Spring | 19

Mechanism of SUMO repression of the transcription factor c-Myb – the role of SUMO acetylation

Andrea Haug

Department of Biosciences
Faculty of Mathematics and Natural Sciences
University of Oslo
Master Thesis Spring 2019

Acknowledgements

The work presented in this thesis was carried out at the Department of Biosciences, Faculty of Mathematics and Natural Science at the University of Oslo, from January 2017 to June 2019.

First and foremost, I would like to thank my supervisor Professor Odd Stokke Gabrielsen for providing me with the opportunity to work on this project as well as welcoming me to his group. The guidance and supervision I have been provided with has been enormous and has made it possible for me to not only learn and improve academically but also be a part of and enjoy a great group.

I would like to specially show my gratitude to my co-supervisor Marit Ledsaak. I want to thank Marit for always being available and helpful, guiding and being patient with me. Marit has been an irreplaceable and supportive pillar that has helped me and encouraged me throughout the practical laboratory work as well as during the writing process. She has been enthusiastic and supportive at every step of this process.

Further, I would like to thank all members of the c-Myb group, both past and present. A special thank you to Roza for enthusiastically helping me in the laboratory, Signe for sharing valuable knowledge so freely and Fernando for always providing with helpful advices and insights.

I would also like to thank my fellow students in the group, Guro, Dina, Jan Ove, Pradip, Melanie and Guro M. Being in a similar situation and supporting each other along the way has been an invaluable experience. I would also like to thank my closest friends, outside the academic circle, Maria, Pia, Maren and Ingunn. They have been a great source of encouragement and motivation, as well as providing me with much needed support.

Lastly, I would like to thank my mother Maria and my partner in crime Sindre, who despite not having any clue to what my academic degree entails, has been curious, supportive and loving from the very start.

Oslo, June 2019

- Andrea Haug

Abstract

The transcription factor and oncoprotein c-Myb is an important master regulator of gene expression in hematopoietic cell lineages. The activity and function of c-Myb is regulated by a large network of protein interactions and post-translational modifications. One such modification is attachment of small ubiquitin like modifiers (SUMO), significantly affecting the transactivation activity of c-Myb.

The c-Myb protein interacts with SUMO in different ways, both by covalent attachment as well as trough non-covalent interactions. Research in our lab has revealed that SUMO conjugation and binding are important regulatory mechanism for c-Myb function (Ledsaak, Bengtsen et al., 2016; Molvaersmyr, Saether et al., 2010; Saether, Pattabiraman et al., 2011). SUMOylation of the c-Myb transcription factor is associated with repression, implicating that relief of SUMO-mediated repression may present a significant contribution to gene activation. Until recently, removal of SUMO appeared to be the only molecular strategy to obtain relief from SUMO-mediated repression. However, a paper from Stefan Müllers lab established an acetylation switch, capable of regulating SUMO-dependent interaction networks (Ullmann, Chien, Avantaggiati, & Muller, 2012). They identified specific acetylations of SUMO within the SUMO-SIM interface that disrupt and prevent SUMO-binding to partners such as PML, Daxx and PIAS. This acetyl-dependent switch is now believed to act as an alternative mechanism for derepression.

In this study, we investigated whether the defined acetylation switch applied to the transcription factor c-Myb and indeed confirmed its capacity of alleviating SUMO-mediated repression to some degree. This was achieved by using acetyl-mimicking and abolishing mutants of SUMO1 and 2 in gene reporter assays, validating a relief on SUMO-mediated repression in both c-Myb and non c-Myb context. Using coimmunoprecipitation assays, we demonstrated that the acetylation switch selectively regulates binding to partner proteins, functioning as a fine-tuning mechanism for binding specificities.

Table of Contents

1 Introduction

This first chapter will provide a general overview and knowledge of the topics discussed and investigated in this thesis, building a base for the reader to understand and interpret the achieved results in chapter three, as well as the discussion in chapter four. Basic introduction of the eukaryotic genome will be presented, as well as the transcription and epigenetic regulation, which will be briefly covered. Subsequently, the proto-oncogenic transcription factor c-Myb, and the small ubiquitin like modifiers- SUMO, as well as the interaction between the two, will be more thoroughly reviewed. Lastly, the aims of the study will be presented at the end of the chapter.

1.1 The eukaryotic genome

The entire genetic information of an organism is the definition of a genome. Composed of four nitrogen-containing bases: cytosine (C), guanine (G), adenine (A) and thymine (T), forming base pairs via hydrogen bonds, the DNA (deoxyribonucleic acid) is structured as a double helix with two sugar-phosphate backbones coiled around each other (Watson & Crick, 1953). As the template of heredity stretches up to 3 billion base pairs (in humans), the double helix is compacted in order to fit the small nucleus with a size of 6 μm (Alberts Bruce, 2002). In eukaryotes, different levels of DNA condensation provide the right level of tightness, ranging from the more open chromatin-state in between cell divisions to the more condensed chromosomes during cell division. The first and basic unit that DNA is primarily packaged into, is the nucleosome. A stretch of 146 base pairs (bp) of DNA is wrapped around (with 1.7 turns) a histone octamer, which consists of 2 copies of histones H2A, H2B, H3 and H4. The beads on a string structure is brought closer together by binding of the internucleosomal DNA sequences to a linker histone H1, forming what is referred to as a chromatosome. Binding of linker DNA to the linker histone H1 allows the structure to bind tighter and form a 30nm fiber, which can then in turn loop around scaffolding protein to form transcriptionally active euchromatin. The 30nm fiber generates loops that are on average 300nm in length and can be further compressed and eventually forming a full compacted chromosome. The human genome contains 23 pairs of chromosomes, where the primary 22 are autonomous, while the last 23rd pair are the sex chromosomes (Alberts Bruce, 2002; van Driel, Fransz, & Verschure, 2003).

1.1.1 The epigenome

Even though DNA is the carrier of the genetic information of an organism, the way it is expressed can be influenced by epigenetics. Epigenetics is defined as the modification and changes in the gene expression, independent of alterations of the genetic code itself. The presence of an additional layer of control on top of the DNA sequence, allows for determination of which genes are to be active and inactive, thus influencing the development of the organism as well. The epigenetic modifications compromise of covalent modification of the DNA bases, posttranslational modification on amino acids of the N-terminal tails of histones and histone variants (Dupont, Armant, & Brenner, 2009). The epigenetic regulation of gene expression is a reversible process and is governed by two classes of regulatory enzymes: the ATP-dependent chromatin remodeling enzymes and histone modifier enzymes (Arrowsmith, Bountra, Fish, Lee, & Schapira, 2012). The former class of enzymes are able to change and alter the position, occupancy or the histone composition of a nucleosome using energy from ATP hydrolysis. The enzymes are categorized into four families with distinct remodeling capabilities: SWI/SNF family, ISWI family, CHD family and INO80 family (Aalfs & Kingston, 2000; Mayes, Qiu, Alhazmi, & Landry, 2014). The other class of enzymes are histone modifiers, exerting their function by generating post-translational modifications on amino residues of N-terminal histone tails. These can be methylation, acetylation, phosphorylation, sumoylation and ubiquitination that promote local changes in the chromatin and manipulate the accessibility of the DNA to the transcriptional machinery (Bannister & Kouzarides, 2011). Alteration of the histone tails in turn recruit proteins and enzymes that recognize the modification marks, and appropriate downstream functional effects take place.

1.1.2 Transcription

The first step of gene expression is transcription; the synthesis of a single stranded complementary ribonucleic acid (RNA), from a DNA template. Transcription is primarily initiated by transcription factors (TF), some of which are pioneer TF, able to bind to nucleosomal DNA and recruit chromatin remodeling complexes. As the chromatin remodelers modulate and manipulate the accessibility of the DNA, additional TF, enzymes and co-factors are able to bind and the assembly of the transcriptional machinery takes place. RNA polymerase II is the enzyme catalyzing the transcription of DNA and synthesizes the messenger RNA (mRNA), which is subsequently spliced to RNA and further translated to proteins. Mistakes in transcription regulation and processing can lead to grave errors in the gene expression pattern, consequently generating faulty proteins that lead to tumorigenesis and other defects in the organism.

1.2 The transcription factor c-Myb

The transcription factor c-Myb is a master regulator of proliferation and differentiation of hematopoietic cells. The *c-myb* gene was originally identified as the cellular homolog of the truncated *v-myb* found in avian myeloblastosis virus (AMV) and the E26 virus in chicken (Lipsick & Wang, 1999). The former is causing rapidly fatal monoblastic leukemia *in vivo* (Baluda & Reddy, 1994), and the latter causing fatal erythroblastosis *in vivo* as well as transforming multipotent hematopoietic progenitors in culture (Graf, McNagny, Brady, & Frampton, 1992).

c-Myb is a member of the *myb*-gene family together with A-Myb and B-Myb, encoded by *MYB, MYBL1* and *MYBL2* genes, respectively. The three members share sequence homology and recognize the same DNA binding motif, but differ in binding affinity (Bergholtz, Andersen et al., 2001). Furthermore, the proteins are expressed in different tissues at varying levels (Oh & Reddy, 1999). There is a second class of Myb-related proteins that recognize and bind DNA sequences that are quite distinct from the ones recognized by c-Myb, plausibly due to a divergent DNA binding domain (Ness, 1996).

Studies have shown that members of the Myb family are present in a range of eukaryotic organisms including animals, plants, fungi, as well as some invertebrates, such as nematodes (Lipsick, 1996, 2010). All Myb proteins are localized in the nucleus, regulating the expression of genes by bridging mechanisms, mediating interaction between DNA and transcription machinery (Ness, 1996). In general, as cells terminally differentiate and withdraw from the cell cycle, *myb*-expression levels are reduced and switched off (Weston, 1998). Myb targets a range of genes that are linked to oncogenicity, either connected to differentiation or proliferation, as well as apoptosis (Lei, Rushton, Davis, Liu, & Ness, 2004; F. Liu, Lei, O'Rourke, & Ness, 2006; Ramsay & Gonda, 2008).

The c-Myb protein consists of three functional domains: an N-terminal DNA binding domain (DBD), a centrally located transactivation domain (TAD) and a C-terminally regulatory domain (CRD), generating a translational product of 75 kDa with 640 amino acids (Sakura, Kanei-Ishii et al., 1989). Additionally, a 89 kDa c-Myb protein exists, arising from an alternatively spliced mRNA of *MYB*, where a region of 363 base pairs is included between exon 9 and 10. This region, commonly called exon 9A is also present in A and B-Myb (Dasgupta, Linnenbach, Giaccia, Stamato, & Reddy, 1989; Dasgupta & Reddy, 1989; Woo, Sopchak, & Lipsick, 1998). A- and B-Myb proteins have a size of 95 and 93 kDa, respectively. The three family members contain a highly conserved DBD, followed by a TAD in A and c-Myb, whereas B-Myb contains an acidic region. The CRD is less conserved between the three proteins, having a negative effect on transactivation function in A and c-Myb and positive effect in B-Myb(Oh & Reddy, 1999).

The viral Myb proteins, v-Myb^{AMV} and v-Myb^{E26} are truncated versions of c-Myb. v-Myb^{AMV} lacks 71aa from its N-terminus, 199 aa from its C-terminus (Gerondakis & Bishop, 1986) and is additionally supplemented with 6 and 11 aa from viral *gag* and *env* genes (Lipsick & Wang, 1999; Oh & Reddy, 1999), thus generating a product of 45 kDa. The v-Myb^{E26} protein has a size of 135kDa, and is

a gag-Myb-Ets-1 fusion protein (Lipsick & Wang, 1999). Both variants have increased transcriptional activation and transformation potential (Zhou & Ness, 2011).

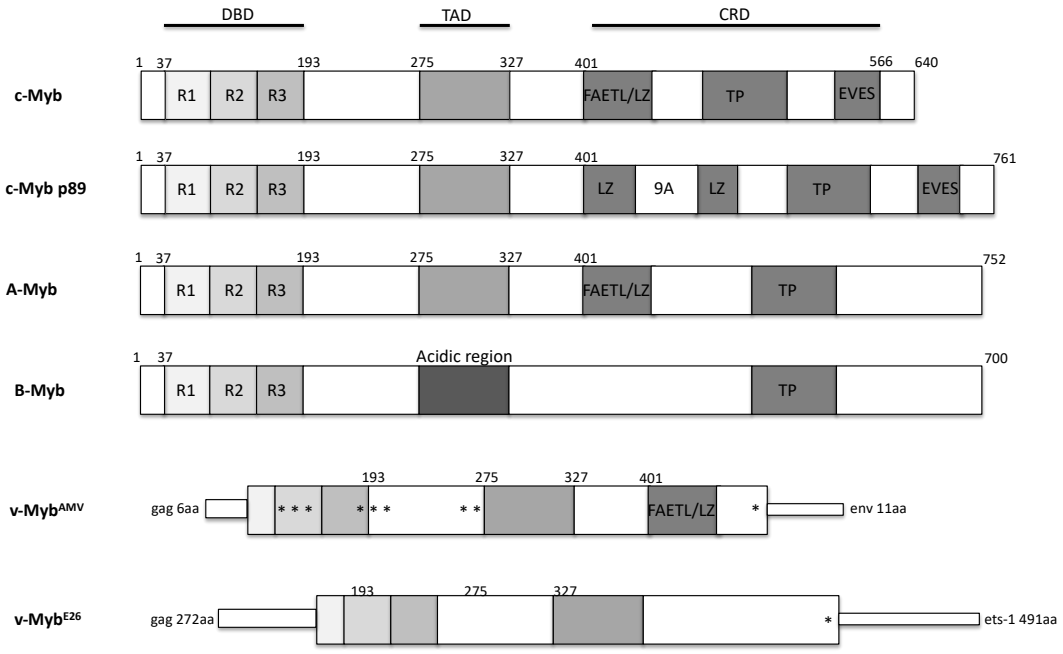


Figure 1.1: **Overview of the vertebrate and viral Myb proteins.** The N-terminal part of c-Myb contains the DNA binding domain (DBD), which is highly conserved between A-, B- and c-Myb. A- and c-Myb DBD is followed by a trans-activating domain (TAD), while in B-Myb DBD is followed by an acidic domain. The C-terminal regulatory domain (CRD) is less conserved between the proteins, with exception of Threonine-Proline rich region (TP). c-Myb and A-Myb additionally contains the regulatory region, FAETL/Leu zipper (LZ), followed by EVES region in c-Myb. The viral Myb proteins v-Myb^{AMV} and v-Myb^{E26} encodes C- and N-terminal truncated version of the protein. In v-Myb^{AMV}, 71 N-terminal amino acids (aa) are replaced by 6 viral gag aa, and 198 C-terminal aa are replaced by 11 env (envelope) aa. v-Myb^{E26} lacks 80 N-terminal aa, replaced by 272 gag aa, as well as 277 C-terminal aa are replaced by 491 Ets-1 aa. Numbers above the indicate the length and position of the domains/regions. Obtained from Ramsay et.al 2008 and modified.

1.2.1 The structural and functional domains of c-Myb

The DNA binding domain of c-Myb

The gene encoding the transcription factor c-Myb is composed of 15 exons, with exons 4-6 encoding the DNA binding domain (O'Rourke & Ness, 2008). Furthermore, the DBD consists of three tandem 52 amino acid pseudo-repeats, designated R1, R2 and R3 (Tanikawa, Yasukawa et al., 1993). Each repeat has three tryptophans, spaced out by 18 or 19 aa, representing a characteristic property of the DBD by forming a hydrophobic core that is essential for DNA binding (Kanei-Ishii, Sarai et al., 1990; Saikumar, Murali, & Reddy, 1990). In the

events where mutagenesis of tryptophan residues to glycine or proline have been implemented, the DNA binding activity of c-Myb has been abolished, whereas replacement with an aromatic residue has retained some activity function (Saikumar, Murali et al., 1990). Further work by NMR (nuclear magnetic resonance) spectroscopy has revealed that each repeat consists of three α-helices, where the second and third helices display a helix-turn helix related structure (HTH) , linking the three repeats by flexible linker regions (Gabrielsen, Sentenac, & Fromageot, 1991; Ogata, Hojo et al., 1992). R1, R2 and R3 binds to DNA in an orientation where each repeat has one helix into the major groove of the DNA. Together, the repeats cover the DNA only on one face, leaving it accessible to protein interactions from the other side (Tanikawa, Yasukawa et al., 1993).

Binding of c-Myb to DNA relies on R2 and R3, while R1 aids in stabilizing the interaction between the protein and the recognition sequence through contact with the phosphate backbone (Tanikawa, Yasukawa et al., 1993). The recognition sequence is presented as YAACNGNN (Y is C/T, and N is any nucleotide) where the first half-site YAAC is recognized by a recognition helix in R3. (Ording, Bergholtz, Brendeford, Jamin, & Gabrielsen, 1996; Prouse & Campbell, 2012). Upon DNA binding of R3, a disordered structure in R2 undergoes a conformational change and folds into a recognition helix, generating a full helix-turn-helix (HTH) motif, recognizing and binding to the second more loosely defined half-site (Jamin, Gabrielsen, Gilles, Lirsac, & Toma, 1993; Myrset, Bostad et al., 1993). It is assumed that the pre-folded R3 domain requires a unique sequence for binding, while the more flexible nature of R2 allows it to change and adapt a broader spectrum of sequences (Ording, Kvavik, Bostad, & Gabrielsen, 1994). The R1 repeat stabilizes the DNA-protein complex trough electrostatic interactions, and deletions of R1 lowers the stability of the complex fix-six folds (Tahirov, Sato et al., 2002). Additionally, linker regions between R2 and R3 further stabilize the complex through appropriate positioning of the repeats in orientation to DNA (Hegvold & Gabrielsen, 1996).

The amino terminal prior of R1, R2 and R3 holds an casein kinase II (CKII) phosphorylation site. All N-terminal deletions, removing the phosphorylation site, influences DNA binding, increasing the transcriptional activation(Dini & Lipsick, 1993; Luscher, Christenson, Litchfield, Krebs, & Eisenman, 1990).

The DBD of c-Myb shares sequence similarity to the SANT domain, frequently found in several chromatin modifying enzymes. SANT domains are known to interact with histone tails and drive processes such as histone acetylation, deacetylation and ATP-dependent chromatin remodeling. Interestingly, c-Myb DBD has been shown to interact with histone tails of H3 and H4 in addition to DNA binding (Aasland, Stewart, & Gibson, 1996; Boyer, Langer et al., 2002; Ko, Ko, Chen, & Lipsick, 2008; Mo, Kowenz-Leutz, Laumonnier, Xu, & Leutz, 2005).

The transactivation domain of c-Myb

The central transactivation domain (TAD) is found downstream of the DBD, consisting of 52 aa (in human c-Myb), ranging from 275 to 327 aa. This region is defined as the minimal fragment of c-Myb necessary for transactivation of gene

expression (Kalkbrenner, Guehmann, & Moelling, 1990; Sakura, Kanei-Ishii et al., 1989; Weston & Bishop, 1989). Similarly to transactivation domains of other transcriptional factors (TF), TAD contains clusters of acidic and hydrophobic patches (Sakura, Kanei-Ishii et al., 1989). This acidic patch is highly conserved among c-Myb and A-Myb proteins, but to a lesser degree in B-Myb(Ganter & Lipsick, 1999). Additionally, TAD encompasses a LXXLL motif (L is leucine and X is any other aa), which interacts with kinase inducible interacting domain (KIX) of transcriptional co-activators p300 and CBP (CREB binding protein), increasing the activity of c-Myb (Chakravarti, LaMorte et al., 1996; Hanstein, Eckner et al., 1996). CPB and p300 are vital for the transforming capacity of c-Myb. Mutants carrying mutations in c-Myb interfering with this interaction blocks transformation and myeloid differentiation (Pattabiraman, McGirr et al., 2014; Pattabiraman, Sun, Dowhan, Ishii, & Gonda, 2009).

Two SIMs (SUMO interacting motifs) have been identified in c-Myb. SIM1 in the R2 repeat of DBD and SIM2 in the N-terminal end of TAD. Interestingly, both SIMs are mutated in AMV v-Myb. SIM1 binds preferentially to SUMO1, while SIM2 in TAD binds preferentially to SUMO2/3 (Saether, Pattabiraman et al., 2011). Binding of SUMO (small ubiquitin like modifiers) trough SIMs lowers the transactivation activity of c-Myb as SUMO has a repressive function. The SIM motif, its function and SUMO protein will be addressed in section 1.3.3.

The C-terminal regulatory domain of c-Myb

The C-terminal regulatory domain (CRD) extends from residue 401 to 566 aa and contains three sub-domains: FAETL/LZ, TP and EVES. Initially, the CRD was thought to be a negative regulator of transcriptional activation, reasoned by changes in transformation and increased transcriptional activity in events of truncation (Hu, Ramsay, Kanei-Ishii, Ishii, & Gonda, 1991; Sakura, Kanei-Ishii et al., 1989). However, it has also been reported that the domain additionally harbors an SUMO-regulated activation function (SRAF), in absence of SUMO conjugation, that acts as an internal repressor in the wild-type protein (Molvaersmyr, Saether et al., 2010).

The three subdomains of CRD function independently of each other. The FAETL sequence consists of a 10 aa stretch EFAETLQLID and is part of a proposed leucine zipper (LZ) that lies close to a cluster of phosphorylation sites. FAETL is required for transcriptional activity of c-Myb and oncogenic transformation in v-Myb, functioning independently of the nearby phosphorylation sites as well as structure of LZ (Fu & Lipsick, 1996). The LZ component is a short heptad that may form an amphipathic alfa-helix with hydrophobic residues at every seventh position (leucine, isoleucine or methionine). Mutation leading to distortion of the alfa-helix, decreases is hydrophobicity and increases transcriptional activity of c-Myb by blocking association of inhibitory proteins with LZ (Kanei-Ishii, MacMillan et al., 1992; Weston, 1998)Favier & Gonda, 1994).

The second sub-domain of CRD is the TP domain, a proline and threonine rich region, containing several acetylation sites at $K^{471/480/485}$ (Tomita, Towatari et al., 2000) as well as phosphorylation sites such as T^{448} and S^{458} (Aziz, Miglarese et al., 1995) regulating the activity of c-Myb.

The last subdomain of CRD consists of the EVES domain. Similarly to the TP domain, EVES domain includes several sites for post-translatorial modifications, such as phosphorylation at $EVES^{532}$ (Aziz, Miglarese et al., 1995) and SUMOylation at $K^{503/527}$ (Bies, Markus, & Wolff, 2002; Dahle, Andersen et al., 2003). Additionally, an intramolecular binding of EVES motif to the DNA binding domain has been proposed based on limited evidence (Dash, Orrico, & Ness, 1996). However, our lab has not been able to reproduce evidence for this model. The EVES domain is highly conserved in all vertebrae c-Myb proteins, but lacks and A- and B-Myb proteins (Dash, Orrico et al., 1996).

1.2.2 The biological functions of c-Myb

The transcription factor c-Myb is a key regulator of stem and progenitor cell populations during hematopoiesis. Furthermore derived lineages are either selectively reduced or enhanced, provoking severe consequences for the organism (Ramsay, 2005; Ramsay & Gonda, 2008). c-Myb is predominantly expressed in hematopoietic cells and vital for normal hematopoietic development (Sullivan, Feeley, Guerra, & Boxer, 1997), playing a direct role in lineage fate selection, cell cycle progression and differentiation of myeloid, B- and T- progenitor cells (Lorenzo, Brendeford et al., 2011).

Hematopoiesis is initiated within the yolk sac in mammalian embryos and occurs in several waves. The first wave, referred to as the primitive wave, generates primitive erythrocytes, which then develop to red blood cells, in order to oxygenate the tissue and stimulate rapid growth of the embryo. This process is c-Myb independent (Fernandez & de Alarcon, 2013; Tober, McGrath, & Palis, 2008). The second wave, termed definitive hematopoiesis, requires c-Myb in order to normally generate myeloid and erythroid progenitor cells, previously migrated from the yolk sac into the fetal liver. Evidently, knockout of the gene in mice is lethal at E15, due to failure of definitive hematopoiesis in liver (Mucenski, McLain et al., 1991).

Generally higher levels of c-Myb are required for self-renewal and proliferation of progenitor cells, whereas lower levels of c-Myb are detected in differentiating and maturing cells. In cases of c-Myb overexpression, normal differentiation is suppressed (Greig, Carotta, & Nutt, 2008; Ramsay & Gonda, 2008). Although c-Myb is heavily present in hematopoietic cells in the bone marrow, the transcription factor also resides in progenitor cells of the colonic crypt as well as in neural progenitor cells in adult neurogenic zones (Thompson, Rosenthal et al., 1998) (Malaterre, Mantamadiotis et al., 2008). This suggests that the transcription factor is able to regulate different genes in different situations as required.

The onco-protein c-Myb has a wide variety of target genes, that include those linked lineage specific functions as well as cell cycle progression and survival (Ramsay & Gonda, 2008). Hence it is not surprising that it is also linked to a wide variety of cancers upon mutation, truncation or overexpression (Introna, Golay et al., 1990; Ness, Marknell, & Graf, 1989). c-Myb has been reported to play a role

in a series of leukemic cancers (Pelicci, Lanfrancone, Brathwaite, Wolman, & Dalla-Favera, 1984) (Clappier, Cuccuini et al., 2007; Lahortiga, De Keersmaecker et al., 2007; O'Neil, Tchinda et al., 2007; Pelicci, Lanfrancone et al., 1984), breast cancers (Drabsch, Hugo et al., 2007; Kauraniemi, Hedenfalk et al., 2000; Persson, Andren et al., 2009), colon tumors (Hugo, Cures et al., 2006; Thompson, Flegg, Westin, & Ramsay, 1997; Torelli, Venturelli et al., 1987), pancreatic tumors (Wallrapp, Muller-Pillasch et al., 1997), glioblastomas (Thompson, Flegg et al., 1997), melanomas (Dasgupta, Linnenbach et al., 1989; Walker, Silliman, Dayton, & Lang, 1998), head and neck tumors (Persson, Andren et al., 2009).

1.2.3 Regulation of c-Myb

MYB gene expression as well as c-Myb protein activity is tightly controlled and regulated. Gene expression levels are subject to control mechanisms on DNA and RNA level, while the translated protein is exposed to several modifications such as phosphorylation, acetylation, ubiquitination and SUMOylation.

Regulation of *MYB* gene expression

The expression of *MYB* is regulated by a range of transcription factors as well as c-Myb itself. Several regulatory sites in the 5´ flanking sequence of the gene have been identified, causing both positive and negative regulation in different cell lineages. Two positive regulatory regions with potential Ets binding sites and a core sequence GGA are known to be selective to Molt4 T-cells, but not DHL-9 B cells and K562 myeloid cells (Sullivan, Feeley et al., 1997). Additionally, *MYB* promoter region includes two c-Myb binding sites that negatively effects its own expression in T-cells but not K562 (Guerra, Withers, & Boxer, 1995). Furthermore, transcription factors WT1 and EGR-1 negatively regulate expression of c-Myb in T-cells and B-cells, by binding to 5´ and 3´of the promoter region, respectively (McCann, Sullivan, Guerra, Arcinas, & Boxer, 1995). Some members of Jun-family of transcription regulators are also known to regulate and control *MYB* expression in presence of an Ap-1 like element (Nicolaides, Correa et al., 1992). c-Myb is also suggested to autoregulate its own expression in order to maintain a steady level in critical phases of the cell cycle, by contributing to amplification of mitogenic signaling (Nicolaides, Gualdi, Casadevall, Manzella, & Calabretta, 1991).

Another mechanism of *MYB* regulation is by transcriptional elongation arrest, that takes effect within intron 1, at approx. 1,7kb downstream of the transcription initiation site (Hugo, Cures et al., 2006). This arrest halts the RNAPII and leads to generation of a RNA stem loop structure, that serves as a docking site for elongation control proteins. It is proposed that this elongation block is alleviated by recruitment of elongation factor P-TEFb via binding of NFkB p50, effectively allowing transcription of the gene to resume (Bender, Thompson, & Kuehl, 1987; Pereira, Hugo et al., 2015). This model of control mechanism was challenged by another study, suggesting that several enhancers within close range of the TSS of *MYB* form an active chromatin hub containing *MYB* promoter and the first intron, providing a high concentration of PolII,

transcription and elongation factors around the gene. Upon differentiation, *MYB* is downregulated when the active chromatin hub is destabilized, due loss of intergenic transcription factors at the region(Stadhouders, Thongjuea et al., 2012).

Apart from autoregulation and other transcription factors, the *MYB*-gene is subject to regulation by microRNA (miRNAs). miRNA binds to the 3´untranslated region (UTR) of *MYB* gene, thus targeting the mRNA for degradation, transcriptional repression or decay (Zhang, Wang, & Pan, 2007). MiR-150 is an established regulator of c-Myb, having two conserved binding sites at the 3´UTR of c-Myb RNA (Xiao, Calado et al., 2007). Other miRNAs shown to have binding at 3´UTR of c-Myb are miR-107 and miR-15a. Interestingly, c-Myb directly regulates miR-15a expression, by having several binding sites in the miR-15a promoter, thus establishing an autoregulatory feedback loop between the two (H. Zhao, Kalota, Jin, & Gewirtz, 2009).

