, et al. Interdependence of PRC1 and PRC2 for recruitment to polycomb response elements. *Nucleic Acids Res*. 2016;44(21):10132-10149.

12. Krumlauf R. Hox genes in vertebrate development. *Cell*. 1994;78(2):191-201.

13. Connelly KE, Dykhuizen EC. Compositional and functional diversity of canonical PRC1 complexes in mammals. *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*. 2017;1860(2):233-245.

14. van den Boom V, Rozenveld-Geugien M, Bonardi F, et al. Nonredundant and locus-specific gene repression functions of PRC1 paralog family members in human hematopoietic stem/progenitor cells. *Blood*. 2013;121(13):2452-2461.

15. Lewis EB. A gene complex controlling segmentation in drosophila. In: *Genes, development and cancer*. Springer; 1978:205-217.

16. Bracken AP, Kleine-Kohlbrecher D, Dietrich N, et al. The polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. *Genes Dev*. 2007;21(5):525-530.

17. Sheppard KE, McArthur GA. The cell-cycle regulator CDK4: An emerging therapeutic target in melanoma. *Clin Cancer Res*. 2013;19(19):5320-5328.

18. Buchwald G, van der Stoop P, Weichenrieder O, Perrakis A, van Lohuizen M, Sixma TK. Structure and E3-ligase activity of the ring-ring complex of polycomb proteins Bmi1 and Ring1b. *EMBO J*. 2006;25(11):2465-2474.

19. Wang H, Wang L, Erdjument-Bromage H, et al. Role of histone H2A ubiquitination in polycomb silencing. *Nature*. 2004;431(7010):873-878.

20. Cao R, Wang L, Wang H, et al. Role of histone H3 lysine 27 methylation in polycomb-group silencing. *Science*. 2002;298(5595):1039-1043.

21. van den Boom V, Maat H, Geugien M, et al. Non-canonical PRC1. 1 targets active genes independent of H3K27me3 and is essential for leukemogenesis. *Cell reports*. 2016;14(2):332-346.

22. Ritossa F. A new puffing pattern induced by temperature shock and DNP in drosophila. *Cellular and Molecular Life Sciences*. 1962;18(12):571-573.

23. Velichko AK, Markova EN, Petrova NV, Razin SV, Kantidze OL. Mechanisms of heat shock response in mammals. *Cellular and molecular life sciences*. 2013;70(22):4229-4241.

24. Loomis WF, Wheeler S. Heat shock response of dictyostelium. *Dev Biol*. 1980;79(2):399-408.

25. Kampinga HH, Bergink S. Heat shock proteins as potential targets for protective strategies in neurodegeneration. *The Lancet Neurology*. 2016;15(7):748-759.

26. Feric M, Vaidya N, Harmon TS, et al. Coexisting liquid phases underlie nucleolar subcompartments. *Cell*. 2016;165(7):1686-1697.

27. Hadjiolov A. *The nucleolus and ribosome biogenesis.* Vienna: Springer; 1985.

28. Olson MO. Nucleolus: Structure and function. In: *eLS.* John Wiley & Sons, Ltd; 2001. 10.1002/9780470015902.a0005975.pub2.

29. Boulon S, Westman BJ, Hutten S, Boisvert F, Lamond AI. The nucleolus under stress. *Mol Cell*. 2010;40(2):216-227.

30. Iliakis GE, Pantelias GE. Effects of hyperthermia on chromatin condensation and nucleoli disintegration as visualized by induction of premature chromosome condensation in interphase mammalian cells. *Cancer Res*. 1989;49(5):1254-1260.

31. Wang Y, Guan J, Wang H, Wang Y, Leeper D, Iliakis G. Regulation of dna replication after heat shock by replication protein a-nucleolin interactions. *J Biol Chem*. 2001;276(23):20579-20588.

32. Vanderwaal RP, Maggi LB,Jr, Weber JD, Hunt CR, Roti Roti JL. Nucleophosmin redistribution following heat shock: A role in heat-induced radiosensitization. *Cancer Res*. 2009;69(16):6454-6462.