Post-translatorial modifications of c-Myb

Along with being regulated at transcriptional level, c-Myb is regulated by post-translatorial modifications (PTM), that affect different aspects of the protein, in terms of activity, function, DNA binding, protein stability, and transcriptional activation. The majority of these modifications are present in the CRD of the c-Myb and are therefore lost in the viral counterparts of the protein.

Phosphorylation

c-Myb is frequently phosphorylated at Ser^{11} and Ser^{12} by casein kinase II (CKII), abolishing its ability to bind to its target sequence, MRE, both *in vitro* and *in vivo*. It is proposed that this phosphorylation promotes structural changes, by folding the N-terminal region over so the DNA binding domain is masked (Luscher, Christenson et al., 1990). Similar outcome is obtained by protein kinase A phosphorylation of Ser^{116} (Andersson, Kowenz-Leutz et al., 2003). Another phosphorylation target is Ser^{532} (Miglarese, Richardson, Aziz, & Bender, 1996), regulating both activity and stability of c-Myb by generating a form that is more susceptible for degradation (Weston, 1998). Interestingly, the exact phosphorylation can promote binding of the Pin1 isomerase, leading to a net increase of c-Myb transactivation activity (Pani, Menigatti et al., 2008). Additionally, p38MAPKδ seems to have the capacity to control c-Myb degradation by phosphorylation of Thr^{354}, Thr^{486}, Ser^{556} and Thr^{572} (Pani & Ferrari, 2008). This suggests that phosphorylation of c-Myb is cellular or genomic context-dependent.

Acetylation

c-Myb is acetylated at the CRD by the histone acetyltransferase p300 both *in vitro* and *in vivo* , resulting in enhanced c-Myb transcriptional as well as DNA binding activity. The identified acetylation sites are Lys^{471}, Lys^{480} and Lys^{485}, suggested to induce a conformational change in the protein due to neutralization of the positive charge on Lys. It is noteworthy to mention that both the

bromodomain and the HAT domain are required for successful acetylation of c-Myb (Tomita, Towatari et al., 2000). Additional acetylation of Lys^{438} and Lys^{441} occurs, mediated by the C/H2 domain of the CREB-binding protein (CBP), enhancing c-Myb binding affinity for the KIX domain of CBP. In similar fashion to p300 acetylation, CBP acetylation leads to higher DNA binding capacity and enhanced transcriptional activity of c-Myb (Sano & Ishii, 2001).

Ubiquitination

c-Myb has a relatively short half-life of 50 minutes, providing a fast off switch for when the transcription factor no longer is needed. Degradation of c-Myb is facilitated by ubiquitination and phosphorylation at several sites, followed by degradation in a ATP-dependent manner by the S26 proteasome complex (Bies & Wolff, 1997). In a Wnt-1 signaling pathway, the protein kinases NLK (nemo-like kinase) and HIPK2 (homeodomain interacting protein kinase 2) binds to and phosphorylates c-Myb at multiple sites. The phosphorylated protein is likely recognized by a E3 ubiquitin ligase, promoting covalent attachment of ubiquitin, which is then degraded in a proteasome pathway (Kanei-Ishii, Ninomiya-Tsuji et al., 2004). Interestingly, mutations of Ser, Thr and Pro to Ala as well as CRD truncated version of c-Myb, partly impairs its phosphorylation, ubiquitination and degradation (Bies & Wolff, 1997; Kanei-Ishii, Ninomiya-Tsuji et al., 2004; Kitagawa, Hiramatsu et al., 2009).

SUMOylation

c-Myb can be SUMOylated by both SUMO1 and SUMO2/3 proteins. Covalent attachment of the SUMO proteins takes place at Lys^{503} and Lys^{527}, located in the EVES subdomain of the CRD region, which is frequently truncated in oncogenic activation of c-Myb (Lipsick & Wang, 1999). Interestingly, SUMOylation of c-Myb is initiated at Lys^{527} which is required for SUMOylation of Lys^{503} (Bies, Markus et al., 2002). SUMOylation of the transcription factor represses its transcriptional activity and mutation of these sites causes a large enhancement of Myb-dependent transactivation (Dahle, Andersen et al., 2003). Furthermore, the level of SUMOylation is enhanced by the E2 conjugation enzyme Ubc9 (ubiquitin conjugation enzyme 9) and the E3 ligase PIASy (protein inhibitor of activated STAT) (Dahle, Andersen et al., 2003). SUMOylation of c-Myb by SUMO1 is predominant, but conjugation of SUMO2/3 is greatly enhanced in stress induced conditions such as heat, osmotic and metabolic stress (Sramko, Markus, Kabat, Wolff, & Bies, 2006). Furthermore, our lab has reported that SUMOylation of c-Myb leads to a strongly reduced synergy between factors bound at multiple sites in a promoter. This is linked to a SUMO-dependent switch in the function of CRD. When sumoylation is abolished, CRD switches into being activating, providing the factor with a second activation function (AF). Thus, c-Myb harbors two AFs, one that is constitutively active and one in the CRD being SUMO-regulated (SRAF) (Molværsmyr et al. 2010).

Two SUMO interacting motifs (SIM) are present in c-Myb. SIM1 in the R2 repeat of DBD binding SUMO 1 and SIM2 in the N-terminal end of TAD binding

SUMO2/3, promoting protein-protein interactions between c-Myb and SUMO conjugated substrates.

1.2.4 Interaction partners and targets of c-Myb

Transcription factors seldom function by themselves, but in combination with partners, co-factors, co-repressors and other TFs in order to exert proper regulation and expression of genes. This section will present a selection of interaction partners of c-Myb as well as briefly mention targets of c-Myb.

Targets of c-Myb

As already mentioned, the transcription factor c-Myb is highly expressed in stem and progenitor cells and acts as an important regulator of hematopoietic cell development. Identification of target genes of the transcription factor can shed light on the wide function and influence it has during development (Lorenzo, Brendeford et al., 2011). c-Myb target genes are generally categorized by function and are sorted to into "housekeeping" genes, cell type specific genes for differentiated cells and genes linked to oncogenicity related either to proliferation or differentiation (Ramsay & Gonda, 2008).

Several methods such as knockdown and null mutant cell lines, induced expression techniques, DamID, ChiP and ChIP sequencing have been used to identify potential targets of c-Myb. Several studies have shown that c-Myb controls expression of transcription factors important for proper hematopoiesis by binding to their promoters: IZF1 (IKAROS family zinc finger 1), STAT5A (signal transducer and activator of transcription 5A), GATA2 (GATA binding protein 2), LMO2 (LIM domain only protein 2), MYADM (Myeloid associated differentiation marker) and KLF1 (Kruppel lie factor 1) (Bianchi, Zini et al., 2010; Brandt & Koury, 2009; Lorenzo, Brendeford et al., 2011; McCormack & Rabbitts, 2004).

Digital genomic foot printing (DGF) has also been used to gauge c-Myb occupancy in the genome, using six different hematopoietic cell lines (Bengtsen, Klepper et al., 2015). c-Myb footprints were detected +/- 10kb from the TSS for 39% of the top 100 c-Myb targets, and expansion of analysis range to +/-100 kb within TSS, increased the detection to 72% for the human erythroid K562 cell line. Additionally, c-Myb footprints overlap with ChiP-seq peaks for active methylation marks such as $H3K4me^1$, $H3K4me^3$, H3K9ac and the repressive mark $H3K27me^3$. Other co-regulatory proteins such as ETS1, SIN3A, p300, CHD3 are found to interact directly or indirectly with c-Myb and enhance its activity (Bengtsen, Klepper et al., 2015; L. Zhao, Glazov et al., 2011).

Interaction partners of c-Myb

CPB and p300

The histone acetyltransferases CPB (CREB binding protein) and p300 are members of the CBP gene family and share high sequence homology (Arany,

Sellers, Livingston, & Eckner, 1994). The proteins interact with c-Myb and enhance its transcriptional activity by recruiting the transcription factor to Myb binding sites of target genes (Pattabiraman, McGirr et al., 2014). Both proteins have a KIX domain that binds to the TAD-domain of the c-Myb and function as co-activators by increasing its activation potential (Dai, Akimaru et al., 1996; Sano & Ishii, 2001). Additionally, acetylation of Lys of c-Myb by C/H2 domain of CBP and p300 increases c-Myb binding affinity to the HATs as well as increasing DNA binding activity (Sano & Ishii, 2001; Tomita, Towatari et al., 2000). It is suggested that interaction between c-Myb and CBP/p300 is important for normal hematopoiesis, as mutations in the KIX domain of p300 disrupts its binding to c-Myb and leads to defects such as B-cell deficiency, anemia, thrombocytosis and megakaryocytosis (Kasper, Boussouar et al., 2002). Mutations in c-Myb disrupting interaction with CBP/p300 have showed to suppresses myeloid transforming ability (Pattabiraman, Sun et al., 2009). It appears that c-Myb also has the ability to recruit CBP/p300 to closed chromatin, which in turn acetylates histones, thus loosening the structure and allowing c-Myb to bind to its recognition sequences at nucleosome-free regions (Fuglerud, Ledsaak, Rogne, Eskeland, & Gabrielsen, 2018).

UBC9 and PIAS1

SUMOylation of c-Myb involves interaction with E2 SUMO conjugation enzyme Ubc9 (ubiquitin carrier protein 9) and E3 SUMO ligase PIAS1 (protein inhibitor of activated STAT). The Ubc9 protein is vital for the SUMOylation cycle but is also capable of binding to the EVES-region in the CRD of c-Myb (Bies, Markus et al., 2002). The PIAS family consist of PIAS1, PIAS2 (PIASx), PIAS3 and PIAS4 (PIASy) and exert multiple functions. The protein family regulates cytokine signaling and inhibit activity of STAT transcription factors (Schmidt & Muller, 2003). They also function as SUMO E3 ligases, enhancing Ubc9-mediated SUMOylation of substrate proteins (Kotaja, Karvonen, Janne, & Palvimo, 2002). Additionally, PIAS proteins can operate as both negative and positive transcriptional coregulators. One of the mechanisms PIAS proteins regulate transcription is through delocalization of transcriptional regulators to subnuclear compartments (Sharrocks, 2006). PIAS1 proteins are shown to interact with FLASH proteins as well as c-Myb in a complex, driving transcriptional activation (Alm-Kristiansen, Lorenzo et al., 2011). Interestingly, PIAS has the ability to switch from activator to repressor functions, depending of SUMO-status of c-Myb, acting as an inhibitor of activated c-Myb and activator of repressed c-Myb (Ledsaak, Bengtsen et al., 2016).

CHD3

CHD3 (Chromo helicase DNA binding protein 3) is a component of the ATP-dependent chromatin remodeling complex NuRD (Nucleosome Remodeling and Deacetylase complex) (Basta & Rauchman, 2015). CHD3 acts as a co-transcriptional activator of c-Myb, binding at several sites in the transcription factor; N-terminally to the DBD and C-terminally to the leucine rich FAETL region in CRD of c-Myb. It is suggested that the chromatin remodeling activity of CHD3 is used by c-Myb to open promoters, thus leading to a gene activation

(Saether, Berge et al., 2007). CHD3 has also been shown to interact with SUMO protease SENP1, linking SUMOylation and deSUMOylation to the chromatin remodeling process (Rodriguez-Castaneda, Lemma et al., 2018).

FLASH

FLASH (FLICE associated huge protein) is a nuclear protein involved in a myriad functions, such as S-phase regulation, apoptosis signaling and co-regulation of transcription (Alm-Kristiansen, Lorenzo et al., 2011). The protein acts as a co-activator of c-Myb by binding to its DBD and co-localizing with active RNA polymerase II in foci, thus enhancing expression of c-Myb target genes (Alm-Kristiansen, Saether et al., 2008). FLASH itself can be SUMOylated at Lys^{1813} and interacts with the SUMO conjugating enzyme E2 Ubc9 as well as SUMO ligase E3 PIASy, regulating the transcriptional activity of the protein (Alm-Kristiansen, Norman, Matre, & Gabrielsen, 2009). Interestingly, the SUMO ligase E3 PIAS1, interacts both with FLASH and c-Myb, forming a complex that provides a strong enhancing effect of c-Myb mediated gene activation (Alm-Kristiansen, Lorenzo et al., 2011).

HIPK1 (homeodomain interacting protein kinase 1), Pim1 (serine/threonine kinase 1), the histone acetyltransferase TIP60 and histone methyltransferase MLL are some amongst the many interaction partners of c-Myb (Jin, Zhao et al., 2010; Matre, Nordgard, Alm-Kristiansen, Ledsaak, & Gabrielsen, 2009; Winn, Lei, & Ness, 2003; H. Zhao, Jin, & Gewirtz, 2012).

1.3 SUMO

SUMO (Small ubiquitin like modifiers) proteins are small proteins initially identified as ubiquitin like interaction partners of RanGAP1, allowing the modified form of RanGAP1 to localize to nuclear pores, versus the unmodified version that strictly resided in the cytoplasm (Matunis, Coutavas, & Blobel, 1996). The proteins are involved in reversible post-translatorial modifications of substrates, regulating their activity, stability, localization as well as modulating protein-protein interactions. The dynamic process of SUMOylation is important in many nuclear structures and functions in the cell, affecting a wide variety of mechanisms and processes and interacting with intrinsic signaling pathways (X. Zhao, 2018). Formation of PML nuclear bodies, DNA replication, cell division, chromosome segregation, DNA damage response, nuclear trafficking and heterochromatin formation are some of the many cellular processes SUMO is involved in (Lallemand-Breitenbach & de The, 2010; Maison, Bailly et al., 2011; Seeber & Gasser, 2017; Wan, Subramonian, & Zhang, 2012). The protein has a similar 3D structure to ubiquitin, and attaches to the same binding site, thus competing for the modification of the substrates despite having different functional consequences (Gill, 2004).

1.3.1 SUMO isoforms and structure

The SUMO-family in humans consists of four members, SUMO1, SUMO2, SUMO3 and SUMO4, each encoded by their respective *SUMO*-genes, residing on different chromosomes. SUMO1 shares 45% sequence homology to SUMO 2 and SUMO3, while the latter two are 95% identical to each other and therefore often referred to as SUMO2/3 (Eifler & Vertegaal, 2015). Different sequence identity may suggest the proteins have distinct function, as well as unique expression pattern in cells and tissues. SUMO1 is predominantly expressed in all cells under normal circumstances, while SUMO2/3 are preferably expressed and conjugated to proteins under stress conditions (Eifler & Vertegaal, 2015; Hay, 2005; Hilgarth, Murphy et al., 2004). SUMO4 is found to have strong expression in human kidney cells, thus raising the question whether SUMO proteins can have tissue specific functions (Dohmen, 2004).

The secondary and tertiary structure of SUMO was solved by NMR spectroscopy in 1998. The secondary structure was determined to have five antiparallel β-sheets with the exception of β1 to β5 as well as two α-helices in the following three dimensional structure: ββαββαβ. The arrangement of the β-sheets and α-helices is akin to the three dimensional structure of ubiquitin, despite the relatively low sequence homology of 18% (Bayer, Arndt et al., 1998; Dohmen, 2004). Contrastingly to the ubiquitin fold, the SUMO proteins contain a 20 amino acids flexible N-terminal arm, containing many strongly charged residues as well as pointing away from the protein core, suggesting its involvement in facilitation of protein-protein interactions (Bayer, Arndt et al., 1998; H. L. Su & Li, 2002). C-terminally, SUMO has two conserved Gly residues that are suggested to play a vital role in conjugation of SUMO to substrates. Consequently, the C-terminal tail has to be cleaved off in order to expose the Gly residues, prior to SUMO conjugation (Kamitani, Nguyen, & Yeh, 1997).

The SUMO conjugation site has been identified to consist of ΨKxE, where Ψ is a hydrophobic amino acid, K is Lys to which SUMO is covalently attached to, X represents any amino acid and E is glutamic acid. (Rodriguez, Dargemont, & Hay, 2001). Mutations in the consensus sequence such as Ψ to Ala reduces SUMO conjugation while mutating Lys to Glu or Ala abolishes all SUMO conjugation to Ubc9 (Sampson, Wang, & Matunis, 2001). Interestingly, SUMO2/3 contains a SUMO consensus motif in the N-terminal tail, enabling them to generate polymeric chains both *in vivo* and *in vitro.* This self-conjugation is possible through Lys^{11} which resides in the ΨKxE motif and mutation of the said residue abrogates the polymeric chains assembly (Tatham, Jaffray et al., 2001). The polySUMO chains are recognized by STUbLs (SUMO targeted ubiquitin ligases) containing SIMO motifs and are commonly targeted for degradation (Bruderer, Tatham et al., 2011).

1.3.2 SUMO-conjugation and deconjugation

The mechanism of SUMO conjugation to the target substrate involves an enzymatic pathway, often referred to as the SUMO cycle. It involves several enzymes that are explicit for SUMO proteins, despite having similarities to the ubiquitin system (Dohmen, 2004). The process involves four enzymatic steps: maturation, activation, conjugation and ligation. In the first step, the pre-mature SUMO protein must be C-terminally cleaved off by specific SUMO proteases in order to expose the diglycine motif, forming mature SUMO protein. This step is identical for SUMO 1,2 and 3 but not for SUMO4, that harbors a proline residue (Pro^{90}), preventing its C-terminal cleavage and maturation (Eifler & Vertegaal, 2015; Hilgarth, Murphy et al., 2004; Song, Durrin, Wilkinson, Krontiris, & Chen, 2004). After maturation, the mature SUMO protein has to be activated by the SUMO-activating enzyme E1, a heterodimeric protein composed of Aos1 (SAE1) and Uba2 (SAE2). The activation process is ATP dependent, generating a SUMO adenylate via ATP hydrolysis, that proceeds to form a thioester bond with the cysteine in the active site of Uba2. An adenoviral protein Gam1, though to promote cell survival, can obstruct with the E1 enzyme activity, thereby inhibiting SUMOylation of proteins *in vivo*, and leading to transcriptional activation of their related promoters (Boggio, Colombo, Hay, Draetta, & Chiocca, 2004). Activations are followed by conjugation, where SUMO is transferred to the SUMO conjugating enzyme E2 known as Ubc9, forming a thioester bond with a conserved cysteine of the enzyme (Hilgarth, Murphy et al., 2004). Furthermore, the E2 Ubc9 enzyme can form a noncovalent complex with SUMO 1, 2 and 3 at its N-terminal region, quite far from the active Cys-residue. Mutations and alterations to the surface of Ubc9, affecting the non-covalent interaction with SUMO, reduced both the thioester bond formation as well as conjugation activity, suggesting that the N-terminal region is of importance in the SUMO-pathway (Q. Liu, Jin et al., 1999; Tatham, Kim et al., 2003). Conjugation is followed by the final step, ligation. In this step, the carboxyl-group of glycine residue on the C-terminal of SUMO forms an isopeptide bond with the amino-group of a lysine residue in the target protein (Meulmeester & Melchior, 2008). *In vitro*, ligation requires only SUMO, ATP, E1 activating enzyme and E2 conjugating enzyme (Bernier-Villamor, Sampson, Matunis, & Lima, 2002), whereas *in vivo*, E3 SUMO ligases mediate this process. E3 SUMO ligases are defined by their ability to bind to the E2 conjugation enzyme as well as target protein, stimulating the transfer of SUMO from the former to the latter (Hilgarth, Murphy et al., 2004). Three types of SUMO ligases have been identified, all structurally different: PIAS (protein inhibitor of activated STAT) family, RanBP2 (Ran-binding protein 2) and Pc2 (Polycomb protein family). Apart from acting as a E3 SUMO ligases, PIAS proteins have other functions such as regulation of the JAK-STAT signaling pathway, co-regulators of transcription as well as being involved in DNA double stranded DNA break repair (Alm-Kristiansen, Lorenzo et al., 2011; S. Liu, Fan et al., 2013; Sharrocks, 2006). The protein family has RING like zinc finger domain, essential for the ligase activity as it directly binds to E2 enzymes and SUMO target proteins. The nucleoporin RanBP2 lacks the RING domain but is involved in SUMOylation of several target proteins, such as RanGAP1, SP100 and topoisomerase II (Dawlaty, Malureanu et al., 2008; Pichler, Gast, Seeler, Dejean, & Melchior, 2002). The region responsible for its E3 ligase activity is defined by

two 50-residue repeats, IR1 and IR2 as well as a spacer M region, where IR1 is the main interactor of Ubc9 while IR2 interacts with SUMO1 but not SUMO2. This suggests a distinct mechanism of SUMOylation, where RanBP2-Ubc9 binding via IR1 is associated with SUMO2/3 conjugation, while SUMO1 conjugation is associated with IR2 region (Tatham, Kim et al., 2005). The last type of E3 SUMO ligases is the Pc2, as component of the Polycomb chromatin-modifying complex that mediates transcriptional repression. Pc2 has a C-terminal region that is able to bind both Ubc9 and the target substrate CtBP (C-terminally binding protein). Contrastingly, a N-terminal region is found to exhibit E3 ligase activity *in vitro*, despite being only able to bind Ubc9. Presumably *in vivo*, the C-terminal domain recruits both the E2 enzyme and target protein to polycomb bodies, while the N-terminal SUMOylates CtPB (Hay, 2005; Kagey, Melhuish, Powers, & Wotton, 2005).

SUMOylation of proteins is reversible and the SUMO cycle can be reset by catalytically deconjugating SUMO from its target protein by SUMO specific proteases (SENP). All known SUMO proteases are classified as cysteine isopeptidases, and function by cleaving the isopeptide bond between the terminal glycine of SUMO and lysine of the target protein. A family of six SUMO specific proteases is identified: SENP 1, 2, 3, 5, 6, 7, all having different preference in processing and deconjugation as well as inclination for the different SUMO paralogs (Eifler & Vertegaal, 2015). Both SENP1 and SENP2

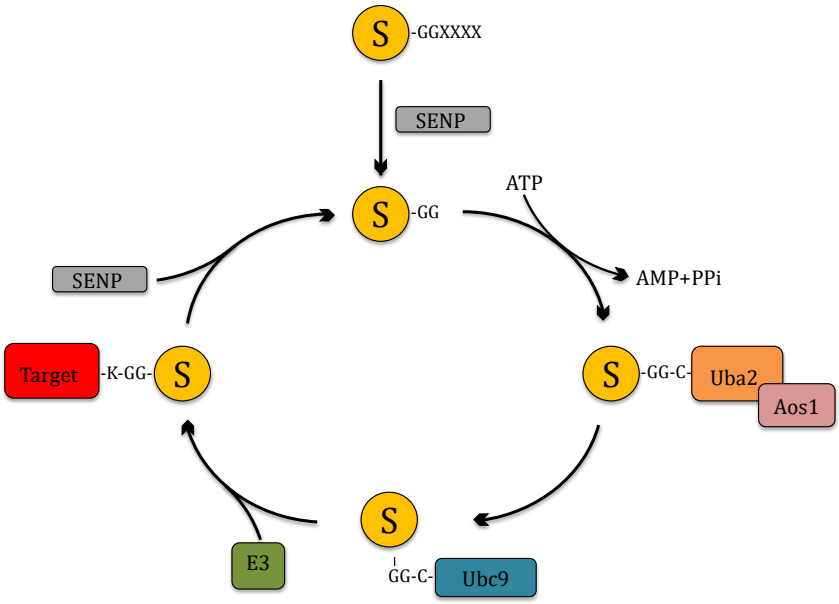


Figure 1.2: **The mechanism of reversible SUMOylation.** Before conjugation, immature SUMO in yellow must be C-terminally cleaved off by SENP isopeptidases, in order to reveal the GG-motif. Mature SUMO is activated by covalent attachment to E1 heterodimer Aos1–Uba2 in an ATP-dependent reaction, which results in a thioester bond between the C-terminal Gly residue and Cys in Uba2. SUMO is then transferred to the catalytic Cys residue of the E2 enzyme Ubc9 and together with E3 ligase, SUMO is covalently conjugated to Lys residue on its target protein. SUMO is deconjugated from the target by SENPs, cleaving of the modification and resetting the cycle. Obtained from Ronald, T. Hay 2005; Ruth Geiss-Friedlander, Frauke Melchior 2007, and modified.

deconjugate the SUMO 1, 2, 3 paralogs with equal efficiency, yet SENP1 favors SUMO1 processing over the other two paralogs (Sharma, Yamada, Lualdi, Dasso, & Kuehn, 2013; Shen, Dong, Liu, Naismith, & Hay, 2006). Interestingly, SENP2 is capable of deconjugating SUMO1 from substrate, but favors SUMO2/3 deconjugation. It also seems that the remaining members of the family have a strong preference for SUMO2/3 processing and deconjugation (Hickey, Wilson, & Hochstrasser, 2012). Furthermore, SENP6 and SENP7 are highly involved in SUMO 2/3 polychain deconjugation (Hickey, Wilson et al., 2012; Lima & Reverter, 2008). SENPs are commonly localized in different cellular compartments, implying having a role in balancing protein sumoylation (Hilgarth, Murphy et al., 2004).

1.3.3 Non covalent SUMO-interaction

Apart from being able to covalently bind to a substrate as described in previous section, SUMO proteins are capable of interacting non-covalently with proteins carrying a SUMO interacting motifs, commonly referred to as a SIMs. The SIM motif for SUMO1was first described to be Ser-X-Ser, where X could be any amino acid, flanked by hydrophobic amino acids on one side and acidic acids on the other (Minty, Dumont, Kaghad, & Caput, 2000). However, later on, it was established by NMR spectroscopy that the flanking hydrophobic core was essential for SUMO-SIM binding, and the new consensus was modified to Val/Ile-X-Val/Ile-Val/Ile (V/I-X-V/I-V/I), binding all SUMO paralogs (Song, Durrin et al., 2004). Interestingly, several proteins that bind to SUMO covalently such as PIAS, RanBP2 and Uba2 are found to contain SIM motifs as well (Gareau, Reverter, & Lima, 2012; Kaur, Park, Pandey, Azuma, & De Guzman, 2017).

The SIM motif forms a β-strand, interacting non-covalently with the β2-strand of SUMO, in either parallel or antiparallel fashion (Hecker, Rabiller, Haglund, Bayer, & Dikic, 2006). Acidic residues bordering the hydrophobic core in the SIM motif can affect and enhance the binding affinity by forming electrostatic interactions with the basic interface of SUMO. In SUMO1, these include Lys^{37}, Lys^{39} and Lys^{46} as well as the complementary Lys^{33}, Lys^{35} and Lys^{42} in SUMO2 (Ullmann, Chien et al., 2012). In similar manner, phosphorylation of serine and threonine nearby the SIM motif introduces negative charges, enabling the residues to interact with positive charged residues in SUMO proteins (Kerscher, 2007).

The functional consequence of SUMO-SIM non covalent interaction is the propagation of protein-protein interactions between SUMOylated proteins and SIM-carrying proteins. This can promote formation of protein-complexes as well as affect their spatial distribution. A prominent example is the SUMO-SIM dependent formation of PML nuclear bodies, where PML proteins are dually affected by SUMO, by being SUMOylated as well as carrying a SIM-motif that enables recruitment of SUMOylated partners, SIM-containing partners as well as partners containing both a SUMO and a SIM (Lallemand-Breitenbach & de The, 2010; Maroui, Kheddache-Atmane et al., 2012).

1.3.4 Biological functions of SUMO

The posttranslational modification of SUMOylation is involved in many functions and mechanisms, playing a vital role in cellular processes. Many SUMO targets are proteins involved in signaling transduction, transcriptional regulation, DNA damage repair, genome structure and integrity, chromosomal segregation and subcellular localization (Dohmen, 2004; Gill, 2004). Even though SUMO conjugation is a specific modification on target proteins, SUMOylation can be viewed as a global influencer in different cellular contexts with a broad spectrum of biological effects. This section will briefly touch upon some amongst many of the functions SUMO is involved in.

Nuclear structures

Droplet like and membraneless structures are common in the nucleus, separating its content from the surrounding solution due to a higher concentration of multivalent molecules- forming intra and inter- molecular interactions. (Banani, Lee, Hyman, & Rosen, 2017). Structures such as PML bodies, Cajal bodies , Polycomb bodies, nucleolus are found to have an abundance of SUMO proteins, suggesting that SUMO-SIM interactions may act as one of the driving forces that contribute to their formation (X. Zhao, 2018). In PML nuclear bodies, the PML proteins dimerization is followed by recruitment of SUMO E2 enzyme Ubc9 and PML SUMOylation, which in turn attracts interaction partners containing SIM motifs. Thus PML proteins are suggested to act as docking platforms for partner recruitment (Sahin, de The, & Lallemand-Breitenbach, 2014). Specific SUMOylation and deconjugation linked to nucleolus suggests that the SUMO targets specific proteins involved in ribosomal biogenesis and can thus influence both its formation and function (Westman, Verheggen et al., 2010).

Transcriptional activity

SUMO is associated with transcriptional activity via the ability to SUMOylate transcription factors (TF) and has been shown to have both negative and positive effects. A possible mechanism may be that SUMO conjugation to a transcription factor may affects its ability to bind to DNA, either by blocking or promoting its association with specific promoters (Lyst & Stancheva, 2007). Thus, SUMOylation of transcription factors regulates gene expression by controlling their association with chromatin. Additionally, SUMO has several mechanisms of TF control, by either recruiting co-repressors such as histone deacetylases or co-activators like p300 and CBP. SUMO generally competes with other modifications on the same Lys-residue, such as ubiquitination that leads to degradation or acetylation, promoting gene activation. By conjugation to Lys on a TF, generally SUMOylation has negative transcriptional effect. Additionally, SUMO conjugation can block or interfere with phosphorylation of nearby residues, necessary for complete activation of target genes (Rosonina, Akhter, Dou, Babu, & Sri Theivakadadcham, 2017). Even though SUMOylation is generally associated with repression of transcription, there are exceptions to this paradigm. Studies have shown SUMO1 to be present upstream of transcription start sites (TSS) on many

active housekeeping genes, including genes encoding translation factors and ribosomal proteins (H. W. Liu, Zhang et al., 2012). In *S. Cerevisiae*, SUMOylation of the transcription factor Rap1promotes interaction with TFIID and recruitment of RNA polymerase II, initiating transcription (P. Chymkowitch, A. P. Nguea et al., 2015; Chymkowitch, Nguea, & Enserink, 2015). Similarly, in HeLa cells, SUMO1 binds to the chromatin at promoters of ribosomal protein genes (RPG) via the scaffold associated factor B (SAFB), stimulating recruitment and binding of RNA polymerase II to promoters of PRG (H. W. Liu, Banerjee, Guan, Freitas, & Parvin, 2015).

DNA damage repair

Many proteins involved in double stranded break (DSB) and nucleotide excision repair (NER) are SUMO targets and are modified in response to DNA damage (Sriramachandran & Dohmen, 2014). In base excision repair (BER), the thymine DNA glycosylase (TDG) responsible for uracil and thymine removal in G:U and G:T base pairs, is a SUMO target. SUMO modification of TDG enhances its dissociation from the DNA after removal of the erroneous nucleotide by reducing its binding affinity with the abasic site, which is the rate limiting step in the repair mechanism (Hardeland, Steinacher, Jiricny, & Schar, 2002; Muller, Ledl, & Schmidt, 2004). Interestingly, in double stranded breaks, SUMO is involved in chromatin organization and relocates the DBS to the nuclear pore. Both mono and poly SUMOylation allows the DBS to shift out of heterochromatin and recruit repair factors (Seeber & Gasser, 2017).

Crosstalk with signaling pathways

Crosstalk between the SUMO pathway and other pathways enables the cell to fine-tune responses to external and internal changes and stimuli as well regulate signal-dependent protein activity. DNA damage checkpoints, protein kinase signaling pathways, Wnt signaling, cytokine signaling are amongst some of the many signaling pathways SUMO interacts with (Muller, Ledl et al., 2004). In DNA damage checkpoints, SUMOylation and phosphorylation occurs in parallel, although independent of each other. The crosstalk is both positive and negative, in addition to a substrate overlap. SUMOylation of the ATRIP, the regulatory partner of ATR (ATM and Rad3 related) kinase, enhances the checkpoint response by boosting its interaction with other checkpoint factors (Wu, Ouyang et al., 2014). In contrast, reduction in the ATR kinase function increases SUMOylation of target proteins, suggesting the crosstalk is context-dependent and bidirectional influential (X. Zhao, 2018).

SUMOylation as a guardian of cell identity

A recent study has shown that SUMO modification functions as a safeguard in cellular identify, by occupying highly distinctive chromatin types in somatic and pluripotent cells (Cossec, Theurillat et al., 2018). By using both *in vivo* and *in vitro* cell fate conversion systems, the role of SUMOylation in cell plasticity was investigated. Hyposumoylation trhough knockdown of Ubc9 significantly

increases the level of pluripotency factors, such as Nanog and facilitates reprogramming of mouse embryonic fibroblasts (MEFs) to induced pluripotent stem cells (iPSC). Additionally, suppression of sumoylation induces conversion of iPSC to 2-cell-embryo-like (2C-like) cells. Analysis of RNA-seq data for knockdown of several members of the sumoylation machinery revealed enrichment in genes specific to 2C-like embryos, indicating their ability to regulate the 2C-like reprogramming. ChiP-seq for SUMO revealed its presence at enhancer elements in MEFs, suggesting its contribution to gene activation in somatic cells. Contrastingly, in embryonic stem cells (ESCs), SUMO is largely present in heterochromatin regions, exerting gene suppression in pluripotent cells. These key findings reveal how SUMO acts as tether on chromatin to stabilize somatic and pluripotent barriers that define different cell states (Cossec, Theurillat et al., 2018; Di Stefano & Hochedlinger, 2018).

1.3.5 Regulation of SUMO

SUMOylation is a highly dynamic process with a high turnover rate. The substrates undergo rapid modification by SUMO that is followed by equally rapid deconjugation. Although only a small amount of the total substrate protein is SUMOylated at any given time, the modification is vital to ensure normal functions in the cell. Large scale changes in SUMOylation levels can lead to alteration of multiple processes. It is thus important to ensure proper and coordinated regulation of SUMOylation, enabling rapid responses to changes in external and internal stimuli (X. Zhao, 2018).

One of the most common ways to regulate SUMOylation levels is trough SUMO-targeted ubiquitin ligases E3 (STUbLs). STUbLs are able to recognize polySUMO chains generated by SUMO 2/3, thus linking the SUMO modification to the ubiquitination/proteasome system (Sriramachandran & Dohmen, 2014). One of the most extensively studied ubiquitin E3 ligases is RNF4. The small nuclear protein has a RING finger domain as well as three identified SIMs (Tatham, Geoffroy et al., 2008). The ubiquitin E3 ligase binds to the SUMO-chains and catalyzes ubiquitin conjugation to the SUMO, targeting the protein complex for degradation (Joazeiro & Weissman, 2000; Sun, Leverson, & Hunter, 2007). Additionally, STUbLs targets SUMO E1, E2 and E2 pathway enzymes as well. One of the most appropriate utilization of the STUbLs is the degradation of PML-RARA (retinoic acid receptor alpha) fusion proteins in acute promyelocytic leukemia (APL). Treatment with arsenic induces PML modification by SUMO, generating SUMO-chains on the PML-RARA proteins, which in turn are recognized by STUbLs and tagged with ubiquitin for degradation (Lallemand-Breitenbach, Jeanne et al., 2008; Tatham, Geoffroy et al., 2008).

Additional control of SUMOylation levels can be brought forth by altering the enzymatic localization of the SUMO pathway enzymes E1, E2, E3 and SENP. SUMOylation plays an important role in DNA double strand break repair by promoting relocation of the broken ends to the nuclear pore and recruiting repair factors. Thus strategic targeting of SUMO E2 and E3 enzymes to DNA

damage sites upregulates SUMOylation levels, favoring repair mechanisms (Galanty, Belotserkovskaya et al., 2009; Seeber & Gasser, 2017).

Post-translatorial modification of the SUMO pathway enzymes is yet another mechanism of regulating the SUMOylation levels. Phosphorylation, acetylation and sumoylation can influence the SUMOylation rate, thus changing the SUMOylation levels (X. Zhao, 2018). Phosphorylation of SUMO E2 Ubc9 at Ser^{71} by CDK1/cyclin B enhances SUMOylation activity by promoting thioester bond formation between the SUMO E2 enzyme and SUMO protein (Y. F. Su, Yang, Huang, Liu, & Hwang, 2012). Acetylation of the same E2 enzyme at Lys^{65} has the opposite effect, downregulating SUMOylation of substrates (Hsieh, Kuo et al., 2013). SUMOylation of Ubc9 at Lys^{14} causes a shift in target specificity, by either decreasing or increasing target modification; reduction in SUMOylation of RanGAP1 and in contrast enhancing SUMOylation of the transcription factor Sp100 (Knipscheer, Flotho et al., 2008). Interestingly, in budding yeast, sumoylation of the SUMO E2 enzyme promotes SUMOchain assembly (Klug, Xaver et al., 2013).

1.3.6 SUMO and c-Myb

As addressed in section 1.2, the transcription factor c-Myb is an important regulator of hematopoietic cell line development. The transcription factor and the SUMO protein interact together at several locations, both covalently and non-covalently.

The human c-Myb has two SUMOylation sites, both in the EVES domain of the CRD, at K^{503} and K^{527}. The attachment of SUMO protein to the lysine residue has been shown to have a negative effect on the transactivation activity of the factor (Bies, Markus et al., 2002; Dahle, Andersen et al., 2003). Despite having an identical consensus sequence, sumoylation of c-Myb takes place with unequal efficiency, where K^{527} is the principal sumoylation site, while K^{503} acts as the secondary site. An explanation for this phenomenon could be the presence of proline residues at the K^{527} site, creating a favorable environment for SUMO-attachment (Dahle, Andersen et al., 2003). Interestingly, in murine c-Myb, mutation of the complementary principal sumoylation site completely abolished SUMO conjugation, implying its importance for SUMO modification on other lysine residue (Bies, Markus et al., 2002). *In vitro*, K^{503} sumoylation is more efficient subsequently to K^{527}sumoylation. It is possible that a sumoylated K^{527} site enhances recruitment of the SUMO E3 enzymes, promoting sumoylation of the secondary site (Dahle, Andersen et al., 2003). Single mutants, at either K^{503}R or K^{527}R, and double mutants, 2KR, displayed increased transactivation activity in reporter assays, as well as having correlation to a decreasing degree of sumoylation (Dahle, Andersen et al., 2003). This SUMO-conjugation negative mutant behaves as an activator in absence of SUMO, suggesting that c-Myb has an repressor to activation switch that is SUMO-dependent; a SUMO-regulated activation function (SRAF), supporting evidence that the synergistic behavior of c-Myb is influenced by SUMO (Molvaersmyr, Saether et al., 2010).

In addition to two SUMO-conjugation sites, c-Myb harbors two SIMs, located in R2 of the DBD as well as at the N-terminal end of TAD. While the former is not active, the latter SIM is functionally active by having a negative effect on the transactivation potential of c-Myb. (Saether, Pattabiraman et al., 2011). The functional SIM in the TAD resides at the V267NIV location and preferentially binds SUMO2/3. Mutations of the SIM2 motif has been shown to derepress c-Myb activity, independently of the SUMO-conjugation site. Additionally, only the loss of SUMO binding through SIM influences the transforming ability of the oncoprotein by exerting a higher proliferation capacity (Saether, Pattabiraman et al., 2011).

1.3.7 Acetylation of SUMO

As earlier stated, SUMO conjugation to substrates enables them to be recognized by a SIM-containing binding partners, promoting protein-protein interaction that in some cases affects the spatial distribution of the proteins (Ullmann, Chien et al., 2012). This protein-protein binding solely relies on SUMO-SIM non covalent binding, which consists of the hydrophobic β-strand of SIM contacting the β strand of SUMO as well as being strengthened by the negatively charged residues adjacent to SIM motif that generate electrostatic interactions with the interaction points of the SUMO protein (Kerscher, 2007). Thus, any changes in the charges of the residues on either side of the interaction point may influence the binding affinity of the non-covalent attachment. Phosphorylation of serine and threonine adjacent to the SIM motif serves to enhance the electrostatic interactions as it introduces negative charges. The phosphorylated serine residues are shown to interact with K^{39} and K^{35} in SUMO1 and SUMO 2, binding to SUMO E3 ligase PIAS1, but affecting its transcriptional coregulatory function rather than its ligase activity (Stehmeier & Muller, 2009). Acetylation of lysine residues on SUMO may induce a reverse effect, as it neutralizes the positive charge of the amino acid, thus decreasing the electrostatic interaction between SIM and SUMO. Investigation of K^{37} and K^{33} acetylation on SUMO1 and SUMO2 respectively has been shown to affect the SUMO-SIM binding drastically, preventing SUMO binding to SIMs of PML, Daxx and PIAS family members. Interestingly, the acetylation event has not affected SUMO binding to RanBP2. This suggests that acetylation of SUMO not only affect the SUMO-SIM dependent protein-protein interactions, but does so selectively, expanding the dynamics of the SUMO-SIM interactions (Ullmann, Chien et al., 2012).

1.4 Aims of the study

Transcription factors (TF) are key players in gene regulation and central to cancer and epigenetics. As quite advanced proteins, they are able to receive signals, process information and control gene programs accordingly. Thus, flaws in signaling to or expression of TFs can lead to aberrant gene expression and development of cancer. The transcription factor and oncoprotein c-Myb is a

master regulator of proliferation and differentiation of hematopoietic cells. It is also a pioneer factor, possessing the ability to access and open chromatin, as well as to recruit co-factors to the DNA (Fuglerud, Lemma et al., 2017). Loss of c-Myb abolishes blood cell formation and its deregulation disturbs normal cell growth.

Posttranslational modifications such as acetylation, phosphorylation, ubiquitination are mechanisms known to regulate protein functions. Modification of proteins with small ubiquitin like modifiers (SUMO) has been shown to influence fundamental nuclear processes and structures, as well as having an impact on transcription and chromatin organization. Research in our lab has shown that covalent SUMO-conjugation as well as non-covalent SUMO-binding through SIM are important regulatory mechanisms for c-Myb function (Ledsaak, Bengtsen et al., 2016; Molvaersmyr, Saether et al., 2010; Saether, Pattabiraman et al., 2011). SUMOylation of the c-Myb transcription factor is associated with repression, implicating that relief of the SUMO-mediated repression may contribute to gene activation. This derepression and controlled relief of SUMO-mediated repression of transcription is a focus of study in our lab.

Until recently, removal of SUMO appeared to be the only molecular strategy to obtain relief from SUMO-mediated repression. However, a paper from Stefan Müllers lab established an acetylation switch, capable of regulating SUMO-dependent interaction networks (Ullmann, Chien et al., 2012). They identified specific acetylations of SUMO within the SUMO-SIM interface that disrupt and prevent SUMO-binding to partners such as PML, Daxx and PIAS. This acetyl-dependent switch may act as an alternative mechanism for derepression.

The main objective of this study is to address whether the acetylation switch defined by Müllers lab applies to c-Myb. A collection of SUMO acetylation mutants, disrupting and mimicking, has been obtained from Stefan Müllers lab. Whether these mutants affect c-Myb activity compared to normal SUMO will be investigated. If influenced, it is of interest to determine to which extent the acetylation mutants of SUMO bound to c-Myb affect its transactivation activity. Observations in our lab have shown that the acetyl transferase p300 (and HIPK1) partially derepresses SUMO-repressed c-Myb. Thus, we plan to investigate if p300 acetylates SUMO and is capable of influencing c-Myb activity by modifying SUMO directly.

Additionally, it is of interest to examine to which extent the acetylation mutants are able to influence the SUMO-SIM dependent binding between SUMO and partner proteins such as PIAS and CHD3.

In summary, we will in this thesis address the following questions:

1. Is the repressive effect of SUMO on the transactivation activity of c-Myb affected by acetylation mimicking and abolishing mutants of SUMO1?
    a. Will cotransfected p300 enhance the derepression effect?
    b. To which extent is the derepression of SUMOylated c-Myb, caused by acetylated SUMO, possible? Can the activity of c-Myb fused to SUMO be 100% restored?
2. Is the repressive effect of SUMO and its mutants on the transactivation activity of c-Myb affected by the type of transactivation assay used?

3. Will the acetylation mutants of SUMO affect their repressive effect in a non-c-Myb context?
4. Is SUMO acetylated directly by p300? If so, which residues are subject to this modification?
5. How does acetylation of SUMO affect binding of interaction partners? Does acetylation of SUMO regulate SUMO-SIM binding selectively?

These key questions will be answered using activation and repression assays, western blot analysis as well as coimmunoprecipitation assays.

2 Methods

The following chapter describes the various methods and techniques applied in the study. For each technique a short introduction and procedural overview is presented. A complete list of commercial kits, buffers, materials and computer software is listed in the appendix.

2.1 Bacterial methods

The bacterial work in the thesis was performed on *Escherichia coli (E.coli)* cells, specifically the DH5α strain. This strain is defined by 3 different mutations that allow for a maximalization of transformation efficiency. A point mutation is engineered in the recA1 gene in order to influence the activity of the recombinases and inactivate homologous recombination. Mutation in endA1 to inactivate intracellular endonuclease, thus preventing it from degrading plasmids that are to be introduces into the cell line. The last mutation is the introduction of the lacZM15 gene that enables the cell line to select for blue/white screening. For this study, DH5α cells have been transformed with plasmids containing ampicillin resistance gene as a selective mark. When applied to LB agar plates containing ampicillin, only cells with properly introduced the resistance gene will grow.

2.1.1 Preparation of competent DH5α cells

Engineering competent cells increases the probability of uptake and expression of exogenous DNA. This can be done with use of chemicals or electrical pulses.

Procedure

1. Plate 5 μl of frozen DH5α cells on a LB medium, antibiotic-less agar plate and incubate overnight at 37° C.
2. Transfer 10-12 colonies from the agar plate and inoculate in 100ml SOB medium in a 500 ml Erlenmeyer flask, for 3 hours at 37°C at 200-250rpm.
3. Measure OD_{600} after 3 hours and calculate the volume of the culture is needed for a new solution of cells with an OD_{600}= 0.05 in a total volume of 250ml SOB medium.
4. Incubate the solution for 16-18 hours at 18°C with shaking (200-250rpm).
5. Measure OD_{600}. The cell density should be OD_{600} =0.3-0.6.
6. Cool down the cell culture on ice for 10 minutes.
7. Transfer the cell culture to sterile centrifuge tubes, and spin down the cells for 10 minutes at 2500xg at 4°C.
8. Discard the supernatant and resuspend the pellet in 80 ml cold transformation buffer (TB buffer).
9. Cool down the cell culture on ice for 10 minutes.

10. Spin down the cell culture for 10 minutes at 2500xg at 4°C.
11. Discard the supernatant and resuspend the pellet in 20 ml TB buffer.
12. Add 700µl DMSO for a concentration of 3.5% and leave on ice for 5 minutes.
13. Add 700µl DMSO for a final concentration on 7% and leave on ice for 10 minutes.
14. Aliquot the cells into pre-cooled microcentrifuge tubes, 200 µl suspension in each.
15. Transfer the tubes to liquid nitrogen for rapid freezing before storage in the -80°C freezer.

The newly prepared cell culture is expected to lack any antibiotic resistant genes and thus survive only if a plasmid containing a resistance gene is introduced. For confirmation, the cells are plated out on LB agar plates containing ampicillin (100µg/ml), kanamycin (50µg/ml) and incubated overnight at 37°C. Any sign of growing colonies on the agar plates indicates contamination. The competence of the cells is determined by transformation with small plasmids (pUC19) at different concentrations, normally 0.1pg, 1pg and 10pg. The transformation efficiency is expected to be between 10^6- 10^9 cells/µg and is calculated as the number of cells transformed per 1µg of plasmid.

2.1.2 Storage and growth conditions for bacterial cells

2.1.2.1 Stock culture

A cell stock is prepped by growing bacteria overnight at 37°C in LB medium supplemented with the appropriate antibiotic (ampicillin) and shaking at 250 rpm. 1ml of *E.coli* cells as well as 430µl 50% glycerol is added to a 2ml (freezing) tube and stored in the freezer at -80°C. This stock is viable for years due to hibernation of the cells. When in need of a cell culture, a small amount of frozen cells can be added to LB medium with appropriate antibiotics and grown overnight at 37°C with shaking (250rpm).

2.1.2.2 Growth conditions for E.coli DH5α-cells

LB medium and LB agar plates containing selected antibiotics such as ampicillin (100µg/ml) or kanamycin (50µg/ml) are best suited for cultivating DH5α-cells. For colony growth the cells are applied to LB agar plates and incubated for 16-20 hours at 37°C. When isolation of plasmid is sought, the cells are cultivated in LB medium, 3ml for mini prep and 200ml for midi prep with appropriate antibiotics and the culture is incubated for 16-20 hours with constant shaking at 250 rpm and 37°C.

2.1.3 Bacterial transformation by heat-shock

Transformation of bacteria is a method of horizontal gene transfer, where foreign genetic material is able to pass through cell membrane of bacterial host cells. This process is dependent on bacterial cells being competent. Transformation can occur naturally in some bacterial strains, while other must

be artificially made competent (described in 2.1.1). The genetic material introduced, in this case plasmids, are required to contain an antibiotic resistance gene for selection of positive cells. Additionally, it should also contain a bacterial origin of replication and a multiple cloning site (MCS).

The process is initiated by addition of plasmid DNA to competent cells, followed by incubation on ice that will ensure the adhesion of genetic material to the cell membrane. Heat shock of bacteria at sub-lethal temperature induces formation of pores on the membrane, through which plasmid can pass and enter the cell. Re- incubation on ice will ensure tightening of the pores. Lastly the bacterial cells are spread on agar plates containing the appropriate antibiotics that will prevent bacterial cells that do not have the selective marker from surviving. Colonies containing the integrated plasmid are grown and selected for further use.

Procedure

1. Thaw the appropriate amount of competent *E.coli* cells on ice. Pre-chill empty 1.5 ml microcentrifuge tubes.
2. Pipet 1-5μl of plasmid DNA (1-50ng/μl) to a pre-chilled microcentrifuge tube.
3. Add 50μl aliquots of *E.coli* cells.
4. Mix carefully and leave on ice for 20-30 minutes.
5. Heat shock the mixture at 42°C for 90 seconds.
6. Return on ice for 2-3 minutes.
7. Transfer the mixture to LB agar plates with the appropriate antibiotic that corresponds to the resistance gene of the plasmid.
8. Spread the mixture using glass rods or glass spheres.
9. Incubate for 16-20 hours at 37°C.

2.2 Mammalian cell methods

The following section describes all mammalian cell techniques enforced in this study, outlining the cell lines used, the method of their treatment and ministration, as well as the preparation for further use in protein assays.

Contaminations from bacteria, fungi and other cell lines are at high risk when working with mammalian cell lines. Consequently, all work regarding mammalian cell lines is performed under sterile condition in order to prevent contamination. Handling of mammalian cells is implemented in Class II laminar flow hoods that are strictly used for only mammalian cell lines and are prior of use, sterilized by UV light. All solutions and equipment are disinfected with 70% ethanol and additionally all solutions are autoclaved at 121° for 20 minutes.

2.2.1 Mammalian cell lines

The mammalian cell work in this study was performed using four cell lines: HEK 293-c1, HEK 293-c4, CV-1 and COS-1. HEK293-c1cells are derived from the human embryonic kidney cell line HEK293, and contains an integration of a 5xGal4 luciferase reporter, described in 2.4.5. The basal activity line of the 5xGal4 luciferase reporter is low, making the cell line a good candidate for use in activation reporter gene assay. Equivalently derived from the HEK 293 cell line, the HEK 293-c4 cells with an integrated 5xGal4 luciferase reporter has a higher basal activity, thus making the cell line suitable for use in suppression reporter gene assays. The CV-1 cells are adherent kidney fibroblasts from male adult African green monkey *Cercopithecys aethiops*. The COS-1 cell line is derived from CV-1 cells and modified to express a defective mutant of Simian Virus 40 (SV40) as well as produce large T antigen, responsible for inducing replication of constructs carrying a SV40 promoter.

2.2.2 Storage and growth conditions

Stocks of HEK293-c1, HEK 293-c4, CV-1 and COS-1 cell lines are sustained in sterile vials in a specific medium and stored in nitrogen tanks. The medium in which the cell line is preserved contains DMSO, a protective agent preventing formation of ice crystals of the medium, that could potentially damage the cells. Upon a demand for cell cultivation, the cell line is removed from cryopreservation, thawed and cultured in pre-warmed at 37° C appropriate medium.

2.2.2.1 Sub culturing of adherent mammalian cells

In order to maintain the cell line, sub culturing is necessary and has to be implemented according to the specific requirements of the cell line. Adherent cells need to be passaged when the available growth surface has been filled or when the cell culture media is spent. Passaging is the method of removing the media and detaching the cells from the cell surface they are adhered to by enzymatic degradation. Prior to subcultivation, cells are washed with phosphate buffer saline (PBS) to remove traces of medium. The cell are treated with trypsin, a serine protease, breaking down the protein matrix that adhere the cells to each other and to the flask. Trypsin is in a solution with EDTA. EDTA prompts cell detachment by binding Ca^{2+} ions, disabling calcium-dependent adhesion molecules such as cadherins. Loose cadherin interaction between cells will allow for a better and more efficient trypsin degradation. After complete cell dissociation, fresh media is administered to inhibit trypsin, as it contains trypsin inhibitors. The cell volume is split into appropriate fractions and added new fresh media, allowing for further stimulation of cell growth.

All cells in this study were grown and maintained in T-75 culture flasks in 12 ml growth medium supplemented with 10% FBS and 1% P/S. Cultured cells were kept at 37° C in incubators with humidified air containing 5% CO_2. Sub cultivation occurred every 48-72 hours, depending on the confluence level, in

order to prevent overgrowth and cell death. Upon reaching 30 passages, the cells were discarded.

Procedure

1. Pre-warm medium and trypsin in 37°C water bath for 30 minutes.
2. Disinfect all utensils and working area with 70% ethanol. Place all of the necessary equipment into the flow hood to minimize hand movement.
3. Examine the cells under light microscope and determine the ratio of splitting.
4. Remove and discard the spent cell culture media from the flask.
5. Add 10 ml of 1xPBS and gently risk the cell layer. Remove and discard 1xPBS.
6. Add 2.5 ml Trypsin solution and incubate for 4 minutes at 37 °C.
7. Add 9.5 ml of the appropriate medium supplemented with FBS and P/S to the flask. Pipette up and down until the suspension is homogenous.
8. Remove cells for counting if the cells are to be seeded, se section 2.2.4.
9. Dilute the cells to the appropriate ratio for the specific cell line.
10. Add pre-warmed fresh medium to achieve a total volume of 12 ml.
11. Return the cells to the 37°C incubator in humidified air containing 5% CO_2 until the next subcultivation.

Passage specifications for cell lines

1. HEK-293c1 and HEK 293-c4: For subcultivation in 48 hours, dilute cell suspension 1:4. For subcultivation in 72 hours, dilute cell suspension 1:6. Add 1.2 µl puromycin (10µg/µl) in T75 culture flasks, for a final concentration of 1µg/ml.
2. CV-1: For subcultivation in 48 hours, dilute cell suspension 1:6. For subcultivation in 72 hours, dilute the suspension 1:10.
3. COS-1: For subcultivation in 48 hours, dilute cell suspension 1:6. For subcultivation in 72 hours, dilute the suspension 1:10.

2.2.3 Counting cells

Countess® Automated Cell Counter by Invitrogen™ provides a mechanism that allows to differentiate between viable and non-viable cells. The method uses a dye; Trypan blue that selectively stains non-viable cells blue. Viable cells exclude the dye from protruding trough the plasma membrane and appear white.

Procedure

1. Add 10µl Trypan Blue to 10µl cell suspension and mix the volume together.
2. Add 10µl of the suspension to a Countess® cell counting chamber slide.
3. Adjust the focus by the use of the focus wheel to ensure that viable cells are white at the center.
4. Press `count cells` on the Countess® Automated Cell Counter for calculations of viable cells and an estimation of percentage of viability.

2.2.4 Seeding cells

The cells are required to be seeded 24 hours prior to transfection and incubated at 37°C in humidified air with 5% CO_2. In this study, cells were seeded in either 10-15 cm dishes or 6-24 well plates.

Procedure

1. Collect an appropriate volume of cells that are required to be seeded.
2. Count the cell as mentioned in the previous section 2.2.3.
3. From the values of viable cells, calculate the required volume of cell suspension for the correct concentration of cells. Table below outlines the specific requirements for each mammalian cell line.
4. Incubate at 37°C humidified air with 5% CO_2 for 24 hours.
5. After 24 hours, the cells are ready to be transfected, as described in the next section 2.2.5.

Cell line	Plates/dishes	Cells per well /dish	Volume per well/dish
HEK 293-c1	24 wells	$0.34*10^5$	500µl
HEK 293-c4	24 wells	$0.34*10^5$	500µl
CV-1	24 wells	$0.2*10^5$	500µl
COS-1	10 cm dishes	$1*10^6$	10ml

2.2.5 Mammalian cell transfection

The non-viral introduction of genetic material into eukaryotic mammalian cells is referred to as transfection. Previously on, transformation was described in section 2.1.3, which refers to non-viral DNA transfer into bacterial and non-animal eukaryotic cells such as plants. Viral DNA transfer is usually termed as transduction.