33. Nollen EA, Salomons FA, Brunsting JF, van der Want JJ, Sibon OC, Kampinga HH. Dynamic changes in the localization of thermally unfolded nuclear proteins associated with chaperone-dependent protection. *Proc Natl Acad Sci U S A*. 2001;98(21):12038-12043.

34. Ochs R, Lischwe M, Spohn W, Busch H. Fibrillarin: A new protein of the nucleolus identified by autoimmune sera. *Biology of the Cell*. 1985;54(2):123-133.

35. Ishikawa-Ankerhold HC, Ankerhold R, Drummen GP. Advanced fluorescence microscopy techniques—Frap, flip, flap, fret and flim. *Molecules*. 2012;17(4):4047-4132.

36. Wüstner D, Solanko LM, Lund FW, Sage D, Schroll HJ, Lomholt MA. Quantitative fluorescence loss in photobleaching for analysis of protein transport and aggregation. *BMC Bioinformatics*. 2012;13(1):296.

37. Lancaster AK, Nutter-Upham A, Lindquist S, King OD. PLAAC: A web and command-line application to identify proteins with prion-like amino acid composition. *Bioinformatics*. 2014;30(17):2501-2502.

38. Patel A, Lee HO, Jawerth L, et al. A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell*. 2015;162(5):1066-1077.

39. Di Croce L, Helin K. Transcriptional regulation by polycomb group proteins. *Nature structural & molecular biology*. 2013;20(10):1147-1155.

40. Phair RD, Gorski SA, Misteli T. Measurement of dynamic protein binding to chromatin in vivo, using photobleaching microscopy. *Meth Enzymol*. 2003;375:393-414.

41. Blackledge NP, Rose NR, Klose RJ. Targeting polycomb systems to regulate gene expression: Modifications to a complex story. *Nature Reviews Molecular Cell Biology*. 2015;16(11):643-649.

42. Riback JA, Katanski CD, Kear-Scott JL, et al. Stress-triggered phase separation is an adaptive, evolutionarily tuned response. *Cell*. 2017;168(6):1028-1040. e19.

43. Meshorer E, Yellajoshula D, George E, Scambler PJ, Brown DT, Misteli T. Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. *Developmental cell*. 2006;10(1):105-116.

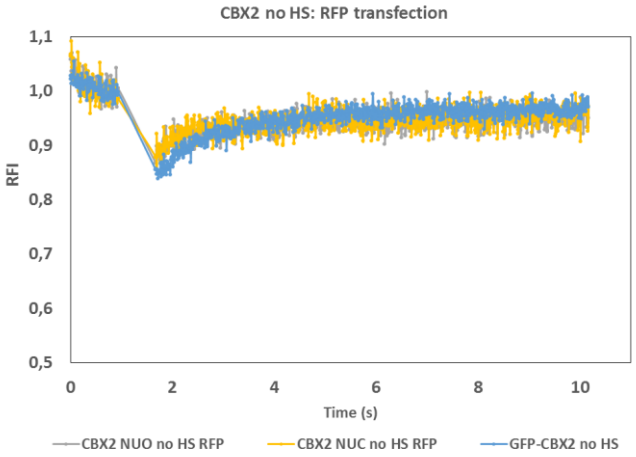
7 BIBLIOGRAPHY

Alberts, B. (2008). *Molecular biology of the cell* (6th ed.): [Hauptbd.]. New York, United States: Garland Science Taylor & Francis.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2012). *Biochemistry* (7th ed.). Basingstoke, United Kingdom: W.H. Freeman.

8 APPENDICES

8.1 APPENDIX 1



FRAP analysis of untreated transfected GFP-CBX2 cells compared to untreated GFP-CBX2 cells. RFP provided visualization of the nucleolus, which allowed for FRAP analysis in both the nucleus and nucleolus in untreated GFP-CBX2 cells. Similar recovery rates were observed.