During transfection, the nucleic acid of interest is normally purified and added to cells that are in the exponential phase of their growth. This allows for genetic material entry into the nucleus under the course of its disintegration and rebuilding. For a more efficient transfection, it is recommended to transfect cells that are 50-80% confluent.

For this thesis, the transfections were performed with the use of the lipopolyplex transfection reagent TransIT®-LT1 from Mirus Bio™. The transfection reagent has a high efficiency plasmid DNA delivery with low toxicity levels and is serum compatible, thus eliminating the need for culture medium change. Its mechanism involves using lipids and polymers to encapsulate the plasmid and

cover the negative charge exerted by the nucleic acids. Consequently, the molecule of plasmid DNA is able to pass into the cell trough endocytosis. TransIT-LT1 is suitable for both transient and stable transfection.

Procedure

1. Inspect the seeded cells under a light microscope.
2. Warm serum-free DMEM at 37°C and TransIT®-LT1 reagent at room temperature for 30 min prior to transfection.
3. Disinfect all the utensils and working area with 70% ethanol and set up the equipment in the flow hood to minimize hand movements.
4. Add serum-free DMEM to plasmid DNA.
5. Add TransIT®-LT1 directly into the medium.
6. After a 20-minute incubation at room temperature, the mixture is added to the cells in the wells in a drop-by drop manner.
7. The lid covering the cells is applied and the mixture is shaken gently to mix the plasmid solution and the cell suspension.
8. The wells are returned to the 37°C incubator with humidified air and 5% CO_2 for 24 hours before cells are harvested.

Transfection parameters for mammalian cell lines

	6 wells	**24 wells**	**10 cm**	**15cm**
DNA	1μg	0.4-0.6μg	5μg	12.5 μg
TransIT®-LT1	2ul	0.8-1.8μl	10μl	25μl
DMEM1	150μl	50μl	1ml	1ml

Cells are ready to be harvested 24 hours after transfection. In this study, cell lysates are prepared to be used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis, luciferase assay and coimmunoprecipitation, all protein assays described in section 2.4.

2.3 DNA methods

DNA handling encompasses a set of techniques that allows for modification of DNA. Examples of these are amplification, purification, digestion, ligation and mutagenesis. These techniques create an opportunity to modify and alter the genetic material towards the desired manner and ultimately study the effects of the changes enforced. In this study, all of the DNA manipulation has been executed accordingly to the procedures described below.

2.3.1 Polymerase chain reaction PCR

The polymerase chain reaction is a method used to amplify short DNA segments (up to 40kbp) in vitro, exploiting the enzymatic activity of the DNA polymerase. The enzymes ability to synthesize complementary strands of DNA, allows for generation of thousands to millions of copies of the submitted DNA sequence.

The PCR method relies on thermal cycling, where repeated cycles of heating and cooling of the mixture allows different temperature dependent reactions to occur. Denaturation, annealing and elongation are the three main steps of the heat cycle. During the denaturation step, the high temperature separates the double stranded DNA (dsDNA) into single stranded DNA (ssDNA) by breaking the hydrogen bonds between the bases. Two primers that are complementary to the flanking segment of the template will anneal to their target sequence when the temperature is lowered and appropriate. DNA polymerase elongates the DNA strand by attaching complementary deoxynucleotides (dNTP) through the primers in a 5´to 3´manner. Template length, primers and enzymes determine the temperature and incubation duration of the different steps of the heat cycle.

The PCR reaction mixture is assembled in a 200µl PCR tube and placed in the PCR machine. During this thesis, the 2720 Thermal Cycler from Applied Biosystem PCR machine was used. After program termination, samples are kept at 4°C, or purified for further use.

The requirements for a PCR reaction

Reagent	**Volume (µl)**
H_2O	36.5
Template DNA (1ng/µl)	1
Forward primer (25µM)	2
Reverse Primer (25µM)	2
BSA 100x (10mg/ml)	0.5
dNTP (5mM)	2
Vent-buffer 10x	5
Vent polymerase (2U/µL)	1
Total	50

Standard PCR program for DNA amplification

Step	**Temperature**	**Duration**	**Reaction**
Step 1	95°C	5 minutes	Denaturation

Step 2	95°C	30 seconds	Denaturation
Step 3	55°C	30 seconds	Annealing
Step 4	72°C	1 minute	Elongation
Step 5	Go to step 2	29 times	Repetition
Step 6	4°C	Forever	Cooling

2.3.2 DNA purification from PCR

For PCR product purification, the NucleSpin® Gel and PCR Clean-up kit from Macherey-Nagel was used. The protocol was followed according to the manufacturer´s instructions. The kit uses binding buffer to adhere the DNA of interest to a silica membrane under the appropriate low salt and pH-conditions. Impurities, primers and buffers are removed by a medium salt wash and the DNA is eluted.

2.3.3 Restriction digestion of DNA

Normally plasmids generated for cloning are developed to contain an origin of replication, selectable markers as well as a multiple cloning site (MCS) that includes a set of recognition sequences for restriction enzymes. These restriction enzymes insert a cut in the polynucleotide chain, allowing single and multiple cleavage of the plasmid. The plasmid is usually separated by agarose gel-electrophoresis procedure described in section 2.3.4.

The nucleotide sequence recognized for cleavage by a restriction enzyme is called a restriction site. Most restriction sites are palindromic and consist of four to eight nucleotides. The restriction enzymes are endonucleases that cleave in the center region of the restriction site, often unevenly on the double strand, to generate complementary single stranded ends, also called “sticky ends”. Sticky ends have an overhang of nucleotides on 5´or 3´end of the single stranded DNA opposed to blunt ends that are generated when the cleavage is executed on the same exact place on both strands.

Restriction enzymes are originally isolated from different organisms and thus have different sets of optimal conditions under which they perform optimally. New England Biolabs (NEB) supplied the restriction enzymes, protocol and optimal condition data used in this study. Normally restriction enzyme concentration is indicated in units/µl. One unit (U) of the restriction enzyme will digest 1µg λ-DNA within one hour in a 50µl reaction volume at optimal conditions.

Recommended amount of DNA for verification is 400-500ng and 2µg when used for further subcloning.

General setup of restriction digest of DNA

Reagent	Amount
H_2O	up to 50µl
Recommended NEB-buffer (10x)	1x
Plasmid DNA	0.5-2.0µg
Restriction enzyme	0.5-1µl
Total	50µl

Incubate for 60-90 minutes at required temperature.

2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis is a method that separates DNA fragments in a sample and is commonly used to isolate specific DNA fragments, visualize and confirm size of specific DNA bands. Agarose is a polysaccharide powder that will in combination with TAE buffer, aggregate and form a gel with channels and pores through which DNA molecules can pass. Exploiting the negative charge of DNA, imparted by its phosphate backbone, application of an electrical field on the gel will drive the movement of DNA fragments across the gel, towards the positively charged electrode. The concentration of agarose in a gel, ranging from 0.5-2.0%, as well as the strength of the electrical field applied, will determine the migration rate and separation of the molecules.

Agarose gels are supplemented with EtBr, a carcinogenic intercalactor that implements itself in between the base pairs of the double helix and fluoresces under UV light.

Procedure for 1% gel:

1. Weigh out 1 mg of agarose powder and add to 100ml 1xTAE buffer in an Erlenmeyer flask.
2. Heat the solution in a microwave until the agarose is dissolved.
3. Cool down the solution to approximately 50°C, under cool water.
4. Add 1 drop of EtBr (428µg/ml) to a final concentration of ~0.5 µg/ml.
5. Prepare a gel tray and pour the solution into the tray. Add a comb to make wells for loading the samples.
6. Leave the gel solidify for 20-30 minutes and remove the comb from the gel (gently).
7. Place the gel into the gel chamber and add 1xTAE-buffer to cover the surface of the gel.
8. Load the samples supplemented with gel loading buffer into the wells and apply electrical field.
9. Run the gel at 100V for 45-60 minutes.
10. Visualize the DNA using the UV lamp from VWR®.

2.3.5 DNA purification from agarose gel

After agarose-gel electrophoresis, distinct bands of DNA can be isolated and purified for further application. The band(s) of interest is removed from the gel by cutting the gel with a clean scalpel blade and dissolved in an appropriate amount of binding buffer at 50°C. The solution is transferred to a silica membrane, which binds DNA while impurities, salts, enzymes, and gel residues are removed by a medium salt wash. Lastly, DNA is normally eluted in a 30µl volume.

For DNA purification from agarose-gel, the NucleoSpin® Gel and PCR Clean-up kit from Macherey-Nagel was used. The protocol was used according to the manufacturer´s instructions.

2.3.6 Ligation of DNA

Fragments of DNA can be joined together covalently by DNA ligases. DNA ligases are enzymes, which operate by catalyzing the formation of phosphodiester bond between the 5´phosphate group of one nucleotide and 3´hydroxyl group in another nucleotide in an ATP-dependent reaction. This mechanism works on both cohesive and blunt ends, although the enzyme concentration may vary for the specific reaction. In this study, DNA fragments of interest were ligated into vectors, further used for transfection. After ligation, the new plasmid can be transformed into bacterial cells for propagation (described in 2.1.3).

In this study, most ligation reactions were performed using T4 DNA ligase, origination from the T4 bacteriophage. New England Biolabs (NEB) supplemented T4 DNA ligase as well as the appropriate buffers. For T4 DNA ligation reactions, the samples were incubated at room temperature from 30 minutes to 2 hours and deactivated at 65°C for 10 minutes. In rare cases, the Quick Ligation Kit supplied by NEB was implemented, using the manufacturers protocol.

The set-up for ligation reaction is found in the table below. For optimal ligation, the physiological conditions, incubation time as well as insert to vector ratio has been taken into the account. The amount of insert needed in the ligation reactions was calculated using the formula bellow. To validate the ligation reaction, a control reaction with no insert was also used.

$$\mathrm{ng}(insert) = \frac{\mathrm{ng}\ (vector) * \mathrm{bp}(insert)}{\mathrm{bp}(vector)} * \mathrm{ratio}\ (5x - 10x)$$

Set-up for ligation reaction using T4 DNA ligase

Reagents	**Amount**
H_2O	to 15µl
T4 DNA ligase buffer (10x)	1µl

Vector DNA	30-50ng
Insert DNA fragment	Calculated
T4 DNA ligase	0.5-1μl
Total	15μl

1. Incubation time: 30 minutes – 2 hours

2. Heat inactivation: 10 minutes at 65°C.

Set-up for ligation reaction using Quick Ligation Kit

Reagents	**Amount**
H_2O	to 20μl
Quick Ligase Reaction Buffer (2x)	10μl
Vector DNA	30-50ng
Insert DNA fragment	Calculated
Quick Ligase	1μl
Total	20μl

1. Incubation time: 5 minutes, followed by cooling on ice. No heat inactivation required.

2.3.7 DNA purification from bacterial cells

For isolation of plasmid DNA, the NucleoSpin® Plasmid and NucleoSnap Plasmid Midi purification kits from Macherey-Nagel were used for mini prep and midi prep methods, respectively. The protocol was followed according to the manufacturer´s instructions.

Both kits use lysis buffers containing sodium dodecyl sulfate (SDS) and sodium hydroxide to achieve alkaline conditions and lysate the cells, ensuring a complete denaturation of DNA and proteins. Neutralization of the total cell lysate results in small circular plasmid DNA recovery. Cell lysate transfer to a silica membrane rescues the plasmid DNA, while debris is removed by a wash of an ethanolic buffer. Pure plasmid DNA is eluted with an elution buffer.

2.3.8 DNA concentration assessment

Measurements of DNA concentration were performed using Nano Drop 2000 UV-vis Spectrophotometer from Thermo Scientific in accordance to the manufacturers protocol. DNA and RNA absorb UV light with a peak at 260 nm. The equipment measures the DNA presence at 260 nm and calculates the DNA concentration by using the Beer-Lambert equation. Background measurement

was performed using the 1-2μl of the buffer the sample was diluted in, followed by application of 1-2μl of the sample.

The degree of purity in the sample is also assessed using the 260 nm/280 nm ratio. The nitrogenous bases in nucleotides have an absorption maximum of UV light at 260 nm, while proteins have an absorption maximum of 280 nm. A ratio of 1.65-1.80 for DNA and respectively a ration of 2.0 for RNA indicate a good degree of purity of the sample.

2.3.9 Sequencing of DNA

DNA sequencing was performed at the GATC Biotech in Germany. A list of used sequencing primers can be found in appendix D.

Sample requirements for shipment to GATC Biotech in Germany

Reagents	**Volume (μl)**
Template (80-100ng/μl)	5μl
Primer (5 μM)	5μl
Total	10μl

2.3.10 DNA mutagenesis

Site- directed mutagenesis (SDM) is a method used for making a precise alteration in a DNA sequence of a gene. The method is implemented in vitro; using custom designed and synthesized oligonucleotides. These are complementary to the relevant region of the gene but contain the desired nucleotide alteration. Oligonucleotides are hybridized to the DNA and used as primers for the strand synthesis reaction, which are allowed to continue all the way around the circular template. The newly synthesized plasmid DNA with the desired mutation is in contrast to its precursor, unmethylated, thus making it distinguishable. The endonuclease DpnI is used to digest the methylated parental DNA, granting access to the product.

In this study, site directed mutagenesis was used to introduce an amino acid alteration in the pCIneoB-GBD2-hcM-SUMO-2KR (233-640) K37 construct: K37Q and K37R. The primers were custom synthesized to contain an altered sequence, changing the lysine (K) amino acid to glutamine (Q) or arginine (R) and the PfuUltra™DNA polymerase from Stratagene with a high fidelity rate was used.

PCR reaction set up

Reagents	**Amount**
H_2O	up to 50μl

Template	25/50ng
Forward primer (100μM)	125ng
Reverse primer (100μM)	125ng
dNTP (5mM)	1μl
Pfu Ultra™Buffer (10x)	5μl
Pfu Ultra™Polymerase (2.5U/μl)	1μl
Total	50μl

PCR program for DNA mutagenesis

Step	**Temperature**	**Duration**	
Step 1	95°C	30 seconds	Denaturing
Step 2	95°C	30 seconds	Denaturing
Step 3	55°C	1 minute	Annealing
Step 4	68°C	10 minutes	Synthesis
Step 5	Go to step 2	18 cycles	
Step 6	68°C	10 minutes	Synthesis
Step 7	4°C	Forever	Cooling

2.4 Protein methods

2.4.1 Preparation of cell lysate for SDS-PAGE and western analysis

A fluid containing the contents of lysed cells is called lysate. In this particular case, the process involves breaking down the cell membrane to expose the intracellular contents for investigation by SDS-PAGE and western blot analysis, methods described in section 2.4.1 and 2.4.5. Cell lysates for reporter assay and co-immunoprecipitation are prepared as specified in section 2.4.5 and 2.4.7.

The cell lysis buffer used in this study to lysate the mammalian cells is 3xSDS loading buffer. This buffer contains Tris-HCl, affecting the acidity and osmolarity of the lysate, SDS detergent that denature proteins and DTT that further denature the proteins by reducing the disulfide bonds. After the cells are lysed by the 3xSDS loading buffer, sonification is applied to physically disrupt the genomic DNA. High frequency sound waves are enforced to disrupt the cell

membrane, ensuring that all cells are lysed as well as to fragment genomic DNA and prevent the samples from being too viscous.

Procedure

1. Remove the cells from the incubator, 24 hours after cell transfection.
2. Remove and discard the cell culture media from the wells and wash the cells with 0.5ml 1xPBS per well for a 24 well.
3. Remove and discard 1xPBS.
4. Add 100µl of 3xSDS loading buffer per well and shake for 5 minutes at 150 rpm.
5. Transfer the cell suspension to microcentrifuge tubes. If the transfection is done in triplicates, collect the lysates of these three wells together.
6. Centrifuge the samples for 2 minutes at 4°C at 3000 rpm.
7. Sonicate the samples using VWR® Ultrasonic Cleaners from VWR™ for 5 minutes.
8. Boil the samples at 95°C for 5 minutes
9. Store the samples in the -20°C freezer until gel analysis.

2.4.2 Separation of proteins by SDS-PAGE

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a method used to separate proteins according to their size by excluding other components such as overall charge and the three-dimensional structure. This is achieved by the use of the anionic SDS detergent that consists of a hydrophobic 12-carbon chain and a polar sulfated head. The hydrophobic chain intercalates into hydrophobic regions of a protein, thus disrupting its folding composition, while the polar head reacts with water and ensures a soluble state for the protein complex. SDS efficiently coats the protein in a negative charged layer, which overrides the original charge of the protein. The SDS-protein complex will move towards the anode, according to the molecular mass of the protein- smaller proteins migrating faster towards the anode than larger ones. Typically, a tracking dye, in this thesis bromophenol blue is added to aid in the visualization of the sample through the gel.

In this thesis, pre-casted 4-12% gradient Bis-Tris Criterion XT gels with 12+2 wells from BioRad was used. By using a gradient gel with an increasing percentage of acrylamide, the proteins are increasingly retarded, subsequently migrating at a slower rate relative to size. Larger proteins require a lower percentage acrylamide-gel, while smaller proteins are better separated in a gel with a higher percentage of acrylamide. The 1xXT-MOPS buffer from BioRad was used as running buffer. Separated proteins were further analyzed by western blotting (section 2.4.4) or stained for visualization (section 2.4.3).

Procedure

1. Prepare the samples of cell lysates beforehand as described in section 2.4.1.
2. Assemble the pre-casted 4-12% Bis-Tris Criterion gel in SDS-PAGE chamber.
3. Dilute the XT MOPS buffer to the appropriate concentration (from 20x to 1x) and fill up the electrophoresis chamber.
4. Check the viscosity of the sample. If the sample is too viscous, sonicate for 3-5 minutes.
5. Load 10µl of the Standard Precision Ladder Duo Color.
6. Load a maximum amount of 45µl sample in each well.
7. Load the remaining empty wells with 3xSDS-loading buffer to avoid a smile-line on the gel.
8. Run the gel for 40-60 minutes at 200V.
9. After electrophoresis, remove the pre-casted gel from the chamber and break the casting to access the gel.

2.4.3 Staining protein bands

Coomassie Blue staining solution was used to visualize protein bands and analyze protein blotting efficiency in polyacrylamide gels. In addition, Ponceau S Red and Brilliant Blue R250 staining solution was used to visualize protein bands blotted in PVDF membrane after Western blotting experiments. Procedures are described as following:

Coomassie Blue

1. Incubate the gel in fixing buffer for 15 minutes with shaking.
2. Stain with Coomassie Blue staining buffer for 40-60 minutes with shaking.
3. Wash the gel in fixing buffer until protein bands are visible.

Ponceau S Red

1. Immerse the membrane in 1x Ponceau S Red staining solution for 5 minutes with shaking.
2. Wash in dH_2O until protein bands are visible.
3. Leave membrane to air dry.

Brilliant Blue R250

1. Wash the membrane with dH_2O for 5 minutes with shaking. Repeat 3 times.
2. Immerse the membrane in Brilliant Blue R250 staining solution for 5 minutes with shaking.
3. Destain the membrane 50%methanol / 7% acetic acid for 5 to 10 minutes with shaking.
4. Rinse the membrane with water, leave the membrane to air dry.

2.4.4 Western blotting

Western blotting is an analytical method, subsequently determining the presence and amount of a protein. This is achieved by taking advantage of the high-resolution electrophoresis done by SDS-PAGE and further transferring the separated sample components onto a mechanically stable membrane. Biomolecules are electrophoresed onto a membrane irreversibly and via hydrophobic interactions. Using an electroblotting apparatus, the gel and the membrane are sandwiched between two electrodes under electrical current. Once the proteins are bound to the membrane, detection and imaging is accomplished by antibody staining that specifically binds to target proteins.

In this thesis, proteins were separated according to their molecular weight by SDS-PAGE as described in section 2.4.2, followed by blotting onto a polyvinylidene diflouride (PVDF) membrane from GE Healthcare. The membrane is incubated in Odyssey® Blocking buffer, blocking any nonspecific antibody binding sites prior to staining with primary antibody overnight. Further on, the membrane is stained with IRDye® secondary antibody from LI-COR® Biosciences, conjugated to a fluorophore, providing a near infrared fluorescent signal. The infrared imaging system Odyssey CLx from LI-COR® Biosciences uses digital fluorescence and provides signal detection that is proportional to the protein concentration that is present in the membrane.

Procedure: Blotting of the proteins

1. Separate the proteins by following the procedure for SDS-PAGE as described in section 2.4.1.
2. Cut the PVDF membrane and 6 pieces of 17 Chr Whatman filter paper from VWR to the size of the gel.
3. Activate the PVDF membrane in methanol for 2 minutes, before equilibrating it in anode(+)-buffer.
4. Assemble the blotting sandwich in the following manner:
    - 3 filter pieces soaked in anode buffer
    - Activated and equilibrated PVDF membrane
    - Polyacrylamide gel
    - 3 filter pieces soaked in cathode buffer
5. Remove air bubbles by pressing lightly on the blotting sandwich with a glass rode.
6. Blot the gel onto the membrane at room temperature for 1 hour at 140mA.
7. When the transfer is complete, confirm the transfer onto the membrane by lifting the layers of the filter papers and the gel.

Procedure: Antibody exposure

1. Transfer the membrane to a small container and block with 1:1 solution of 1xPBS and blocking buffer for 1 hour at room temperature.

2. Dilute the primary antibody in a 1:1 solution of 1xPBS and blocking buffer.
3. Incubate the membrane in primary antibody solution; in a heat-sealed bag overnight at 4°C with shaking.
4. Remove the primary antibody and wash the membrane in 1xTBST. Repeat this step 3 times for 20 minutes at room temperature.
5. Dilute the secondary antibody 1:10 000 in a solution of 1:1 1xPBS and blocking buffer.
6. Incubate the membrane in secondary antibody solution in room temperature for 1 hour. Cover the container with aluminum foil due to light sensitivity of the secondary antibody.
7. Remove the secondary antibody and rinse the membrane 6 times with 1xTBST.
8. Wash the membrane with 1xTBST for 20 minutes at room temperature.
9. Image the blot using the Odyssey CLx imaging system from LI-COR® Biosciences.

2.4.5 Luciferase reporter gene assay

The reporter gene assay can be used to study the activity of the gene of interest as well as the behavioral changes in response to other effectors. In this thesis, luciferase reporter gene assay was implemented to assess the activity of c- Myb transactivation in presence of SUMO1, repressive activity influenced by SUMO2 as well as mutated variants of SUMO1 and SUMO2. The luciferase enzyme catalyzes oxidation of its substrate luciferin, generating energy in form of light. Detection and measurement of bioluminescence can be carried out by using a luminometer.

In this study, reporter gene assays were performed with both stably integrated reporter genes as well as transiently transfected reporter genes. The HEK293-c1 and HEK293-c4 cell lines have stably integrated the luciferase reporter gene under the control of a 5xGal4 responsive elements, thus regulating the expression of the reporter gene in presence of Gal4. The plasmid constructs coding for c-Myb proteins have their DNA binding domain replaced with a Gal4 binding domain, recognizing and binding to the Gal4 elements, subsequently activating transcription of luciferase. The HEK293-c1 cell line has a low basal activity, suitable for analysis of activation, while the HEK293-c4 cell line has a higher basal level, more suitable for repression analysis.

CV1 cell line is dependent on a reporter gene being co-transfected with effector and co-factor plasmids. This study used the pGL-4b-3xMRE(GG)-Myc aab plasmid, with a core promoter from Myc and 3xMyb recognition elements (MRE) that are recognized and bound to by the Myb proteins, activating transcription of luciferase.

Empty vector pCIneo was used as a control as well as equalizer to the total DNA transfected. The cells were seeded as described in section 2.2.4, transfected as described in 2.2.5 and harvested 24 hours after transfection. Experiments were performed in triplicates, as well as in three independent experiments, resulting

in nine repeats of each measurement. Further, parallel experiments were implemented for western blot analysis, described in 2.4.4.

The luminometer used to determine the quantity of bioluminescence in a sample was the TD20/20 luminometer from Turner BioSystems, quantifying the luciferase activity in relative luminescence units (RLU). Promega Corporation supplied both the passive lysis 5X buffer and luciferase substrate.

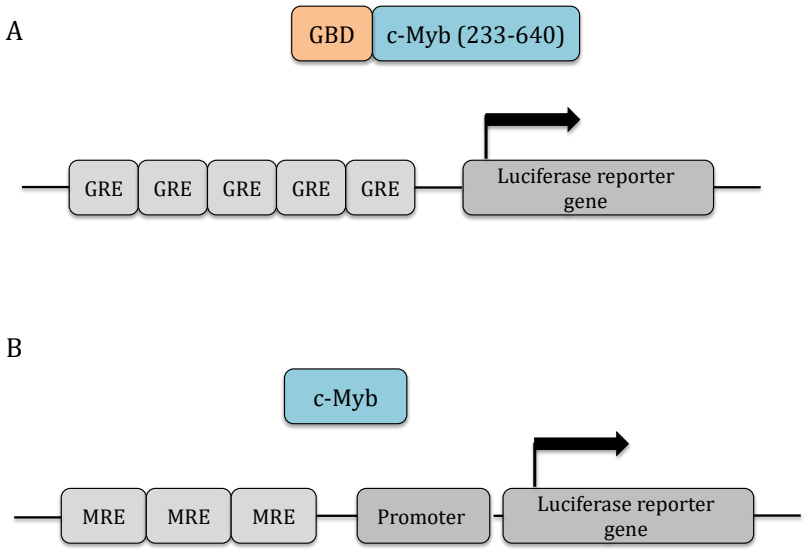


Figure 2.1: **A schematic overview of stable and transient synthetic luciferase reporters. A:** The stably integrated luciferase reporter gene in HEK 293-c1 and HEK293-c4 cell line has five Gal4 responsive elements (GRE) upstream of the reporter gene. Th reporter gene is fully chromatinized. **B:** The transient luciferase reporter gene in CV-1 cell line has three Myb recognition elements (MRE) upstream of promoter of luciferase reporter gene.

Procedure

1. Transfect the cell 24 hours prior as described in section 2.2.5.
2. Thaw luciferase substrate and dilute the 5xpassive lysis buffer (PLB) to 1xPLB.
3. Check the confluence level of the cells in the microscope.
4. Remove the media from the cells and wash the cells with 0.5ml 1xPBS.
5. Remove the 1xPBS and add 100µl of 1xPLB per well.
6. Incubate the cells on oscillator for 15 minutes in room temperature at 150rpm.
7. Transfer the cell lysate to microcentrifuge tubes.
8. Spin down the cell lysate for 2 minutes in room temperature at maximum rpm.
9. Mix 50µl luciferase substrate with 50µl cell lysate in tubes and measure the luminosity of the sample.
10. Save the data for further statistical analyses.

TD-20/20 luminometer parameters for measuring luciferase activity

	Setting
Delay time	2 seconds
Integrate time	10 seconds
Number of replicates	1
Sensitivity level	59.8 %
Mode	STD

2.4.6 Acetylation of SUMO1 by p300

Acetylation is a frequently occurring post-translational modification in proteins, having different biological implications. In this thesis, acetylation of SUMO1 by acetyltransferase enzyme p300 is of interest, in order to determine potential acetylation sites. The standard reaction was performed with recombinant p300 provided by Active Motif, isolated hSUMO1 protein and acetyl-CoA molecules provided by Sigma Aldrich. Coomassie Brilliant Blue staining solution was used for protein-bands visualization and validation of acetylation by mass spectrometry was performed at the Department of Biosciences of University of Oslo.

Procedure

1. Prepare reaction buffer for the acetylation-reaction.
2. Set up the reaction and incubate for 30 minutes at 30°C.
3. Stop the reaction by adding 15µl of 3xSDS loading buffer.
4. Separate the proteins by SDS-PAGE as described in 2.4.2
5. Run the gel for 1 hour at 140mA.
6. After electrophoresis, remove the pre-casted gel from the chamber and break the casting to access the gel.
7. Stain the protein bands with Coomassie Blue dye as described in 2.4.3.
8. Cut the desired gel pieces and transfer to a microfuge tube.
9. Validation of acetylation by mass spectrometry.

Acetylation reaction set up

	H_2O	2x buffer	p300	hSUMO1
1	13.75 µl	15 µl	1.25 µl	-
2	13.6 µl	15 µl	-	1.4 µl
3	12.35 µl	15 µl	1.25 µl	1.4 µl

2.4.7 Co-immunoprecipitation assay

Co-immunoprecipitation is a method used to study protein-protein interactions. The method is based on immunoprecipitation (IP), where proteins are isolated from a sample by using specific antibodies. The antibodies form an immune complex with the protein of interest, which is further on precipitated, isolated and analyzed. The method is further utilized for isolation of protein-protein complexes, where antibodies target a protein known to form complexes, thus pulling the entire complex out of the solution and identifying interaction partners.

In this thesis, COS1 cells were seeded and transfected with the plasmid of interest as described in 2.2.4 and 2.2.5 respectively. For transfection, 10cm dishes were used, in order to attain $1*10^6$ cells that were harvested 24 hours thereafter. Super magnetic iron impregnated 4% agarose beads with attached anti-flag M2 monoclonal antibody from Sigma-Aldrich were used. The M2 antibody binds to proteins containing the FLAG-peptide sequence, thus separating the protein complex from the solution when applied to a magnetic field. Unbound proteins are washed away, allowing the protein complex to be isolated and analyzed.

Preparation of Anti-Flag M2 Magnetic beads

1. Calculate the amount of anti-flag M2 magnetic beads needed for the procedure (20µl per sample).
2. Transfer the appropriate amount of anti-flag magnetic beads to a microcentrifuge tube and place the tube in the magnetic rack.
3. Remove the clear solution from the sample and place the tube on ice.
4. Wash with 1ml PBS w/BSA and spin down shortly at 4^0C.
5. Repeat the washing step with 1ml PBS w/BSA.
6. Repeat the washing step once more with the appropriate amount of KAc lysis buffer.
7. Dissolve the flagbeads in the appropriate amount of KAc lysis buffer (20µl per sample).

Preparation of COS1 cell lysate

1. Aspire medium from cell culture dishes.
2. Detach the COS1-cells from the bottom dish by scraping in 2 x 5ml cool PBS.
3. Transfer the PBS suspension to 50ml falcon tubes.
4. Centrifuge at 900rpm, for 5 min at 4^0C.
5. Discard the supernatant and transfer the pellet to microcentrifuge tubes.
6. Centrifuge for 900rpm, for 5 min at 4^0C.
7. Resuspend the pellet in 400µl KAc lysis buffer.
8. Incubate on rotation for 30 minutes at 4^0C.
9. Centrifuge at 16000rpm, for 10 minutes at 4^0C.

10. Transfer the supernatant to 0.5ml tubes.
11. Remove 5% input sample (20µl of 400µl sample) and add 10µl 3xSDS GLB. Boil the sample for 5 minutes at 95^0C and store in -80^0C freezer until gel analysis.
12. The remaining cell lysate is used for co-immunoprecipitation, described in the next section.

Co-immunoprecipitation procedure

1. Mix the cell lysate with 20µl flag beads and 80µl KAc lysis buffer for each sample in 0.5ml tubes.
2. Incubate the samples on rotation for 1.5 hours at 4^0C.
3. Centrifuge the samples shortly at 4^0C.
4. Transfer the samples to 1.5ml microcentrifuge tubes.
5. Wash the samples by placing them in the magnetic rack, remove the clear solution and add 400µl of KAc lysis buffer.
6. Repeat the washing step.
7. Remove the KAc lysis buffer and add 40µl of 3xSDS GLB.
8. Boil samples at 65^0C for 10 minutes and store at -80^0C until gel analysis.

Gel analysis was performed by using pre-casted 4-12% gradient Bis-Tris Criterion XT gels with 12+2 wells from BioRad, implementing SDS-protein separation procedure described in 2.4.2, followed by Western blot analysis described in 2.4.4, using anti-Flag M2 antibodies from Sigma-Aldrich as primary antibody for the bait protein. Gal antibody was used for SUMO2 detection. Imaging of the blot was performed using the Odyssey CLx imaging system from LI-COR® Biosciences.

2.4.8 Statistical analysis

Statistical methods are used to analyze the data generated from reporter assays in order to present reliable results. The data from the triplicates are compared and normalized in the Microsoft *Excel* software. Normalization of the data minimizes variations of data sets and corrects for factors that may present inaccurate values. These factors may be inconsistencies in pipetting, fluctuation in cell population, transfection efficiency and other conditions that may influence the data achieved.

Analysis of a data set using statistical methods offers the sample average/mean (x), the standard deviation (SD) and the standard error of the mean (SEM). The relative deviation (RD) is calculated by using SD and SEM to determine how varied the data point of the data set is. These calculations were implemented by using the add-in supplemental program Solver. Data sets are presented in descriptive graphs, with error bars representing SEM to illustrate the confidence of the data.

3 Results

The aim of the study was to investigate whether the acetylation switch defined by Stefan Müller *et. al* applied to the transcription factor c-Myb, and if so, to which extent. Further on, to determine whether the acetyl transferase p300 had the capacity to influence the derepression on c-Myb activity brought forth by acetyl mimicking mutants of SUMO. It was also of interest to explore whether the acetylation mutants of SUMO affected their repression properties in a non-c-Myb context. Lastly, the intent was to determine if the acetylation switch on SUMO would affect its binding to interaction partners, as well as observe if this regulation was selective.

This part of the thesis will present the results obtained from all the functional assays, which will be discussed in the next section.

3.1 Plasmid constructs used in the study

The following section will address all constructs used in this study. A selection of mutants and constructs was gifted by Stefan Müller lab as well as being available from other past and present members of Gabrielsen lab. All plasmid constructs were generated by subcloning and mutagenesis. The cloning experiments were planned in advance *in silico*, using the CLC Main Workbench software from CLC bio. The program provided restriction site and primer binding sites identification as well as sequence alignment. All constructs were confirmed by sequencing.

pCIneoB-GBD2-hcM-SUMO-1 2KR [233-640] – expression plasmid for a Gal4-Myb-SUMO fusion protein

We first decided to monitor the effect of SUMO variants in a cell system with an integrated luciferase reporter driven by a Gal4-responsive promoter. For this we needed a plasmid to express a c-Myb Gal4 fusion protein. Furthermore, in order to study the effect of SUMO variants, we decided to use SUMO-fused C-terminally to c-Myb, thus mimicking a 100% SUMOylated protein. The plasmid we needed, called pCIneoB-GBD2-hcM-SUMO-1 2KR [233-640], was generated by subcloning using two available plasmids, pCIneoB-GBD2-hcM-SUMO-1 [233-640] and pCIneoB-GBD2-hcM-TPC-2KR [410-640]. Both plasmids encoded a shortened variant of c-Myb (hcM) and had their DNA binding domain substituted with a GAL4-binding domain (GBD). The latter plasmid included a 2KR mutation in c-Myb. The mutations of lysine residues to arginine in positions K503 and K527 prevented covalent attachment of SUMO protein, which could have interfered with the effects of the fused SUMO.

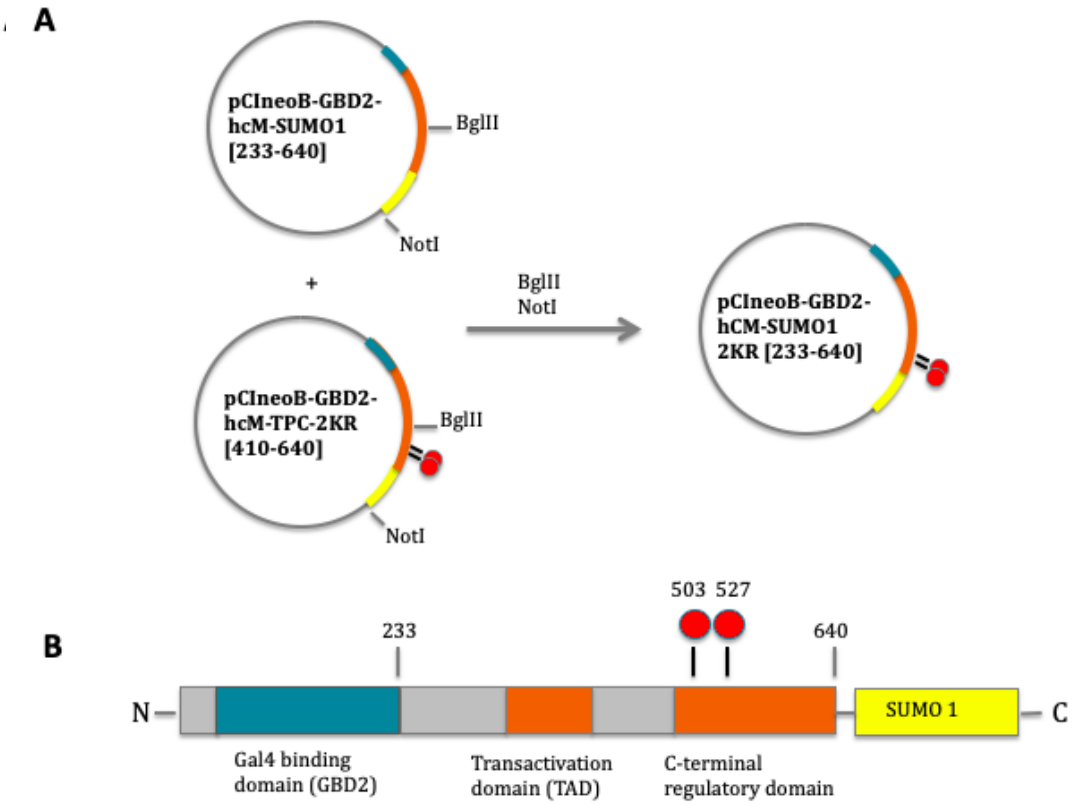


Figure 3.1 **Subcloning strategy for pCIneoB-GBD2-hCM-SUMO-1 2KR [233-640].**

A. Both plasmids pCIneoB-GBD2-hcM-SUMO1 [233-640] and pCIneoB-GBD2-hCM-TPC-2KR [410-640] were digested with restriction enzymes BglII and NotI, as indicated. The former plasmid was used as vector while the latter as insert in order to obtain the fragment of hcM containing the 2KR mutation at position K503 and K527. The fragments were ligated, and positive clones were designated pCIneoB-GBD2-hcM-SUMO1 2KR [233-640]. The final construct was verified by restriction digestion and sequencing.

B. A schematic illustration of the cMyb-SUMO1 construct marking the different domains of the construct, location of the two point mutations at K503R and K527R in c-Myb and location of SUMO.

pCIneoB-GBD2-hcM-SUMO-1 2KR [233-640] K37Q and K37R - expression plasmid for Gal4-Myb-SUMO fusion proteins mutated in SUMO residues

In order to investigate whether the acetylation switch could act as a mechanism of derepression of SUMO-mediated transcriptional repression, acetylation mutants of SUMO were generated. From the original pCIneoB-GBD2-hcM-SUMO1 2KR [233-640] plasmid, a point mutation was introduced, in order to replace Lys^{37} in SUMO-1 with either Glu or Arg. As acetylation of lysine would remove the positive charge of the residue, mutation to glutamine was suggested to behave as an acetyl-mimicking variant of SUMO (Kamieniarz & Schneider, 2009). The latter mutation, to arginine, has a close similarity to the wild type SUMO, keeping the positive charge of the replaced Lys. Similar approach was used in

creation of both constructs, with few exceptions such as using different primers and introduction of different restriction sites for each of the construct. The strategy is illustrated in Fig 3.2 and involved subcloning of a fragment into the cloning vector pBluescript, site directed mutagenesis of this construct, and back-cloning of the mutant fragment into the expression plasmid.

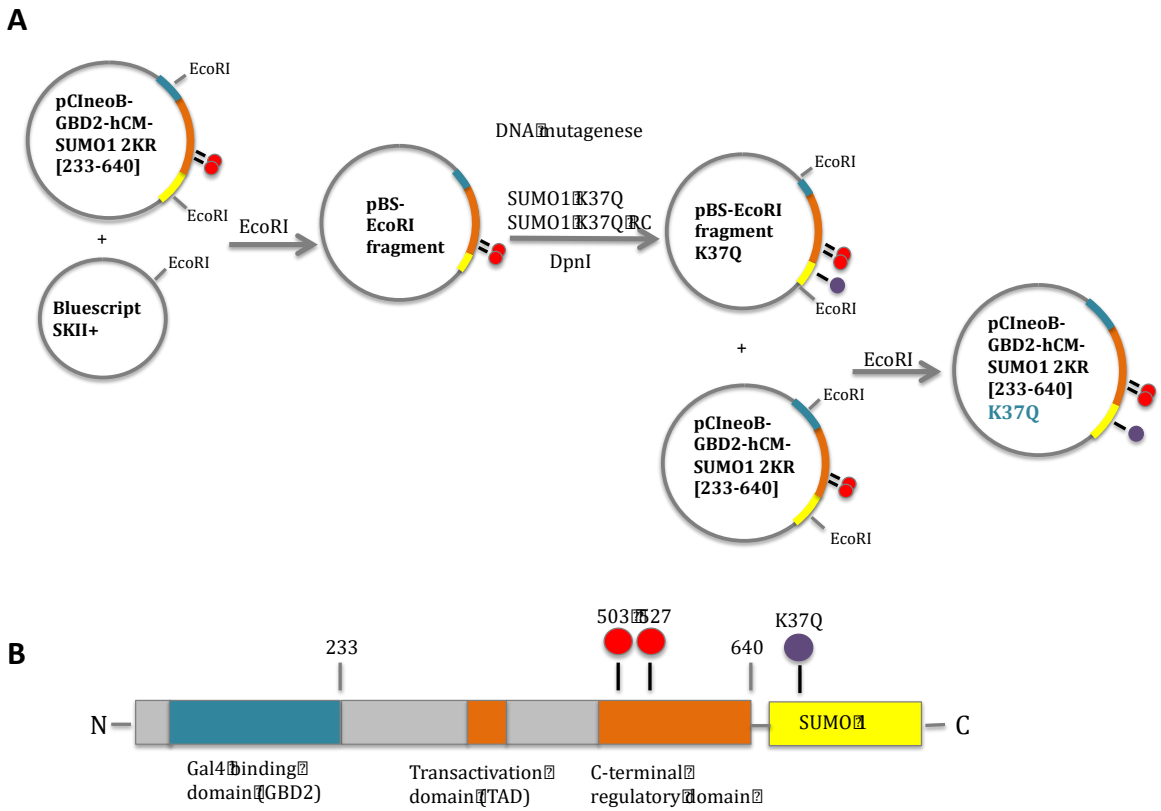


Figure 3.2: **Subcloning strategy for pCIneoB-GBD2-hCM-SUMO1-2KR [233-640] K37Q.**

A. A fragment from pCIneoB-GBD2-hcM-SUMO1 2KR [233-640] was isolated and cloned into a pBluescript vector by restriction endonuclease digestion with EcoRI, resulting in a pBS-EcoRI fragment with parts of the GBD2-domain, hcM-domain and parts of SUMO1. Next, a mutagenese reaction was set up with pBS-EcoRI fragment as a template and appropriate forward and reverse primers for introduction of K37Q and K37R mutations. Two point mutation were introduced in each construct: the K37Q/R mutation and a silent mutation. A restriction site for EcoRII and a K37Q mutation were made in one construct. Equivalently, a restriction site for AleI was introduced along with the K37R mutation on the other construct. After mutagenesis, the template was digested with DpnI and the pBS-EcoRI fragment with the desired mutation in SUMO1 was isolated. Plasmids was further digested with EcoRI, releasing the desired fragment. The fragment was subcloned back into the original pCIneoB-GBD2-hcM-SUMO1 2KR [233-640] plasmid. Positive clones were digested with EcoRII and AleI, respectively to confirm the correct insertion, as well as sequencing was performed to verify and identify the correct orientation of the inserted fragment.

B. A schematic illustration of the cMyb-SUMO1 construct marking the location of the two point mutations at K503R and K527R in hcM as well as the K37Q/R mutation in SUMO1.

3.2 Is the repressive effect of SUMO on the transactivation activity of c-Myb affected by acetylation mimicking and abolishing mutants of SUMO1?

As described in section 3.1, three plasmid constructs were generated, all expressing a fusion of a GBD domain, c-Myb domain and SUMO1. One of the constructs contained wt SUMO1, and the other two contained SUMO1 with Lys^{37} modified to either Glu or Arg. The acetyl mimicking construct with $K^{37}Q$ mutation was hypothesized to behave in similar manner as a acetylated K, while the $K^{37}R$ mutant was suggested to act similar to the wt SUMO, due to its intact positive charge.

To answer the first question of whether the acetyl mimicking and abolishing mutants affect the SUMO mediated repression on c-Myb, a selection of different luciferase reporter gene assays were implemented. The results of performed luciferase assays are discussed in their appropriate sections.

Western blot analysis was performed to further validate the presence and confirm equal expression of the transfected plasmids in the cells. The stable 37kDa housekeeping protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. GAPDH is constitutively expressed in cells, thus, equal detection of the protein will represent an even number of cells in the different samples.

3.2.1 Luciferase assay in HEK293-c1 cells containing a stably integrated reporter

A reporter assay was set up in HEK 293-c1cells. This cell line has a stably integrated luciferase reporter in the genome, making it fully chromatinized. As earlier mentioned, 5xGal4 responsive elements reside in the promoter region, upstream of the luciferase gene. Therefore, the constructs used in this assay have their DBD of c-Myb substituted with a Gal4- binding domain. (To not confuse the reader, the constructs are marked with GBD2, as it simply has an alternative MSC site to another series of constructs marked GBD1). The setup of the experiment included wildtype and 2KR versions of c-Myb-Gal4 fusions (lacking SUMO-fusions), as well as wildtype, acetyl mimicking and abolishing mutants of SUMO1. The results are presented in figure 3.3. Additionally, a western blot analysis was performed to support the findings. The results are presented in figure 3.4.

The results from the luciferase assay (figure 3.3) show first that removing the SUMO conjugation sites (2KR versus WT) in c-Myb causes a dramatic increase in the transactivation of the reporter, as previously reported (Bies, Markus et al., 2002; Dahle, Andersen et al., 2003). When SUMO is fused to the derepressed 2KR version of c-Myb, the activity drops again almost to the level of the wildtype. This large difference in output caused by SUMO, makes the system useful to monitor the effect of the SUMO mutants. We see from the figure how the acetyl mimicking SUMO1 with $K^{37}Q$ mutated has a weaker repressive effect on the transactivation activity of c-Myb than wildtype SUMO1. By comparing activity of c-Myb fused to wt SUMO, the activation of c-Myb fused to the $K^{37}Q$ mutant had an increase of 8-

fold. A much smaller increase was present in the c-Myb fused to $K^{37}R$ mutant, but this was however hardly significant. Western blot analysis (figure 3.4) confirmed the presence of proteins expressed. Here, we observed a robust expression of all constructs, apart from wt c-Myb. This is suspected to be due to rapid c-Myb degradation. It may appear that removing the SUMO conjugation sites, not only increases the transactivation activity of c-Myb but increases protein stability as well. However, we observed a lower quantity of the $K^{37}Q$ mutant compared to the wt SUMO and $K^{37}R$ variants. In theory, we could have expected a lower activity for c-Myb-SUMO1 $K^{37}Q$ mutant due to a lower amount protein in the sample. However, this was not the case as the activity levels were higher despite lower protein levels. This will be further addressed in section 3.2.3.

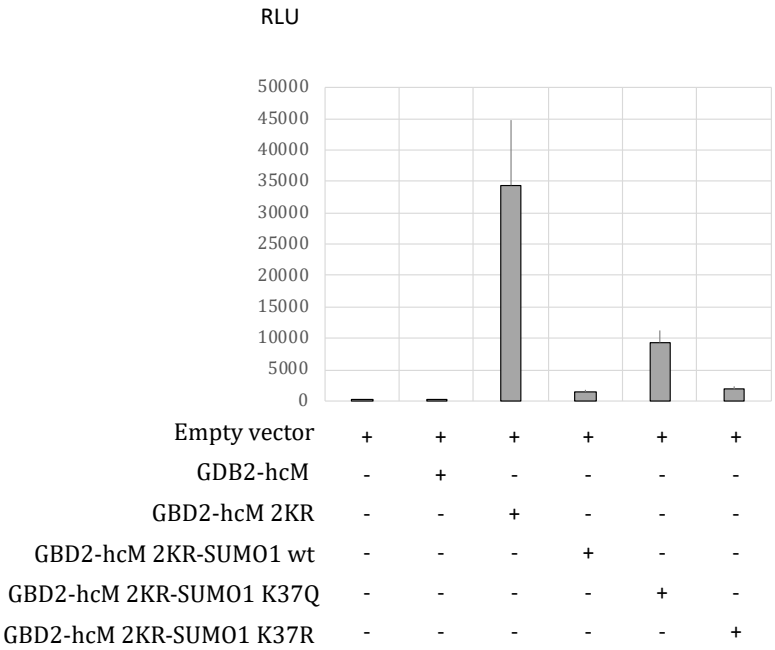


Figure 3.3: **Acetyl mimicking SUMO1 derepresses SUMO-mediated repression and enhances c-Myb activity on a Gal4 promoter in HEK293-c1 cells**. Cells were seeded in a 24 well plate with $0.34x10^5$ HEK293-c1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.4 µg plasmid DNA : varying amounts of empty vector pCIneoB, 0.2 µg pCIneoB-GBD2-hcM-HA [233-640] wt, 0.2 µg pCIneoB-GBD2-hcM 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37Q [233-640] and 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37R [233-640]. 24 hours after transfection, cells were harvested by treatment of 100 µl passive lysis buffer. The luciferase activity was measured using the TD20/20 luminometer from Turner BioSystems. Values are normalized and presented in relative luciferase units (RLU), with the background value set to 1 RLU.

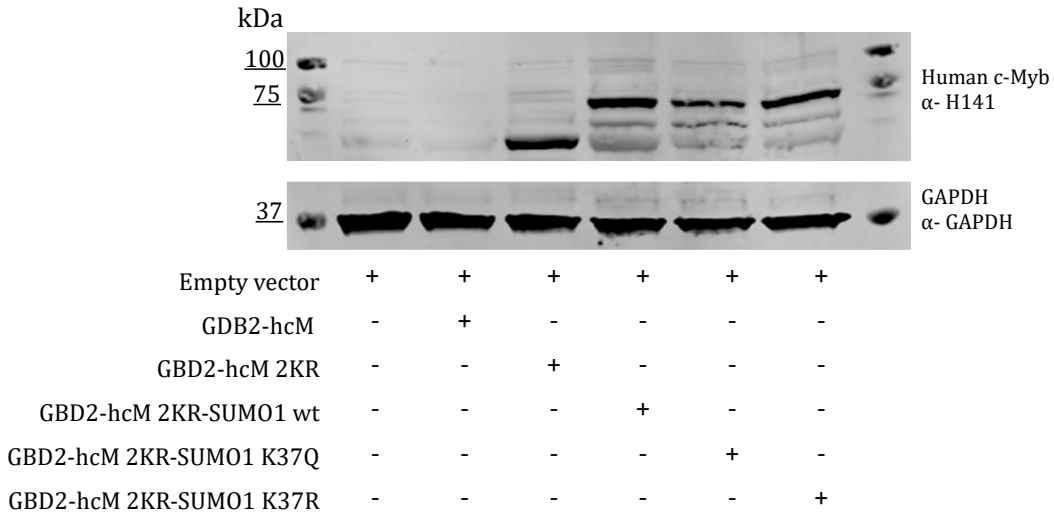


Figure 3.4: **Western analysis of transfected HEK293-c1 cells shows lower protein expression of SUMO1K^{37}Q construct.** Cells were seeded in a 24 well plate with 0.34x10^{5} HEK293-c1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.4 µg plasmid DNA : 0.2 or 0.4 µg empty vector pCIneoB, 0.2 µg pCIneoB-GBD2-hcM-HA [233-640] wt, 0.2 µg pCIneoB-GBD2-hcM-2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37Q [233-640] and 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37R [233-640]. 24 hours after transfection, cells were harvested by treatment of 100 µl 3xSDS GLB, followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:200 α-H141 (rabbit) and 1:4000 α-GAPDH (mouse) overnight. Second incubation with secondary antibodies 1:10 000 IR800(rabbit) and 1:10 000IR680 (mouse) followed by infrared fluorescent signal detection using the Odyssey CLx imaging system from LI-COR® Biosciences.

To clarify the difference in the expression levels of the transfected constructs, we performed a quantification of the protein levels. This was done by measuring the intensity of the western blot signals. The expression of c-Myb constructs was normalized against expression of GAPDH from the same sample. The results are illustrated in figure 3. 5. Here, lower expression of c-Myb-SUMO1 K^{37}Q is clearly recognized when compared to c-Myb 2KR and c-Myb 2KR-SUMO1 wt fusion construct.

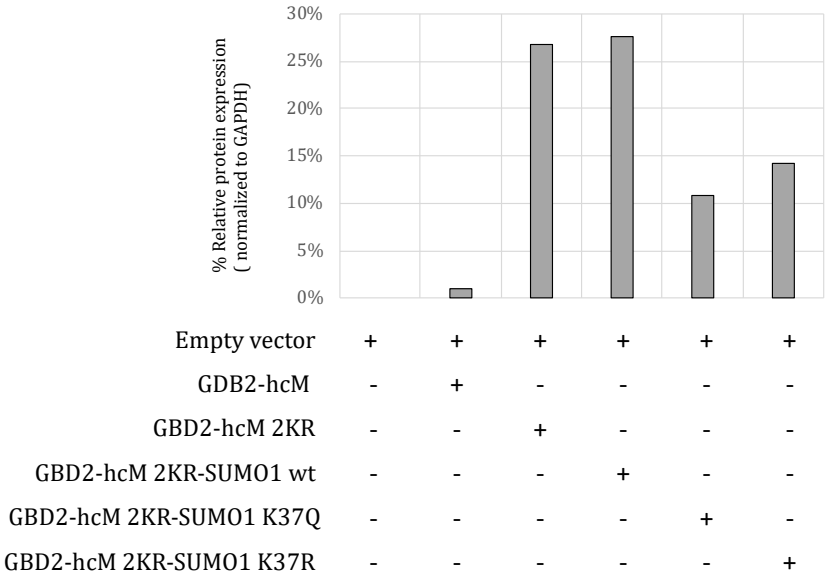


Figure 3.5: **$K^{37}Q$ mutant has lower protein expression compared to $K^{37}R$ and wt SUMO1.**

The intensities of Western blot signals were measured with Image Studio (LI-COR) to quantify protein expression. The expression of c-Myb is normalized against GAPDH expression and presented in a percentage relative.

3.2.2 Does cotransfection with p300 influence the derepression effect?

Based on our observations above and reports that that acetyl transferase p300 has the ability to acetylate c-Myb (Tomita, Towatari et al., 2000) and possibly SUMO (Girdwood, Bumpass et al., 2003), we decided to investigate whether p300 could affect the derepression effect shown in 3.2.1. The same reporter gene assay was set up as in 3.2.1, with the adjustment of adding p300 cotransfection. The results achieved are presented in figure 3.6. Complementary western blot analysis is presented in figure 3.7.

The results in figure 3.6 are similar, if not identical to the results in figure 3.3. The 2KR version of c-Myb has the same high activity as in figure 3.3, due to its inability to conjugate SUMO. The mutated variants of SUMO fused to c-Myb behaved in similar way, with wt SUMO and $K^{37}R$ variant exhibiting a stronger repression effect on c-Myb, compared to $K^{37}Q$. If cotransfection with p300 would have caused higher levels of acetylation in the K37 residue of SUMO1, we should have expected less repressive effect of wt SUMO1 fused to c-Myb in this experiment compared to the previous one. We did not observe such a shift. However, increased levels of p300 may affect many targets and it is not obvious

what the total outcome would be. But this experiment does not give support to the involvement of p300 in acetylation of Lys37 in SUMO1.

Western blot analysis in figure 3.7 presented similar results as in fig 3.4, confirming protein expression of the transfected constructs. The intensity of the bands representing c-Myb-SUMO constructs are weaker compared to the previous western blot (figure 3.4). This is due to a struggle in proper optimalization of concentration of a new antibody. However, here as well, the protein expression of the acetyl mimicking mutant $K^{37}Q$ was lower compared to wt SUMO and the $K^{37}R$ variant. In a similar manner as in 3.2.1, even though levels of the $K^{37}Q$ mutant were lower, its derepression effect was stronger.

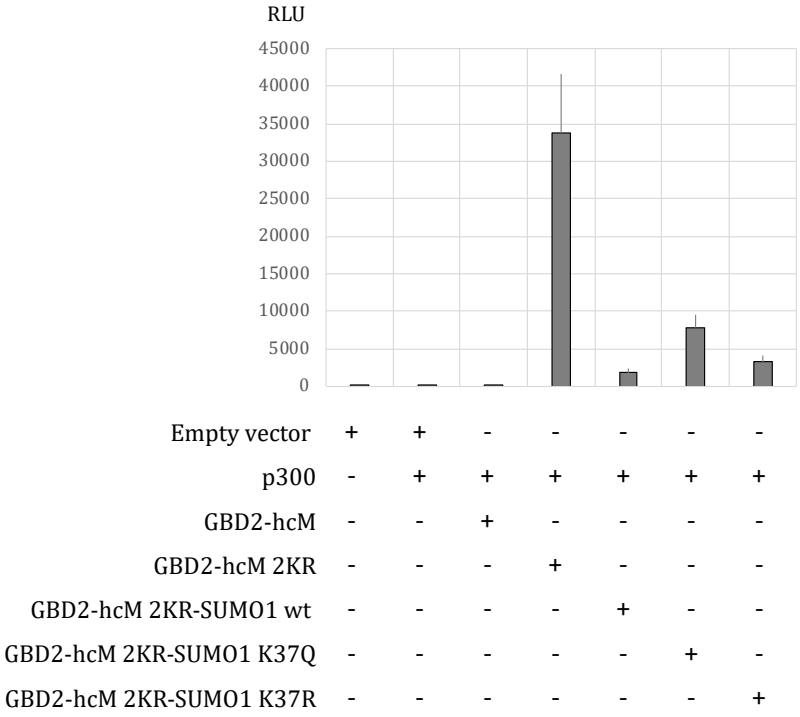


Figure 3.6: **p300 does not influence derepression brought by acetyl mimicking mutant of SUMO1 on c-Myb activity on a Gal4 promoter in HEK293-c1 cells.** Cells were seeded in a 24 well plate with $0.34x10^5$ HEK293-c1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.4 µg plasmid DNA : 0.2 or 0.4 µg empty vector pCIneoB, 0.2 µg pCMVb-NHA-CV5-p300, 0.2 µg pCIneoB-GBD2-hcM-HA [233-640] wt, 0.2 µg pCIneoB-GBD2-hcM 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37Q [233-640]and 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37R [233-640]. 24 hours after transfection, cells were harvested by treatment of 100 µl passive lysis buffer. The luciferase activity was measured using the TD20/20 luminometer from Turner BioSystems. Values are presented in relative luciferase units (RLU), with the background value set to 1 RLU.

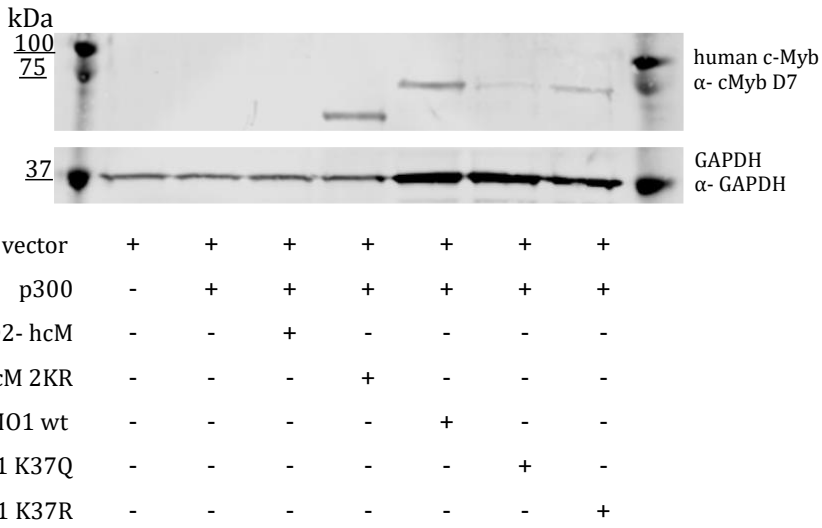


Figure 3.7: **p300 does not influence protein expression in transfected HEK293-c1 cells**. Cells were seeded in a 24 well plate with $0.34x10^5$ HEK293-c1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.4 µg plasmid DNA: 0.2 or 0.4 µg empty vector pCIneo, 0.2 µg pCmvb-NHA-CV5-p300, 0.2 µg pCIneoB-GBD2-hcM-HA [233-640] wt , 0.2 µg pCIneoB-GBD2-hcM 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR [233-640], 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37Q [233-640] and 0.2 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37R [233-640]. 24 hours after transfection, cells were harvested by treatment of 100 µl 3xSDS GLB, followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:200 α-cMyb D7 (mouse) overnight, followed by incubation with secondary antibody 1:10 000 IR800(mouse) for visualization of c-Myb constructs. Subsequently, incubation with primary antibody 1:4000 α-GAPDH (mouse) overnight and secondary antibody 1:10 000 IR680 (mouse) for GAPDH visualization. Infrared fluorescent signal detection was performed using the Odyssey CLx imaging system from LI-COR® Biosciences.

3.2.3 To which extent is the derepression of SUMOylated c-Myb, caused by acetylated SUMO, possible? Can the activity of c-Myb fused to SUMO be 100% restored?

As earlier noted in 3.2.1. and 3.2.2, we observed that expression levels of the $K^{37}Q$ mutant were significantly lower than those of wt SUMO and $K^{37}R$ variant, even when same amounts of construct were transfected. Theoretically, lower expression of the $K^{37}Q$ mutant was expected to lead lower levels of reporter activation. However, we observed higher reporter activation suggesting that the repressive effect of SUMO1 was reduced due to the mutation. Therefore, it was of interest to investigate if the derepression effect could be stronger if the reduced protein level was compensated for. Could the activity of c-Myb fused with SUMO $K^{37}Q$ be 100% restored, given higher or equal expression of the SUMO $K^{37}Q$ variant to the wt SUMO?

To test this hypothesis, a reporter gene assay was set up as in 3.2.1 and 3.2.2 with increasing amounts of the $K^{37}Q$ mutant transfected. The starting point for the acetyl mimicking mutant was equal to that of the wt SUMO construct, increasing to 6-fold at the maximum amount. The results achieved are presented in figure 3.8. Complementary western blot analysis is presented in figure 3.9.

The result in figure 3.8 present the measured reporter activity levels induced by the c-Myb fusion proteins, in combination with different variants of SUMO, as well the effect of increasing amounts of the acetyl mimicking SUMO mutant with $K^{37}Q$. Here, we observed a gradual increase in the reporter activation with the increased amount of $K^{37}Q$ mutant. We concluded that the derepression effect increased with higher input but reached only approx. 40% of the fully derepressed level of 2KR c-Myb, when maximum amount of the construct was added. In a similar fashion to reporter gene assays from 3.2.1 and 3.2.2, the $K^{37}R$ mutant had only a slight derepression effect. Lower values presented in the reporter gene assay in figure 3.8 compared to those in figure 3.3 and 3.6. This is due to lower amount of constructs used in this particular assay compared to the other two. Here, we reduced the amount of co-transfected DNA in half, in order to refrain from introducing too much DNA to the cells.

From the western blot analysis (figure 3.9), we observed a gradual increase in the protein expression levels of the acetyl mimicking mutant $K^{37}Q$, seen by an increase in band intensity. Additionally, band intensity of c-Myb 2KR-SUMO $K^{37}R$ mutant seems to be stronger than that of c-Myb 2KR fused to wt SUMO, even though equal amounts of DNA was co-transfected. Again, band intensities in general are weaker, representing less protein expression, due to a reduction of DNA transfected. Essentially, we concluded that the 6-fold increase in the construct restored 40% of the activity of 2KR c-Myb.

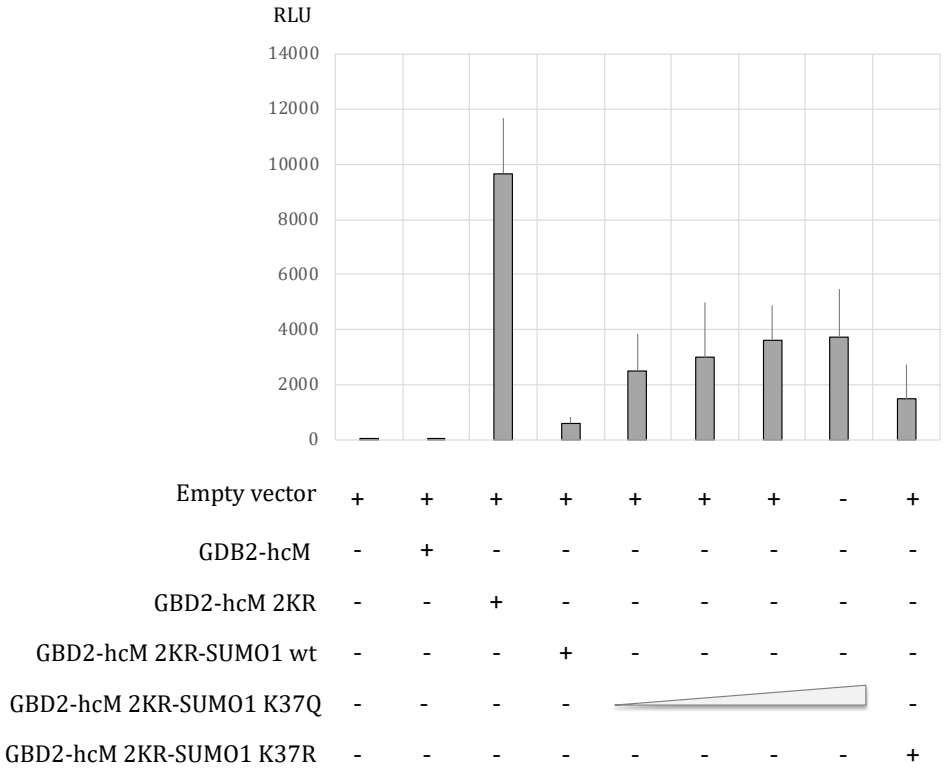


Figure 3.8: **Derepression effect increases with higher input of c-Myb 2KR-SUMO1 K^{37}Q.** Cells were seeded in a 24 well plate with 0.34x10^5 HEK293-c1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.6 µg plasmid DNA: varying amounts of empty vector pCIneo, 0.1 µg pCIneoB-GBD2-hcM-HA [233-640] wt, 0.1 µg pCIneoB-GBD2-hcM 2KR [233-640], 0.1 µg pCIneoB-GBD2-hcM-SUMO1 2KR [233-640], increasing concentrations of 0.1 µg, 0.2 µg, 0.4 µg and 0.6µg of pCIneoB-GBD2-hcM-SUMO1 2KR K37Q [233-640], and 0.1 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37R [233-640]. 24 hours after transfection, cells were harvested by treatment of 100 µl passive lysis buffer. The luciferase activity was measured using the TD20/20 luminometer from Turner BioSystems. Values are presented in relative luciferase units (RLU), with the background value set to 1 RLU.

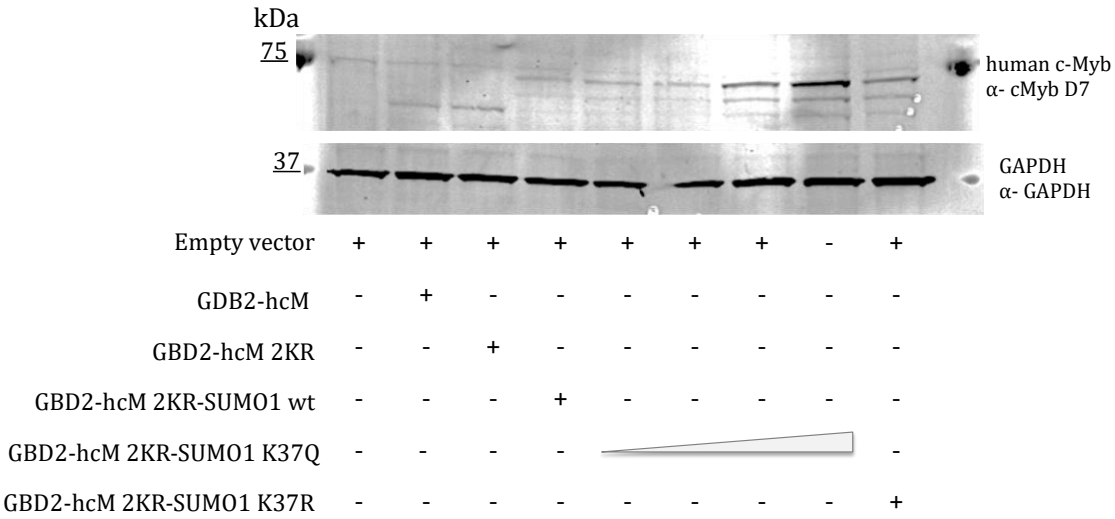


Figure 3.9: **Stronger protein expression with increasing input of c-Myb 2KR SUMO1 $K^{37}Q$ fusion construct.** Cells were seeded in a 24 well plate with $0.34x10^5$ HEK293-c1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.6 µg plasmid DNA: varying amounts of empty vector pCIneo, 0.1 µg pCIneoB-GBD2-hcM-HA [233-640] wt, 0.1 µg pCIneoB-GBD2-hcM 2KR [233-640], 0.1 µg pCIneoB-GBD2-hcM-SUMO1 2KR [233-640], increasing concentrations of 0.1 µg, 0.2 µg, 0.4 µg and 0.6µg of pCIneoB-GBD2-hcM-SUMO1 2KR K37Q [233-640], and 0.1 µg pCIneoB-GBD2-hcM-SUMO1 2KR K37R [233-640]. 24 hours after transfection, cells were harvested by treatment of 100 µl 3xSDS GLB, followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:200 α-cMyb D7 (mouse) overnight, followed by incubation with secondary antibody 1:10 000 IR800(mouse) for visualization of c-Myb constructs. Subsequently, incubation with primary antibody 1:4000 α-GAPDH (mouse) overnight and secondary antibody 1:10 000 IR680 (mouse) for GAPDH visualization. Infrared fluorescent signal detection was performed using the Odyssey CLx imaging system from LI-COR® Biosciences.

3. 3 Is the repressive effect of SUMO and its mutants on the transactivation activity of c-Myb affected by the type of transactivation assay used?

To further analyze if the acetyl mimicking and abolishing mutants of SUMO affected the transactivation activity of c-Myb, we performed a standard reporter assay with transiently transfected effector and reporter constructs for comparison. For this, the CV1 cell line was used, together with a Myb-responsive reporter plasmid, plasmids encoding full length wt and 2KR c-Myb, as well as plasmids for expression of variants of SUMO. The SUMO-constructs were modified at Q94P, enabling conjugation but preventing cleavage and detachment of the SUMO protein. Again, western blot analysis was performed to monitor

changes in steady-states of the proteins. The results are presented in figure 3.10 and 3.11.

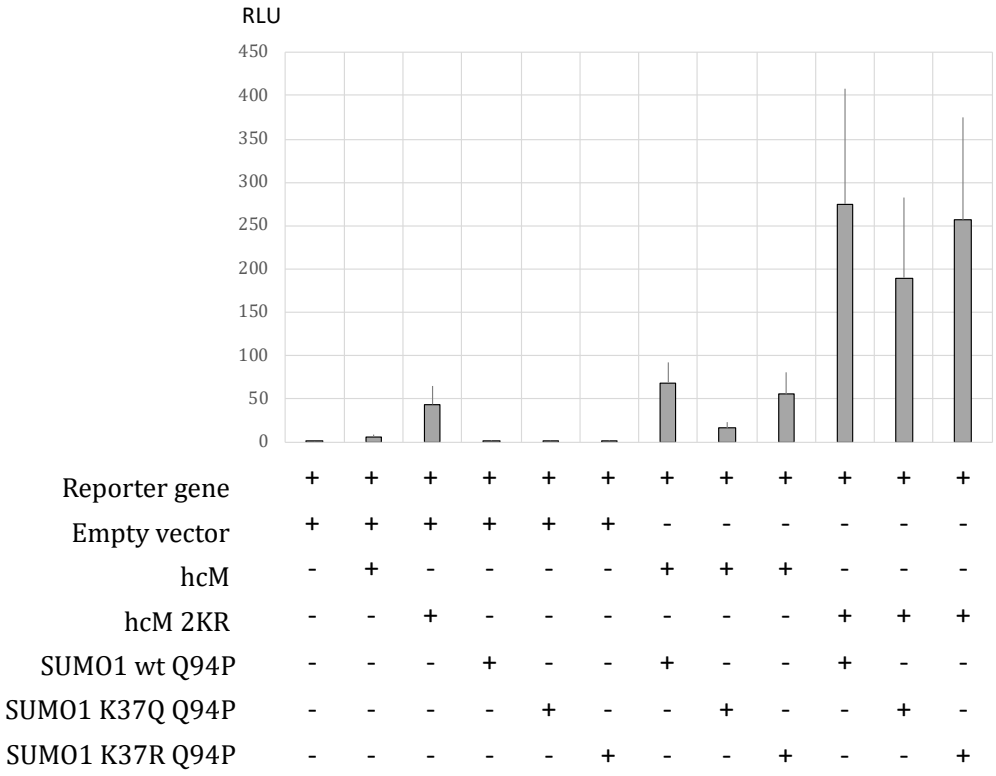


Figure 3.10: **Acetyl mimicking SUMO1 K^{37}Q Q94P strongly represses wt and 2KR c-Myb in transient transfection**. Cells were seeded in a 24 well plate with 0.2x10^5 CV1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.6 µg plasmid DNA, 0.2 µg of each plasmid: reporter gene pGL-4b-3xMRE(GG)-Myc aab, empty vector pCIneo, pCIneo-hcMyb-HA wt, pCIneo-hcMyb 2KR, pCI-Flag-SUMO1-Q94P (GG) wt, pCI-Flag-SUMO1-K37Q-Q94P (GG) and pCI-Flag-SUMO1-K37R-Q94P (GG). 24 hours after transfection, cells were harvested by treatment of 100 µl passive lysis buffer. The luciferase activity was measured using the TD20/20 luminometer from Turner BioSystems. Values are presented in relative luciferase units (RLU).

As expected, we observed first a slight activation with wt c-Myb, which increased after the removal of the SUMO conjugation sites (2KR). Neither of the SUMO constructs caused any activity, as predicted. However, co-transfection with wt SUMO-Q94P and wt c-Myb caused a higher transcriptional activity than that of wt c-Myb alone, which should not have been the case. One would have expected enhanced levels of SUMOylation would have caused increased repression. The same observation was made for c-Myb 2KR, where co-transfection with wt SUMO Q94P increased the transcriptional activity of c-Myb 2KR. Additionally, the results were contradicting the findings from 3.2.1 and 3.2.2. Acetyl mimicking SUMO K^{37}Q Q94P had the strongest repressive effect on transcriptional activity of c-Myb, for both wt and 2KR. In contrast, wt SUMO Q94P had the weakest repression capacity on wt and 2KR c-Myb. The 2KR profile did however clarify the interpretation of these unexpected results. The fact that the 2KR c-Myb, lacking SUMO-conjugation sites, in fact responded significantly to cotransfected SUMO constructs, clearly shows that the changes in reporter

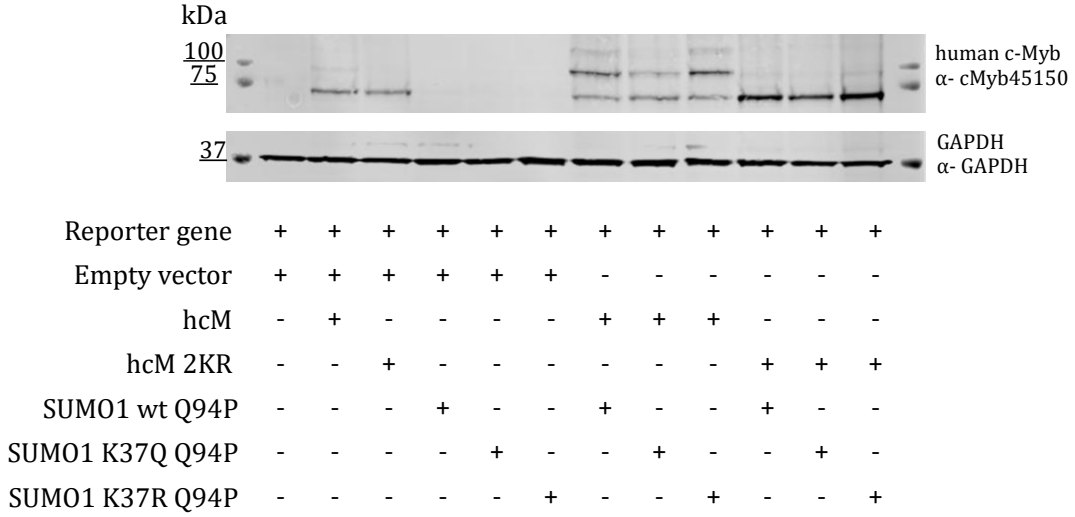


Figure 3.11**: c-Myb 2KR is hihgly stable when co-transfected with wt and mutants of SUMO1 .** Cells were seeded in a 24 well plate with $0.2x10^5$ CV1 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.6 µg plasmid DNA, 0.2 µg of each construct: reporter gene pGL-4b-3xMRE(GG)-Myc aab, empty vector pCIneo, pCIneo-hcMyb-HA wt, pCIneo-hcMyb 2KR, pCI-Flag-SUMO1-Q94P (GG) wt, pCI-Flag-SUMO1-K37Q-Q94P (GG) and pCI-Flag-SUMO1-K37R-Q94P (GG). 24 hours after transfection, cells were harvested by treatment of 100 µl 3xSDS GLB, followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:1000 α-cMyb 45150 (mouse) overnight, followed by incubation with secondary antibodies 1:10 000 IR800(mouse) for visualization of c-Myb constructs. Second incubation with primary antibody 1:4000 α-GAPDH (mouse) overnight, followed by incubation with secondary antibody 1:10 000IR680 (mouse) for GAPDH visualization. Infrared fluorescent signal was detected using the Odyssey CLx imaging system from LI-COR® Biosciences.

activation must be caused by other proteins than c-Myb. Probably, the cotransfected SUMO constructs have led to enhanced SUMOylation of some players in the transcription apparatus, causing these effects. But this also means that we in this assay are not measuring only effects on c-Myb, but the assay is confounded by other SUMO-targets, making it impossible to conclude from.

Western blot analysis (figure 3.11) revealed that wt c-Myb is equally stable as the 2KR variant, when transfected with the reporter gene only. Furthermore, co-transfection of wt c-Myb with SUMO variants displayed 3 bands, two of which are above the 75 kDa mark. These most likely represent mono and di-SUMO attachment at the K503 and K27 residues, showing that the Q94P mutant in fact caused enhanced levels of SUMOylation. In combination with SUMO variants, c-Myb 2KR is highly stable, compared to wt c-Myb. Based on these findings, we concluded that this reporter gene assay was not conclusive and not suitable to properly investigate SUMO- mediated repression of c-Myb.

3.4 Will the acetylation mutants of SUMO affect their repressive effect in a non-Myb context?

In section 3.2 we used activation reporter gene assays in order to observe the changes in activity levels of c-Myb fused to variants of SUMO. Further on, we were interested to explore the repressive effect of SUMO and its mutants in a non-Myb context. For this purpose, a repression reporter gene assay was set up in HEK293-c4 cells. This cell line is similar to the HEK 293-c1 cell line used in 3.2, with 5xGal4 responsive elements upstream of the luciferase gene. Unlike the c1 cell line, the basal activity of c4 cells is higher, making the cell line suitable for use in monitoring repression of the reporter. For this experiment, a range of GBD-SUMO2 constructs were used. It should be noted that we in this setup switched from SUMO1 to SUMO2 since we had available from Stefan Müllers lab a series of Gal-SUMO2 fusion constructs. Because the activity of GBD2 was low, we performed a second reporter gene assay with the addition of GBD2-TAD, expressing a Gal4 DBD fused to the central transactivation domain of c-Myb, to better investigate the suppression effect SUMO and its variants displayed. Results of the suppression luciferase assay are presented in figure 3.12, with complementary western blot analysis in figure 3.13.

Presented in A from figure 3.12, a visible, but not tremendous activity was observed with GBD2. Co-transfection of GBD2 with SUMO 2 variants repressed this activity to under the background values of the reporter gene alone. However, the difference between the repressive effect of wt SUMO 2 and mutants was not easy to determine. Addition of GBD2-TAD increased reporter gene activity significantly, up to 12-fold (B from figure 3.12). The repressive effects of SUMO and its variants was easier to distinguish and showed a repeat of the same trend observed in 3.2.1 and 3.2.2. Acetyl mimicking SUMO2 $K^{33}Q$ mutant had the weakest repressive capacity, while wt and $K^{33}R$ mutant of SUMO 2 shared almost the same repressive effect.

Western blot analysis (figure 3.13) displays strong and even bands for SUMO 2-Gal4 constructs, at approx. 30 kDa (under the GAPDH bands at 37 kDa). GBD2 construct alone is also present at approx. 20 kDa. In figure B, we observed weak but present bands under the SUMO 2-Gal4 constructs, slightly above 20 kDa. We suggest this may be remnants of GBD2-TAD. In contrast to western blot analysis for HEK293-c1 assays, where lower protein expression for the acetyl mimicking mutant of SUMO was observed, expression of the SUMO 2 $K^{33}Q$ seems to be equal to wt and $K^{33}R$ SUMO 2. From these findings, we concluded that the defined acetylation switch could indeed be implemented in a non c-Myb context and may perhaps be used for other proteins as well.

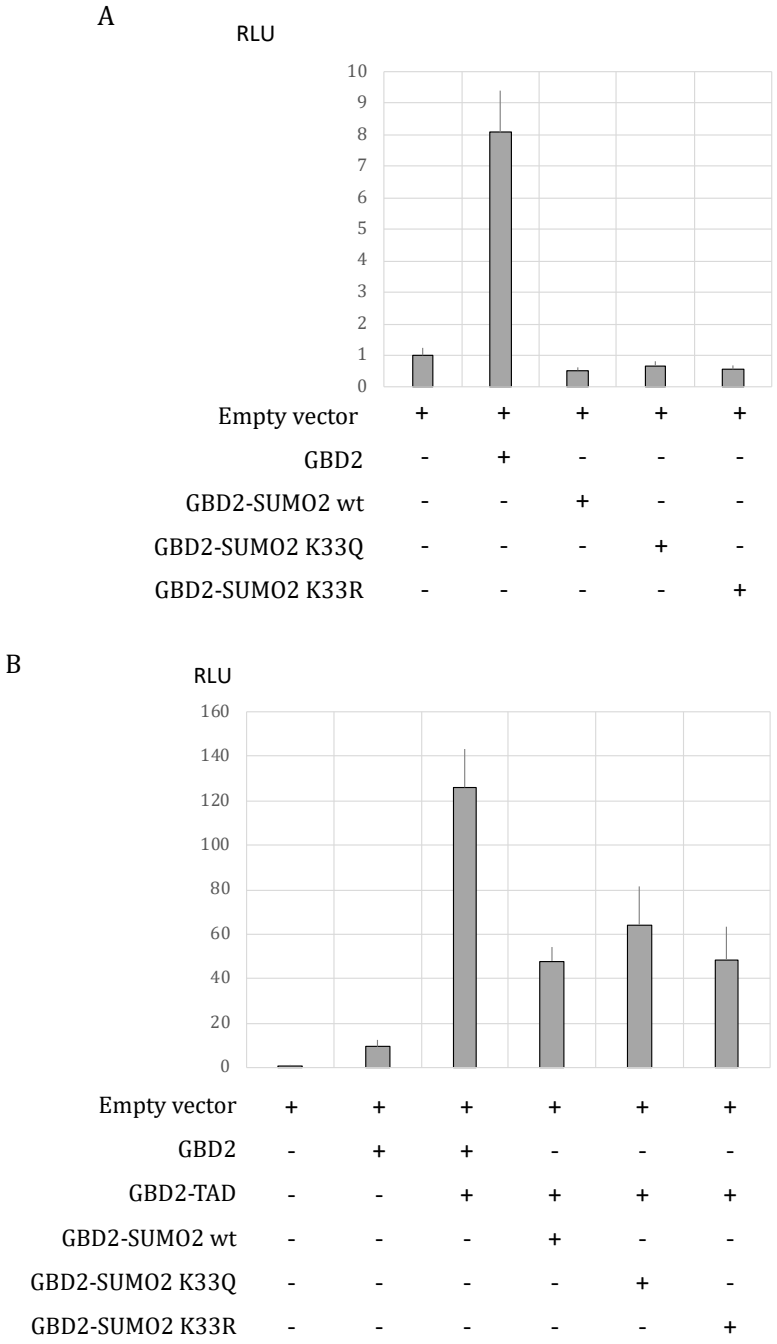
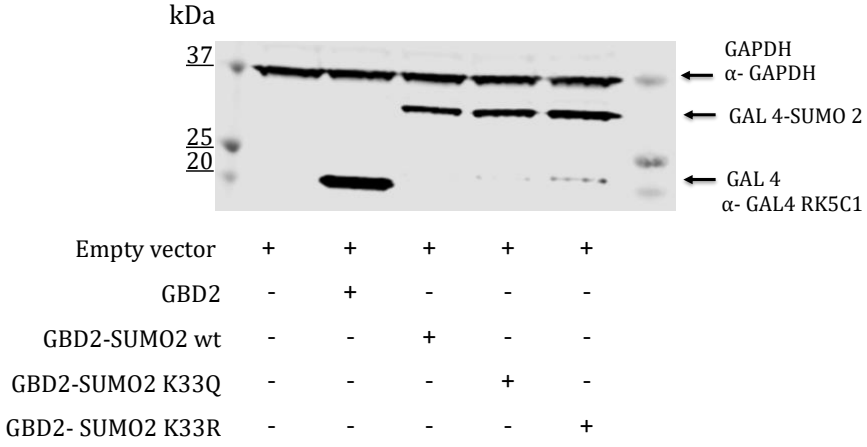


Figure 3.12: **Acetyl mimicking mutant of SUMO2 K^{33}Q represses GBD2 on a Gal4 promoter in HEK293-c4 cells.** Cells were seeded in a 24 well plate with 0.34x10^5 HEK293-c4 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.4 µg plasmid DNA. **A:** 0.2 or 0.4 µg empty vector pCIneo, 0.2 µg pCIneo-GBD2, 0.2 µg pGal4-SUMO2 wt, 0.2 µg of pGal4-SUMO2 K33Q and 0.2 µg pGal4-SUMO2 K33R. **B:** varying amounts of empty vector pCIneo, 0.2 µg pCIneo-GBD2, 0.05 µg pCIneo-GBD2-TAD, 0.2 µg pGal4-SUMO2 wt, 0.2 µg of pGal4-SUMO2 K33Q and 0.2 µg pGal4-SUMO2 K33R. 24 hours after transfection, cells were harvested by treatment of 100 µl passive lysis buffer. The luciferase activity was measured using the TD20/20 luminometer from Turner BioSystems. Values are presented in relative luciferase units (RLU), with background value set to 1 RLU.

A



B

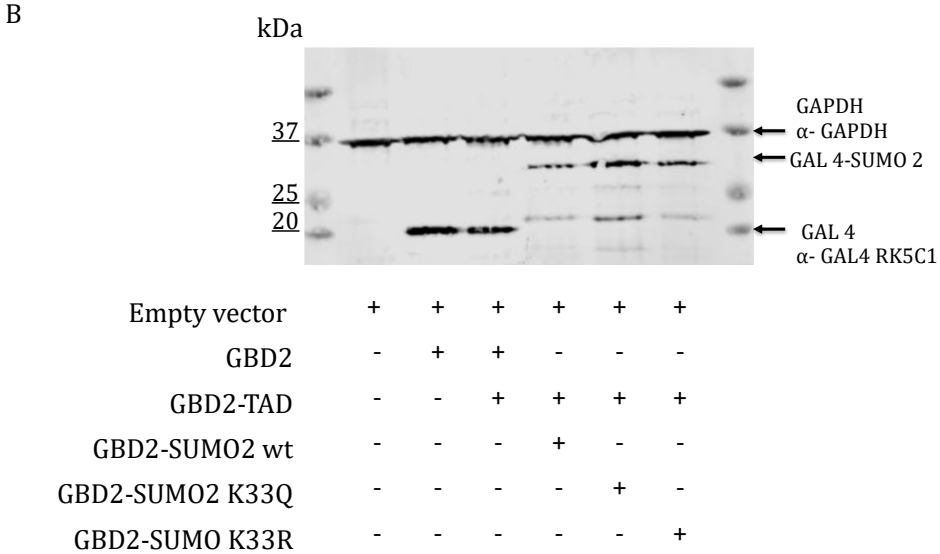


Figure 3.13: **Even protein expression of Gal-SUMO2 constructs in transfected HEK293-c4 cells.** Cells were seeded in a 24 well plate with $0.34x10^5$ HEK293-c4 cells in 0.5 ml medium 24 hours prior to transfection. Transfection was performed in triplicates with total input of 0.4 µg plasmid DNA. **A:** 0.2 or 0.4 µg empty vector pCIneo, 0.2 µg pCIneoB-GBD2, 0.2 µg pGal4-SUMO2 wt, 0.2 µg pGal4-SUMO2 K33Q, and 0.2 µg pGal4-SUMO2 K33R. **B:** varying amounts of empty vector pCIneo, 0.2 µg pCIneo-GBD2, 0.05 µg pCIneo-GBD2-TAD, 0.2 µg pGal4-SUMO2 wt, 0.2 µg of pGal4-SUMO2 K33Q and 0.2 µg pGal4-SUMO2 K33R. 24 hours after transfection, cells were harvested by treatment of 100 µl 3xSDS GLB, followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:1000 α-Gal4 RK5C1 (mouse) overnight, followed by incubation with secondary antibody 1:10 000 IR800 (mouse) for Gal4-SUMO2 visualization. After validation of Gal4-SUMO2, the membrane was again incubated with primary antibody 1:4000 α-GAPDH (mouse) overnight, followed by secondary antibody 1:10 000IR680 (mouse). Infrared fluorescent signal was detected using the Odyssey CLx imaging system from LI-COR® Biosciences.

3.5 Is SUMO acetylated by p300? If so, which residues are subject to this modification?

The study in Stefan Müllers lab referred to several residues of SUMO being subject to acetylation. Amongst these are $K^{37, 39, 46}$ in SUMO1 and $K^{33, 35, 42}$ in SUMO2. Therefore, it was of interest to us to confirm these acetylation events and perhaps identify other residues subject to the modification. For this acetylation experiment, we set up an *in vitro* acetylation assay. Purified recombinant hSUMO1 protein together with recombinant acetyl transferase p300 provided by Active Motif, and acetyl-CoA, was incubated and treated as described in section 2.4.6. Furthermore, the samples were sent to mass spectrometry (MS) and western blot analysis was performed in order to confirm acetylation of the protein.

Unfortunately, we were not able to generate reproducible data from this experiment. Replication of the experiment produced confounding results, with distinct residues modified at each of the separate occasions. Thus, we were not able to confirm whether acetylation of the same residues takes place every time. As the experiment was repeated several times, staining of the polyacrylamide gel after SDS-PAGE was performed. From figure 3.14, we validated the presence of hSUMO1 alone, and together with p300 (10kDa), prior to isolating gel pieces for MS. Western analysis (figure 3.15) displayed presence of acetylated hSUMO1 protein, confirming that the acetylation experiment was partly successful. From the results, we concluded that further optimalization was required in order to

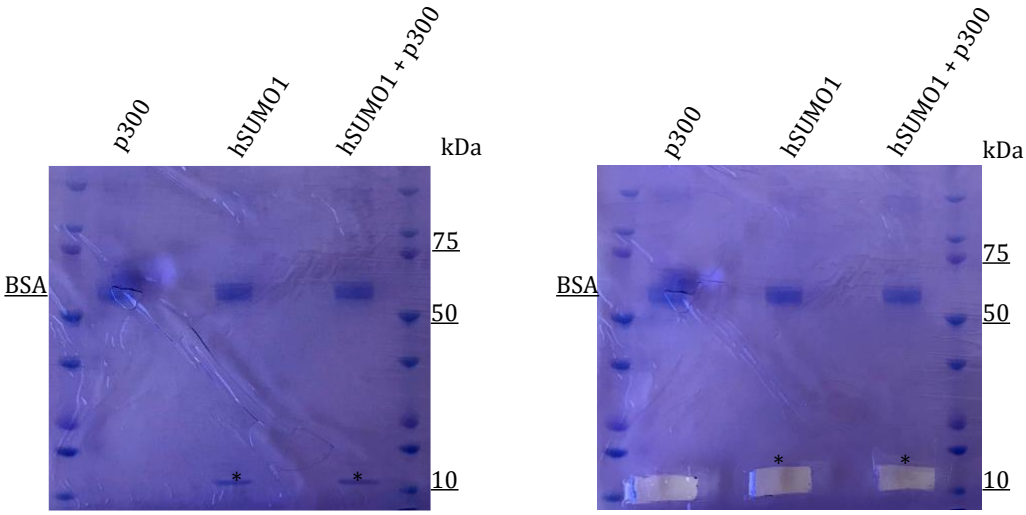


Figure 3.14: **Acetylation of hSUMO1 by p300.** Acetylation reaction with 0.5µg E.coli expressed hSUMO1, 0.5 µg recombinant p300 enzyme and Acetyl-CoA. The reaction was stopped by addition of 15 µL 3xSDS GLB followed by protein separation in a 4-12% gradient Bis-Tris gel for 1 hour at 170V. Staining of gel was performed using Coomassie Blue Staining solution for 1 hour to visualize bands of the hSUMO1 protein at 10 kDa. Additional bands at approx. 66kDa are present and correspond to BSA found in buffer solution. Gel samples were cut out and transferred to microcentrifuge tubes for MS analysis.

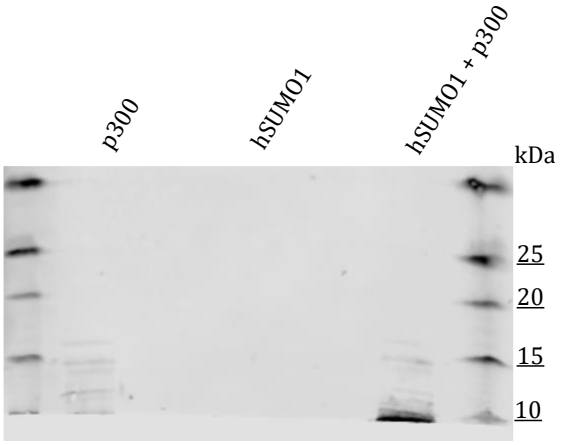


Figure 3.15: **Acetylation of hSUMO1 by p300.** Acetylation reaction with 0.5µg E.coli expressed hSUMO1 and 0.5 µg recombinant p300 enzyme input. The reaction was stopped by addition of 15 µL 3xSDS GLB followed by protein separation in a 4-12% gradient Bis-Tris gel for 1 hour at 170V. Western blotting analysis was performed by incubating the membrane with primary antibody 1:500 panAcetyl (C2) (rabbit) overnight. Second incubation with secondary antibodies 1:10 000 IR800(rabbit) was performed, followed by infrared fluorescent signal detection using the Odyssey CLx imaging system from LI-COR® Biosciences.

obtain desirable results. Due to time constraints, these experiments were not followed further.

3.6 How does acetylation of SUMO affect binding of interaction partners? Does acetylation of SUMO regulate SUMO-SIM binding selectively?

The acetylation switch defined by Stefan Müllers lab implied a negative effect by acetylation of SUMO on SUMO-SIM interactions, thus affecting protein-protein interactions. This disruption was also shown to be selective and only affected binding to specific proteins. Amongst several protein interaction networks, they reported abrogation of SUMO binding to PIAS family members. Here we sought to explore if we, in a similar manner, could confirm and extend these results. Therefore, in addition to PIAS1, the experiment was repeated with the CHD3 protein, to explore whether binding to SUMO would be affected by acetylation mimicking mutants. To achieve this, a coimmunoprecipitation assay was set up for both PIAS1 and CHD3. Again, wt SUMO2 as well as mutant variants of SUMO2 were used. The separate experiments are further discussed in the next sections.

3.6.1 Validation of protein interaction between SUMO2 and PIAS1 and effects of acetylation mutants of SUMO2

The coimmunoprecipitation experiment was set up with COS1 cells. Flag-tagged Pias1 was used together with variants of Gal-SUMO2 constructs. For the experimental set up, 5% of the sample was used as input, and the remaining 95% was incubated with anti-flag tagged magnetic beads that would bind to the flag-tagged Pias1 protein. Thus, in theory, the magnetic beads should remove the Pias1-SUMO2 protein complex from the solution as the proteins bind to each other through SUMO-SIM interactions. Use of the acetyl mimicking variant of SUMO2 should inhibit some of this interaction, according to findings in Stefan Müllers lab. To confirm protein expression, western blot analysis was performed. Antibodies against Flag and GBD2 were used to identify Pias1 and SUMO2. The results as presented in figure 3.16.

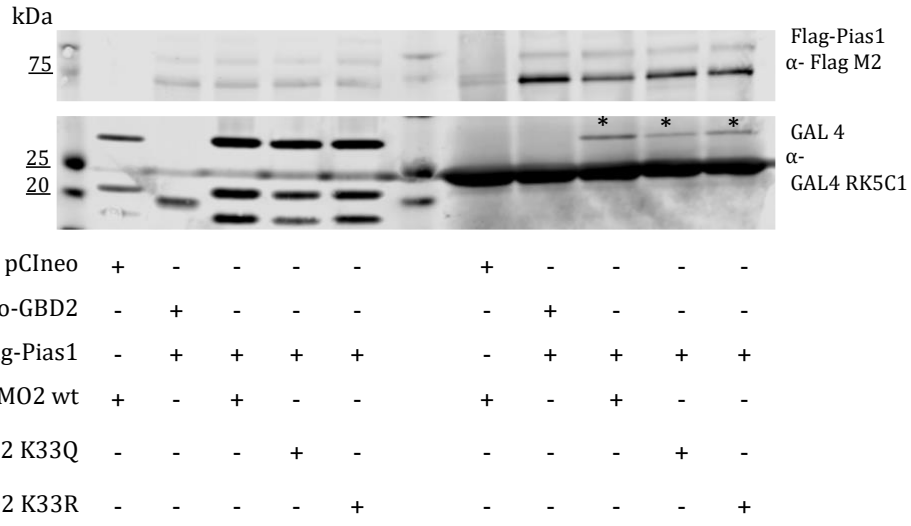


Figure 3.16: **Western blot analysis of coimmunoprecipitation in COS1 cells displays a weaker protein interaction between SUMO2 $K^{33}Q$ and PIAS1.** Cells were seeded in 10cm plates with $1*10^6$ COS1 cells in 10 ml medium, 24 hours prior to transfection. Transfection was performed with total amount of 5 µg DNA with following plasmids: 2,5 µg 3xFLag-Pias1, 2,5 µg pCIneo, 2,5 µg pCIneo-GBD2, 2,5 µg pGal4-SUMO2 wt, 2,5 µg pGal4-SUMO K33Q and 2,5 µg pGal4-SUMO K33R. 24 hours after transfection, cells were harvested by treatment with Kac lysis buffer. Coimmunoprecipitation was performed by incubation of the samples with magnetic flagbeads for 1,5 hours at 4^0C. All samples were added 3xSDS GLB followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:1000 α-Gal4 RK5C1 (mouse) overnight and secondary antibody 1:10 000 IR800 (mouse) for Gal4-SUMO2 validation. This was followed up by a second staining with primary antibody 1:10 000 α-Flag M2 (mouse) overnight and secondary antibody 1:10 000IR680 (mouse) for validation of Flag-PIAS1. Infrared fluorescent signal was detected using the Odyssey CLx imaging system from LI-COR® Biosciences.

From figure 3.16, we successfully confirm the interaction between the Pias1 and SUMO2 protein. This is presented by the star markings (*). Bands at 72 kDa confirm presence of PIAS1, in both input and output, indicating an equal amount of protein in the sample. Staining with Gal-antibody confirmed presence of Gal4-SUMO2 constructs, with strong expression in input and weaker in the output, as expected. This is due to the fact that PIAS1 would pull SUMO2 through protein interaction. Further on, the results show that interaction between Pias1 and the acetyl mimicking $K^{33}Q$ SUMO2 is weaker, compared to the wt SUMO as well as the wt mimicking $K^{33}R$ SUMO2 variant. The strong bands at approx. 20 kDa are believed to be Gal-constructs with cleaved SUMO2. Results from Stefan Mullers lab showed total abrogation of interaction between acetyl mimicking SUMO2 $K^{33}Q$ and PIAS2, whereas we observed partial abrogation. Nevertheless, our observed results are in somewhat accordance with their reported findings (Ullmann, Chien et al., 2012).

3.6.2 Validation of protein interaction between SUMO2 and CHD3 and effects of acetylation mutants of SUMO2

The same set up as described in 3.6.1 was used for the following coimmunoprecipitation investigating the CHD3-SUMO2 interaction. Our lab has unpublished results investigating and mapping the interaction between the chromatin remodeler CHD3 and both SUMO1 and SUMO2. Again, flag-tagged CHD3 protein was used with the different variants of SUMO2 constructs. Western blot analysis was performed to confirm presence of the CHD3 proteins, as well as GBD-SUMO2 proteins. Results are presented in figure 3.17.

In similar manner as to previous results, successful validation between CHD3 and SUMO2 variant was implemented. Strong bands at 250 kDa represent the CHD3 protein and slightly stronger bands are observed in mutant variants of SUMO2 ($K^{33}Q$ and $K^{33}R$) compared to wt SUMO2. Again, what we assume are Gal-constructs with cleaved off SUMO2 are present at approx. 20 kDa in the input. Gal-SUMO2 constructs at approx. 30 kDa are present, confirming successful transfection. Bands marked with stars (*) indicate the interaction of CHD3 and SUMO2 variants through SUMO-SIM. In contrast to the results in figure 3.16, we observed that the interaction between CHD3 and the acetyl mimicking SUMO2 $K^{33}Q$ was not influenced. A weaker band is displayed for the wt SUMO2 and CHD3 interaction, but this may be a consequence to the weaker band of CHD3 at 250 kDa, indicating lower protein quantity compared to other samples. From these results, we concluded that the interaction between CHD3 and SUMO2 $K^{33}Q$ as well as $K^{33}R$ is not impaired, compared to the wt SUMO2.

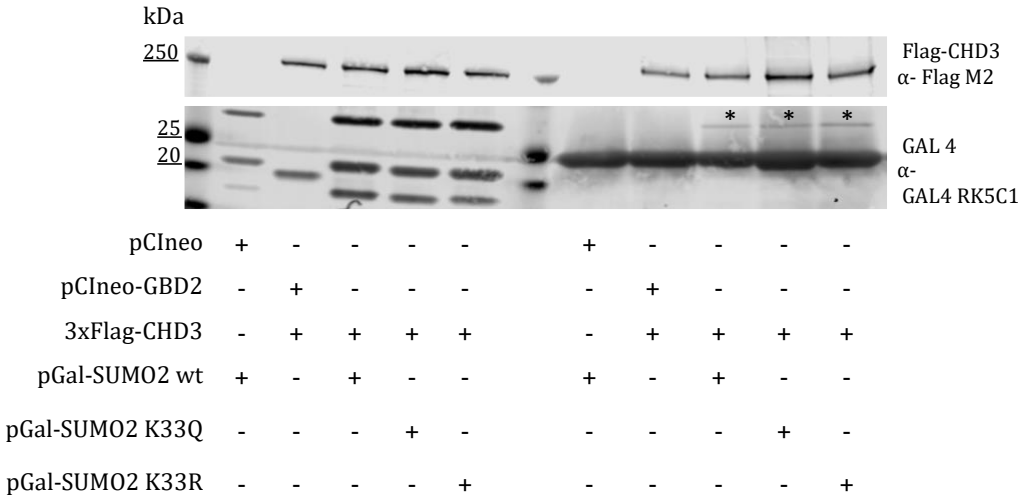


Figure 3.17: **Western blot analysis of coimmunoprecipitation in COS1 cells displays an even protein interaction between wt and mutants of SUMO2 and CHD3.** Cells were seeded in 10cm plates with $1*10^6$ COS1 cells in 10 ml medium, 24 hours prior to transfection. Transfection was performed with total amount of 5 µg DNA with following plasmids: 2,5 µg 3xFLag-CHD3, 2,5 µg pCIneo, 2,5 µg pCIneo-GBD2, 2,5 µg pGal4-SUMO2 wt, 2,5 µg pGal4-SUMO K33Q and 2,5 µg pGal4-SUMO K33R. 24 hours after transfection, cells were harvested by treatment with KAc lysis buffer. Coimmunoprecipitation was performed by incubation of the samples with magnetic flagbeads for 1,5 hours at 4^0C. All samples were added 3xSDS GLB followed by SDS-PAGE electrophoresis for protein separation. Western blotting analysis was performed by incubating the membrane with primary antibody 1:1000 α-Gal4 RK5C1 (mouse) overnight and secondary antibody 1:10 000 IR800 (mouse) for Gal4-SUMO2 validation. This was followed up by a second staining with primary antibody 1:10 000 α-Flag M2 (mouse) overnight and secondary antibody 1:10 000IR680 (mouse) for validation of Flag-CHD3. Infrared fluorescent signal was detected using the Odyssey CLx imaging system from LI-COR® Biosciences.

4 Discussion

The background for this thesis was a study published in 2012 by Stefan Müller, where an acetylation switch on SUMO was identified and established as a mechanism to selectively regulate SUMO-SIM interactions. Thus, the main goal in this study has been to investigate whether the defined acetylation switch in SUMO affected the transcription factor c-Myb in any way. Further on, several sub questions regarding the interaction between SUMO and c-Myb activity have been explored. Additionally, we have investigated how SUMO-SIM interactions are selectively affected by acetylation mimicking mutants. In this chapter, the results from the previous chapter are discussed in more detail.

4.1 Novel findings in interaction and functional assays

We performed several reporter gene assays in order to establish how acetylation mimicking and abolishing mutants of SUMO influenced the transactivation of c-Myb in comparison to wt SUMO. Using several types of cells in combination with activation and repression assays allowed us to generate independent results of similar origin. By obtaining similar data from different experiments, we were able to confirm and strengthen our findings.

4.1.1 c-Myb activity is affected by acetyl mimicking and abolishing mutants of SUMO 1

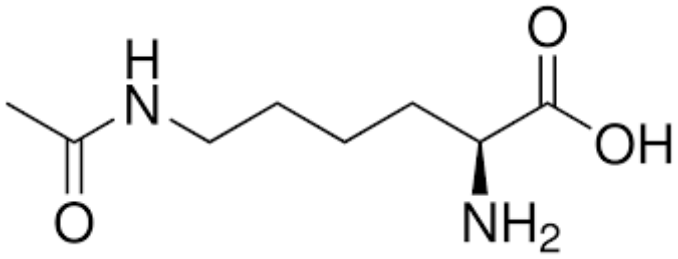
In section 3.2.1, the transactivation activity of c-Myb was examined after being fused to wt, acetyl mimicking or acetyl abolishing mutants of SUMO1. Here, we observed a derepression effect brought forth by the acetyl mimicking mutant. However, the acetyl abolishing mutant of SUMO1 showed no significant derepression ability and was repressing the activity of c-Myb to a similar degree as the wt SUMO1. From the data acquired by western blot analysis (figure 3.4), we observed a difference in band intensities of SUMO1 mutants compared to the wild type. Both mutants of SUMO1 had lower protein expression levels compared to wt SUMO.

The findings of the activity assay raise a series of questions. How is the acetyl mimicking mutant of SUMO1 able to induce an increase in the activity of c-Myb? Why is the acetyl abolishing mutant incapable of producing the same effect? And what could be the reason behind the reduced protein quantity in the mutant versions of SUMO1?

A possible explanation behind the observed effect is that the repression induced by a fused SUMO moiety causes recruitment of some SIM-containing factors with repressive effect. From Stefan Müller lab, we know that SUMO1^{K37Ac} (and SUMO2^{K33Ac}) weakens SUMO-SIM interaction, thus it is not surprising that K^{37}Q variant of SUMO1 alleviates SUMO-mediated repression on the transactivation activity of c-Myb. The same is not true for the K37R mutant.

The use of acetylation mimicking and abolishing mutants deserves a comment. The lysine residue has a long side chain containing four methylene groups and an amino group. The residue also harbours a positive charge and is classified as aliphatic and basic amino acid. In the same sub group of amino acids, arginine is similar in structure, with a side chain of three aliphatic carbons ending in a guanidine group. Thus, the two amino acids are typically thought to be similar in 3D structure and charge. The difference between them is that only lysine becomes acetylated. Acetylation of lysine neutralizes the positive charge of the residue. Addition of the acetyl group also lengthens the tail of the residue and, thus changing its structure in space (Kamieniarz & Schneider, 2009). Glutamine, a polar but neutral amino acid, has a structure that is resembling the one of acetylated lysine, as illustrated below in figure 4.1. Thus, we believe that in this case, the acetyl mimicking mutant of SUMO with glutamine substituting lysine, may be the closest resemblance to acetylated SUMO in 3D structure. Respectively, the mutant variant of SUMO with arginine substituting lysine, acts as the closest match to the wild type SUMO but where the possibility of acetylation is abolished.

A



B

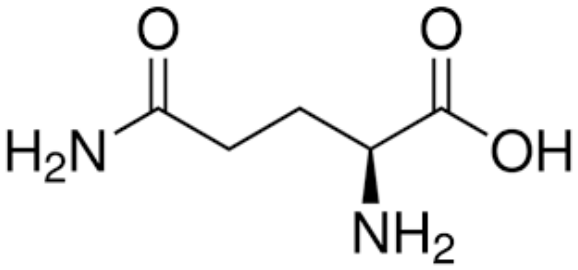


Figure 4.1: Illustration of **A:** Acetylated lysine residue **B:** Glutamine residue

We noticed that these mutations had an effect on the steady-state level of the fusion-proteins. Despite an even GAPDH expression, quantification of protein expression of c-Myb with mutants of SUMO1 revealed reduced expression compared to c-Myb with wt SUMO1 (figure 3.4 and 3.5). How and why this phenomenon was observed in several experimental reporter gene assays is still unknown. That SUMO already in its normal conjugated position affects the stability of c-Myb is seen from the observation that the 2 KR mutation of c-Myb showed significantly increased stability of the protein. Apparently, SUMO conjugation contributes to the stability of c-Myb, promoting its rapid degradation. If this is the case, it may explain the weak expression of wt c-Myb. However, fusion of wt SUMO1 to the SUMO-free 2KR c-Myb does not destabilize

the protein similarly. Apparently, the mutant SUMO1 version however had some destabilizing effect.

4.1.2 p300 does not have a significant effect on the derepression effect brought by SUMO mutants

The p300 protein is a known interactor of both c-Myb and SUMO. The protein can act both as a enhancer and repressor of transcription, often linked to transcriptional activation of c-Myb by recruiting it to potential target sites (Pattabiraman, McGirr et al., 2014). p300 additionally harbours two SUMO consensus sequences in its CRD, capable of SUMO binding both *in vivo* and *in vitro* (Girdwood, Bumpass et al., 2003). It is also suggested that p300 may influence the derepression effect by acetylating SUMO, weakening SUMO-SIM interaction.

In our experiments, it was of interest to explore whether the acetyltransferase activity of p300 could enhance or inhibit the derepression effect established in section 3.2.1. Therefore, we performed a corresponding activation reporter gene assay supplemented with p300. A small to insignificant change in the activity of c-Myb with different mutants of SUMO was observed, as shown in figure 3.6. Otherwise, the result followed the same trend as findings from 3.2.1. We concluded that p300 did not have any effect on the derepression brought by acetyl mimicking mutant of SUMO1.

We predicted that p300 would not be able to have any effect on SUMO1 mutants ($K^{37}Q$ and $K^{37}R$) as these residues are not subjects to acetylation. However, it may be capable of exerting its function in wt SUMO1. In saying that, we did not observe a difference in the activity levels of c-Myb fused to wt SUMO1 in this assay compared to the previous (without p300). A reason for this could be due to the unnatural fusion of SUMO1 to c-Myb, affecting of preventing SUMO acetylation.

Recent findings have led to believe that c-Myb also acts as a potential recruiter of p300 to regions of closed chromatin and that this effect is tripled with the use of SUMO-negative mutant (2 KR) (Ledsaak, Bengtsen et al., 2016). However, they also show that PIAS1 acts as a corepressor of activated c-Myb (2KR mutant) and inhibits recruitment of p300 to chromatin-bound c-Myb. Thus, this may have been a possible mechanism of interference in this particular experiment.

4.1.3 Acetyl mimicking mutant of SUMO does not fully derepress c-Myb transactivation activity

Observed findings from section 3.2.1 and 3.2.2 repeatedly exhibited a reduced protein expression of SUMO1 mutants in the reporter gene assays. Despite lower expression, significant derepression due to the acetyl mimicking mutant of SUMO1 was observed. Therefore, we speculated whether the derepression effect

would increase, given higher protein levels of SUMO1 mutants. We therefore performed a reporter gene assay with increasing quantities of the $K^{37}Q$ mutant of SUMO1. The results (figure 3.8) showed a correlation between $K^{37}Q$ levels and c-Myb activity. Higher levels of acetyl mimicking mutant led to higher levels of reporter activation, confirming an increase in derepression. However, the hypothesis of 100% derepression, if the proteins level of wt and mutant were equal, was not validated, as activity levels of c-Myb only increased from approx. 20% to 40%, compared to activity of the c-Myb 2KR.

Several explanations may be put forward as to why derepression of c-Myb was less extreme than assumed. One of them may be as plain as the fact the acetyl-mimicking mutant of SUMO is in fact, just acetyl mimicking and not acetylated SUMO. It could be possible that the same experiment performed with SUMO1 $K37^{Ac}$ would lead to a 100% derepression of the transcription factor, leading only to a slight change in the derepression effect with use of the acetyl mimicking variant. It could also be that full derepression would require more than the K37 residue to be acetylated.

In this setup, SUMO is fused C-terminally to c-Myb. It is however unclear how SUMO and mutants exerts its function on c-Myb. We speculate that this may be through a repressive complex dependent on SUMO-SIM interactions. If interaction between SUMO and a putative repressor is somehow SUMO-SIM mediated, other residues in the SUMO-SIM interface may regulate the strength of SUMO binding. Ullmann *et. al* reported identification of additional acetylation sites on SUMO paralogs within the basic SIM-binding interface, such as $K^{37/39/46}$ of SUMO1 or $K^{32/35/42}$ on SUMO2. It may be possible that these contribute to interactions between SUMO and a putative repressor, hindering a full derepression of c-Myb activity.

Lastly, we speculate that 100% derepression is not achievable due to the unnatural set up of the system, where SUMO is fused to c-Myb that is mutated (2KR) in its natural conjugation sites. Another speculation may be that the natural pool of wt SUMO in the cells may be capable of binding to c-Myb in a SUMO-SIM manner or in a complex and repress some of its activity.

4.1.4 The repressive effect of SUMO on c-Myb is influenced by transactivation assay

The previous experiments were performed in the HEK293-c1 cell line, with a fully chromatinized reporter. To further evaluate the derepression effect mediated by the acetyl- mimicking SUMO1 mutant, we performed an activation reporter assays in another cell line; CV1. Specifically, it was of interest to assess whether there would be a difference in the derepression effect with a classical transient transfection assay. Here, separate constructs for c-Myb, c-Myb-2KR and variants of SUMO were used. Additionally, the SUMO constructs were modified (Q94P), enabling them of conjugation but preventing their cleavage from the substrates, thus increasing the level SUMOylation. This would mimic a system slightly more natural, where c-Myb and SUMO were not fused together, such as in the HEK293-c1 cell line.

The results presented in figure 3.10 were unexpected. The first observed abnormality was the increased activity of wt c-Myb co-transfected with wt SUMO, compared to wt c-Myb alone. Secondly, the activity of the transcription factor with variants of SUMO had the opposite trend, where the acetyl mimicking mutants of SUMO1 displayed strongest repression.

Similar results were observed for c-Myb 2KR. When wt and mutants of SUMO were introduced, c-Myb 2KR activity levels increased drastically, despite the lack of SUMO conjugation sites. This led to the belief that SUMO may have attached itself to other proteins influencing the reporter activity, and globally SUMOylated the cells proteome. Also, the natural pool of SUMO proteins in the cells may have interfered with the arrangement of the system in a way that is difficult to evaluate. Further optimalization of the setup is necessary in order to assess the true effect of derepression brought by the acetyl mimicking mutant of SUMO1 on c-Myb.

4.1.5 Acetylation mutants of SUMO exert their effect in a non-c-Myb context

The main goal of this study was to establish to which degree an acetylation switch would influence the transactivation activity of c-Myb. The derepression effect discovered in section 3.2.1 to 3.2.3 was further assessed with different parameters, such as adding p300 and increasing the quantity of SUMO mutants. Subsequently, it was of interest to investigate if the derepression event could also take place in a non c-Myb context. For this, a repression reporter gene assay was performed in the HEK293-c4 cell line. Constructs of Gal-SUMO2 were used as they were readily available, and their repression ability was examined on luciferase activity, activated by a GBD-TAD construct. GBD constructs would induce expression of the luciferase reporter gene, on which the SUMO variants would exert their effect. The original set up included only a GBD-construct. However, low expression of reporter gene was induced, making it difficult to differentiate the functional effect of wt and mutants of SUMO2. Therefore, a GBD-TAD construct was used in order to raise the activity levels of by 10-12-fold. By doing so, a clear estimate of the different suppression levels could be assessed. We observed the same trend, where acetyl mimicking mutant of SUMO2 was indeed capable of alleviating the suppression SUMO has on GDB. These results further confirmed and strengthened our findings from previous reporter gene assays in HEK293-c1 cells. This also suggests that the detected derepression due to the acetyl mimicking mutant of SUMO can be used for other constructs apart from c-Myb. This may open up an opportunity to investigate whether SUMO derepression can be used on a wider range of proteins. As SUMO2 is a paralog of SUMO1, one could expect to obtain similar results if we would have used SUMO1 constructs.

4.1.6 Does p300 acetylate SUMO?

As earlier mentioned, p300 is a protein with several functions and domains. Amongst these is the histone acetyl transferase domain (HAT), responsible for acetylation that is typically associated with gene activation (Goodman & Smolik, 2000). Ullmann *et. al* reported using immunoprecipitated exogenous Flag-tagged SUMO2 from HEK293T cells, further analysed by mass spectrometry (MS), in order to identify acetylation sites. In our case, we used hSUMO1 recombinantly expressed from *E.coli*, readily available. An acetylation reaction with p300 was set up, followed by SDS-PAGE for protein separation. Desired gel pieces were isolated and sent to MS for validation. The procedure was repeated twice, with the addition of sodium butyrate the second time, inhibiting potential activity from histone deacetylases (HDAC). Unfortunately, the result obtained were deficient. Different residues, both expected an unexpected were acetylated, but none of the results were reproducible. Western blot analysis using an acetylated antibody confirmed the presence of acetylated SUMO, but without reproducible data, it was difficult to trust our findings. In order to obtain better results, optimalization was required. These could have been a longer duration of the acetylation reaction and readjustment of protein to enzyme ratio. However, as time to repeat the experiment was lacking, we abandoned the option of further optimalization.

4.1.7 Acetylation mutants of SUMO affect binding of interaction partners selectively

Having performed extensive investigation on how the acetylation switch regulated transcriptional repression in both c-Myb and non c-Myb context, we turned our attention to explore how acetylation mutants of SUMO would affect their binding to interaction partners such as PIAS1 and CHD3. Again, Ullman *et. al* reported failure of acetyl mimicking SUMO1 and SUMO2 to bind to PIAS family members in GST pulldown and yeast two hybrid assays (Ullmann, Chien et al., 2012). In order to validate the interaction between the two proteins by an independent method, we performed a coimmunoprecipitation assay. PIAS1 was chosen in one of the assays with expectations of obtaining similar results as the ones in the Ullmann *et. al* study. CHD3 however, was chosen as it is a chromatin remodelling complex that acts as a co-activator of c-Myb and has been linked to the SUMO-system as well.

Our findings, figures 3.16 and 3.17 presented two different results. When expressed in COS1 cells, pGal-SUMO2^{K33Q} had a reduced ability to be co-immunoprecipitated together with PIAS1. The respective wild type SUMO2 as well the mutant with lysine to arginine substitution showed a stronger binding. Although findings in Müllers lab showed complete abrogation of acetyl mimicking SUMO2 (and SUMO1) to PIAS family members, our results are similar to some degree.

From the results, we assumed that neutralization of the positive charge prevents binding of SUMO2 to SIM containing region of PIAS1. This is also partly

supported by observations in other studies, where the K^{33} in SUMO2 (and K^{37} in SUMO1) forms a salt bridge with aspartic acid in SIM of PIASX and PIAS2 (Namanja, Li et al., 2012). A reasoning as to why our result are slightly divergent may be the use of PIAS1 in contrast to PIAS2. Despite originating from the same family, it has been observed that modifications within the SIM of PIASXα has influenced binding to SUMO1 but not SUMO2 (Hecker, Rabiller et al., 2006). It may be that K^{33} plays a partial role in binding to PIAS1, with other residues contributing to the SUMO-SIM interaction, therefore not fully abolishing the binding of acetyl mimicking mutant of SUMO2 to PIAS1.

Coimmunoprecipitation with Flag-tagged CHD3 gave different results. We observed no difference in binding strength between CHD3 and wt as well as mutants of pGal-SUMO2. The band indicating the interaction between the wild type SUMO2 and CHD3 is visible weaker. However, the intensity of CHD3-band in the same sample is weaker as well, in comparison to the rest of the samples. Hence, we believe that the weaker intensity of the CHD3-band may be due to a smaller quantity of CHD3 in the sample, leading to a weaker wt SUMO2-CHD3 interaction.

CHD3 is a validated partner of sentrin-specific protease (SENP1), which is responsible for desumoylation. Thus, CHD3 is believed to be a link between the SUMO system and chromatin remodelling (Rodriguez-Castaneda, Lemma et al., 2018). Here we confirm that SUMO2 is also capable of binding to CHD3, through SUMO-SIM interactions. Given that the binding strength is not influenced by the acetyl mimicking mutant of SUMO2, it may imply that K^{33} in SUMO2 does not play a vital role in the SUMO-SIM interaction. This may also imply that other residues in within the SUMO-SIM interface may act as bigger contributors to a productive SIM interaction. Together, these contrasting results indicate that acetyl-mimicking mutants of SUMO paralogs modulate SUMO-SIM interactions selectively.

4.2 Methodological considerations

In this study, several reporter gene assays in different cell lines were used. This enabled us to test out hypothesis in several setups with different conditions. For stably transfected constructs, we used an activation luciferase assay in HEK293-c1 cells. As earlier mentioned, this cell line has integrated 5xGal responsive elements upstream of the luciferase reporter gene. This provides an advantage as the reporter gene is not transfected and thus not influenced by poor transfection efficiency. Additionally, regulatory elements, enhancers and promoters are in an endogenous and fully chromatinized setting. In this cell line, we were able to specifically study the interaction between SUMO and c-Myb, using fusion constructs. However, this system was highly artificial as several aspects were altered. These changes are substituting the DBD domain in c-Myb with the DBD for the yeast Gal4 protein, point mutating residues in order to abolish covalent SUMO attachment, and fusing SUMO C-terminally to the transcription factor. Such alterations may affect both protein stability and change the interaction pattern with other proteins or complexes.

To achieve a more natural setting, a reporter assay was performed in CV1 cells. Transient transfection of reporter gene and effector genes can be influenced by poor transfection efficiency, leading to inconsistent results. However, co-transfecting c-Myb and SUMO constructs separately mimics slightly more physiological conditions. In saying that, mutated variants of both c-Myb (2KR) and SUMO (Q94P) constructs were used. Undesired SUMOylation of the proteome occurred, and without a method of differentiating unspecific SUMOylation from the actual results, obtaining meaningful data is impossible.

Subcloning strategies and PCR mutagenesis were methods used to generate mutations in fusion constructs. Altering a protein sequence to ensure or abrogate binding can cause unwanted side effects that are unnatural in physiological conditions. An alternative method to introduce the desired mutations and study their consequences under physiological conditions would be using the CRISPR-Cas9 editing tool in murine models. Clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein-9 nuclease (Cas9) is a genome editing system, which could be used to introduce a single point mutation in SUMO, generating acetyl mimicking $K^{37}Q$ and wild type mimicking $K^{37}R$ mutants of SUMO1. Generation of knock-out and knock-in mice would then provide an opportunity to study interaction between SUMO and c-Myb, as well as a wide range of proteins.

5 Summary of findings

In chapter 1, we outlined the aims of the study in section 1.4. Chapter 3 presented the results, which were further discussed in the previous chapter. This final chapter will summarize and conclude the findings presented in this study. A section for future studies will be presented.

5.1 Conclusions

At the starting point of this study, we hypothesized that the acetylation switch defined by Ullmann *et. al* in Stefan Müllers lab could relieve the SUMO-mediated repression of the transcription factor c-Myb. A key residue in SUMO-paralogs was mutated to mimic both acetylated and wild type states of SUMO. Reporter gene assays were used to investigate the effect of SUMO in both c-Myb and non c-Myb context *in vivo.* Additionally, SUMO-SIM mediated binding of protein partners to mutated SUMO paralogs was explored.

In conclusion, our findings are presented as following:

1. Acetyl-mimicking mutant of SUMO1^{K37Q} is indeed capable of derepression, alleviating the transcriptional activity of c-Myb.

    a. Co-transfected p300 does not influence the derepression effect.

    b. Investigating the capacity of the defined derepression effect revealed that c-Myb activity cannot be fully restored but is derepressed up to 40%.

2. The repressive effect of wt and mutants of SUMO on c-Myb was not possible to study in a classical transient transfection assay with cotransfection of SUMO-variants, probably because this led to a global change in SUMOylation causing indirect effects on the reporter system, precluding meaningful reporter assays.

3. Derepression mediated by acetyl- mimicking SUMO2^{K33Q} mutant is applicable in a non c-Myb context, indicating possible value for a wide range of proteins.

4. Acetyl mimicking mutants of SUMO2^{K33Q} interacts with protein partners selectively, suggesting that the acetylation switch serves as a fine-tuning mechanism for binding specificities.

5.2 Future studies

In this study, the main method used was reporter gene assays, both stable and transient, in order to establish the effect that wt SUMO, acetyl-mimicking as well as acetyl abolishing mutants of SUMO had on the transcription factor c-Myb. Our findings validated a derepression effect, brought forth by an acetyl mimicking variant of SUMO. Ullmann *et. al* had successfully demonstrated that endogenous SUMO2/3 indeed was acetylated at K^{33} (as well as other residues)

under physiological conditions (Ullmann, Chien et al., 2012). However, so far there is no evidence of naturally occurring SUMO^{Ac} conjugated to c-Myb. An interesting approach would be to further explore the presence and level of SUMO^{Ac} conjugated to c-Myb, either validating or rejecting its existence under physiological conditions.

In this study, by using fusion-constructs of c-Myb and SUMO, SUMO function on c-Myb was ensured. However, it is still unclear how naturally conjugated SUMO^{Ac} would affect c-Myb function directly or through altered recruitment of associated components. GST-pulldown assays and co-immunoprecipitation assays could be used in order to identify possible co-factors present in in the complex. Additionally, we suggested that a full derepression of c-Myb activity may be achievable but required acetylation of several residues in SUMO. Reporter assays using constructs of SUMO with several residues mutated could be implemented to assess whether the transactivation activity of c-Myb could be further derepressed.

According to Ullman et al., SUMO is acetylated under physiological conditions and we suggested p300 as a possible candidate for the acetyl transferase responsible. However, our result did not support this hypothesis by showing that cotransfected p300 had no effect on derepression. Thus, identification of the acetyl transferase responsible for acetylation of SUMO could be of interest. One approach could be a systematic knock-down of candidate acetyl transferases. Alternatively, one might design a screen with a library of siRNAs. If identified, knock-down and knock-out experiments could be implemented in order to investigate the consequence this has on SUMO mediated derepression, in both c-Myb and non-c-Myb context.

SUMO has been established as a global influencer, being involved in many important nuclear structures and processes, as described in section 1.3.To study the effects of acetylated SUMO^{Ac} under the ultimate physiological conditions, clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein-9 nuclease (Cas9) genome editing system could be used to generate gene edited mice with desired mutations in SUMO (at one or several residues) and study the global effect this generates, in terms of SUMO-mediated repression, activation, SUMO-SIM interactions with partner proteins as well as cell development and cell identity.

Appendix A

Abbreviations

µ	Micro
Acetyl-CoA	Acetyl coenzyme A
AMV	Avian myeloblastosis virus
A	Absorbance
AU	Absorbance unit
aa	amino acid
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
bp	Base pairs
BSA	Bovine serum albumin
BER	Base pair excision
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
c-Myb 2KR	Non-SUMOylated c-Myb mutant
C-terminal	Carboxyl-terminal
CBP	CREB-binding protein
CREB	cAMP response element-binding protein
CHD3	Chromo-helicase-DNA binding protein 3
CKII	Casein kinase II
CRD	C-terminal regulatory domain (of c-Myb)
COS	CV-1 Origin and carrying the SV40 genetic material
CtBP	C-terminally binding protein
DBD	DNA binding domain in c-Myb
DBS	Double stranded break
Da	Dalton
DMEM	Dulbecco´s Modified Eagle medium
DMSO	Dimethyl sulfoxide
dH_2O	Destilled water
dNTP	Deoxynucleotide
DTT	Dithithreitol
DPBS	Dulbecco's phosphate buffered saline

E1, E2, E3	Enzymes of the SUMO pathways
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetra-acetate
EtBr	Ethidium bromide
EVES	Subdomain of CRD in c-Myb
FAETL/LZ	Subdomain of CRD in c-Myb
FBS	Fetal Bovine Serum
FLASH	FLICE associated huge protein
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GBD	Gal4 binding domain
GLB	Gel loading buffer
GTF	General transcription factor
H1,H2A,H2B,H3,H4	Histone proteins
hcM	Human c-Myb
HA tag	Hemaglutinin tag
HAT	Histone acetyltransferase
HDAC	Histone deactyltransferase
HEK	Human embryonic kidney
HIPK1	Homeodomain-interacting protein kinase 1
HTH	Helix-turn-helix
k	Kilo
kDa	Kilo Dalton
kb	Kilo base pairs
KAc	Potassium acetate
KIX domain	Kinase-inducible domain interacting domain
l	Litre
LB medium	Lysogeny broth
m	Milli
M	Molar
Mb	Mega base pairs
MDM2	Mouse double minute 2
MYB domains	R1, R2 and R3
MRE	Myb-recognition element
mRNA	Messenger RNA

MCS	Multiple cloning site
n	Nano
n.s.	Not significant
N-terminal	Amino terminal
NEB	New England Biolabs
NER	Nucleotide excision repair
NuRD	Nucleosome Remodeling and Deacetylase complex
nm	Nanometre
OD	Optimal density
p	Pico
p300	E1A-associated protein 300
pBS	Bluescript SKII vector
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PIAS	Protein inhibitor of activated STAT
P/S	Penicillin/Streptomycin
Pc2	Polycomb protein family
PML NBs	Promyelocytic leukemia nuclear bodies
PVDF membrane	Polyvinylidene difluoride membrane
PTM	Post-translational modification
R1, R2, R3	Imperfect repeats in DBD of c-Myb
RanBP2	Ran-binding protein 2
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RLU	Relative luminescence unit
rpm	Revolutions per minute
RPG	Ribosomal protein genes
SANT domain	Swi3, Ada2, N-CoR, and TFIIIB domain
SAFB	Scaffold attachment factor B
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
SEM	Standard error of the mean
SENP	Sentrin specific proteases
SIM	SUMO-interaction motif

SRAF	SUMO-regulated activation function
SUMO	Small ubiquitin-related modifier
SUMO	Small ubiquitin-related modifier
TAD	Transactivation domain of c-Myb
TAE-buffer	Tris-acetate-EDTA-buffer
TB-buffer	Transformation buffer
TBS-buffer	Tris buffered saline buffer
TBST-buffer	Tris buffered saline with Tween- 20
TDG	thymine DNA glycosylase
TE- buffer	Tris-EDTA buffer
TF	Transcription factor
TP region	Sub-domain of CRD in c-Myb
U	Unit of enzyme
UBC9	Ubiquitin conjugation enzyme 9
UV light	Ultraviolet light
UV vis spectroscopy	Ultraviolet visible spectroscopy
WB	Western Blot
vs.	Versus
wt	Wildtype
x g	Centrifugal force

Amino Acid Abbreviations		
One letter code	**Three letter code**	**Name**
G	Gly	Glycine
A	Ala	Alanine
V	Val	Valine
I	Ile	Isoleucine
L	Leu	Leucine
S	Ser	Serine
T	Thr	Threonine
C	Cys	Cysteine
M	Met	Methionine
D	Asp	Aspartate
E	Glu	Glutamine

N	Asn	Asparagine
Q	Gln	Glutamine
K	Lys	Lysine
R	Arg	Arginine
H	His	Histidine
F	Phe	Phenylalanine
Y	Tyr	Tyrosine
W	Trp	Tryptophan
P	Pro	Proline
Ψ	Large hydrophobic amino acid residues	
x	Any amino acid residue	

Nucleotide Abberviations	
One letter code	**Name**
A	Adenine
G	Guanine
C	Cytosine
T	Thymine
U	Uracil
R	Purine (A or G)
Y	Pyrimidine (C or T)
N	Any nucleotide
W	Weak (A or T)
S	Strong (G or C)
M	Amino (A or C)
K	Keto (G or T)
B	Not A (G, C or T)
H	Not G (A, C or T)
D	Not C (A, G or T)
V	Not T (A,G or C)

Appendix B

Buffers and media

Buffers were stored at room temperature if not stated otherwise.

Anode (+) buffer

3 g Tris

20 % methanol

Adjust volume to 1000ml with dH_2O

Cathode (-) buffer

500 ml anode (+) buffer

2.62 g e-Amino-n-Caproic acid

100x Complete Protease Inhibitor

1 Complete Protease Inhibitor tablet

500 µl dH_2O

Coomassie Brilliant Blue fixing/destaining buffer

700 ml methanol

350 ml acetic acid

1750 ml dH_2O

Coomassie Brilliant Blue staining buffer

700 ml methanol

350 ml acetic acid

1750 ml dH_2O

1 ts Coomassie Brilliant Blue R

1M DTT

3.09 g DTT

20 ml 0.01 M sodium acetate, pH 5.2

Sterile filtrate, store at -20 °C

0.5 M EDTA

93.06 g Na_2EDTA x 2 H_2O (372.24 g/mol)

400 ml H_2O

Add NaOH pellets for pH 7.0. Dissolve EDTA in solution

Adjust pH to 8.0 with NaOH pellets

Adjust volume to 500 ml with dH_2O

LB medium

10 g Trypton

5 g Yeast extract

10 g NaCl

1000 ml H_2O

Adjust pH to 7.2 with 5 M NaCl solution

Autoclave: 20 minutes at 121°C

Add antibiotics to buffer when setting up a cell culture

Ampicillin: 1 µl of 100 µg/ml to 1 ml LB medium

LB agar medium

400 ml LB medium without antibiotics

6 g agar

Autoclave: 20 minutes at 121 °C

Add antibiotics to buffer when the temperature is below 50 °C:

Ampicillin: 100µg/ml

20 ml LB agar medium is added per petri dish

5 M NaCl

146.1.g NaCl (58.44 g/mol)

500 ml dH_2O

Autoclave: 20 minutes at 121 °C

10 M NaOH

20 g NaOH pellets

50 ml dH_2O

3x SDS gel loading buffer

3.75 ml 0.5 M Tris-HCl pH 6.8

0.69 g SDS

3 ml Glycerol

0.3 mg bromphenolblue

1.75 ml dH_2O

Prior to use, add: 100 mM DTT

SOB medium

20 g Trypton

5 g Yeast extract

0.5 g NaCl

800 ml dH_2O

1 ml 2.5 m KCl

Adjust pH to 7.0

Adjust volume to 1000 ml with dH_2O

Autoclave: 20 minutes at 121 °C

Add 10 ml 2 M $MgSO_4$ through a sterile filter

Add antibiotics prior to use: Ampicillin: 100μg/ml

50X TAE Buffer

121 g Tris

50 ml 0.5 M EDTA

28.5 ml acetic acid

Adjust volume to 500 ml with dH_2O

1x TAE-buffer

50 ml 10x TAE buffer

450 ml dH_2O

10X TBS-T buffer

50 ml 1 M Tris-HCl, pH 8.0

150 ml 5 M NaCl

2.5 ml Tween- 20
Adjust volume to 500 ml with dH_2O

1X-TBS-T Buffer
50 ml 10x TBS-T
450 ml dH_2O

0.5 M Tris- HCl, pH 6.8
30.28 g Tris
400 ml dH_2O
Add x ml HCl for pH 7.8
Adjust volume to 500 ml with dH_2O
Autclave: 20 minutes at 121 °C

XT MOPS buffer
50 ml MOPS
950 ml dH_2O

KAc lysis buffer
20mM HEPES
10%Glycerol
0,05%NP-40
1,5 mM MgCl2
150 mM KAc
1mM DTT
1x complete
1mM PMSF

2x Acetylation reaction buffer
100mM Tris HCl pH 8
0.2mM EDTA, 0.1µg/µl BSA
2mM DTT
40µM Acetyl-CoA

Appendix C

Plasmids

Plasmids marked by asterisks were designed by other members of the Gabrielsen group or gifted to the group.

General

Bluescript SKII+ (pBS)*

pCIneo*

pBS [Bgl]-hcM-HA-2KR-Bgl/Not *

pGL-4b-3xMRE(GG)-Myc aab*

3xFlag-Pias1*

3xFlag-CHD3*

pCIneo-GBD2*

pCIneo-GBD2-TAD*

pCmvb-NHA-CV5-p300*

c-Myb

pCIneoB-GBD2-hcM-HA [233-640] *

pCIneoB-GBD2-hcM-HA [233-640] 2KR*

pCIneo-hcMyb-HA wt*

pCIneo-hcMyb 2KR*

SUMO

pCI-Flag-SUMO1-Q94P (GG) wt*

pCI-Flag-SUMO1-K37Q-Q94P (GG)*

pCI-Flag-SUMO1-K37R-Q94P (GG*

pGal-SUMO2 wt*

pGal-SUMO2 K33Q*

pGal-SUMO2 K33R*

c-Myb-SUMO fusion

pCIneoB-GBD2-hcM-TPC-2KR-SUMO1 [410-640] *

pCIneoB-GBD2-hcM-2KR-SUMO1 [233-640] *

pCIneoB-GBD2-hcM-SUMO1-2KR [233-640]

pCIneoB-GBD2-hcM-SUMO1-2KR [233-640] K37Q

pCIneoB-GBD2-hcM-SUMO1-2KR [233-640] K37R

pBS+pCIneoB-GBD2-hcM-SUMO1-2KR [233-640]-EcoRI fragment

Appendix D

Primer sequences

Primers

Code	Name	Sequence (5´-3´)
S082 fwd	pCIneo	GAC ATC CAC TTT CTC TCC
S033 fwd	hcM-F1230-seq	AA ACT CAG ACT TGG AAA TGC CTT CTT
S183 rev	Rev pBS	GAA ACA GCT ATG ACC ATG
S069	hcM-F1722	AAT GGC ACC AGC ATC AGA AG
S161	hcM-F1064-seq	CAT CTC TGC CAG CGG ATC
S182 fwd	Fwd pBS	GTA AAA CGA CGG CCA GTG
S165 rev	hcM	GTC ATC TGC TCC TCC
I085	SUMO R3	GGA ACC TTG CGG CCG CCT
I060	SUMO-Myb R2	ACA ACT TAA GGA GTC
S068	pCIneo RS	CTC CCC CTG AAC CTG AAA CA

Primers for site-directed mutagenesis

Name	Sequence (5´-3´)
SUMO1 K37Q	GAT AGC AGT GAG ATT CAC TTC CAG GTG AAA ATG ACA ACA CAT CTC
SUMO1 K37Q-RC	GAG ATG TGT TGT CAT TTT CAC CTG GAA GTG AAT CTC ACT GCT ATC
SUMO1 K37R	GAT AGC AGT GAG ATT CAC TTC CGT GTG AAA ATG ACA ACA CAT CTC
SUMO1 K37R-RC	GAG ATG TGT TGT CAT TTT CAC ACG GAA GTG AAT CTC ACT GCT ATC

Appendix E

Materials, equipment and computer software

Material	Producer	Product nr.
Antibiotics		
Ampicillin	Sigma-Aldrich	A9518
Puromycin	Sigma- Aldrich	P9620
Antibodies		
α-Flag (mouse)	Sigma- Aldrich	F3165
α-GAPDH (mouse)	Invitrogen	AM4300
α-H141 (rabbit)	Santa Cruz Biotechnology	sc-7874
α-c-Myb D7 (mouse)	Santa Cruz Biotechnology	sc-74512
α-Gal4 RK5C1 (mouse)	Santa Cruz Biotechnology	sc-510
α-panAcetyl (C2) (rabbit)	Santa Cruz Biotechnology	Sc-8649
Donkey α-rabbit IR Dye 800 CW	LI-COR® Biosciences	926-32213
Donkey α-mouse IR Dye 800 CW	LI-COR® Biosciences	926-32212
Donkey α-rabbit IR Dye 680 RD	LI-COR® Biosciences	926-68073
Donkey α-mouse IR Dye 680 RD	LI-COR® Biosciences	926-68072
Cell culture		
Countess ™ chamber slides	NanoEnTek/ VWR	734-2676
DMEM	Gibco®Invitrogen	41965-039
PBS	Gibco®Invitrogen	14190-094
FBS	Saveen Werner	FBS-11A
Trans-IT®-LT1	Mirus Bio	MIR 2305
Trypan Blue	NanoEnTek/VWR	EBT-001
Trypsin-EDTA	Gibco®Invitrogen	25300-054
P/S Penstrep	Gibco®Invitrogen	15140-122
Buffers		
CutSmart	New England Biolabs®	Supplied
NEB 2.1	New England Biolabs®	Supplied
NEB 3.1	New England Biolabs®	Supplied
Pfu Ultra buffer	New England Biolabs®	Supplied

T4 DNA ligase buffer	New England Biolabs®	Supplied
Vent Polymerase buffer	New England Biolabs®	Supplied
XT MOPS running buffer	BioRad	161-0788
Enzymes		
NotI	New England Biolabs®	R0189S
NotI-HF	New England Biolabs®	R3189S
EcoRI	New England Biolabs®	R0101S
BglII	New England Biolabs®	R0144S
DpnI	New England Biolabs®	R0176S
Pfu Ultra Hotstart DNA Polymerase	Stratagene	600390
T4 DNA ligase	New England Biolabs®	M0202S
Vent Polymerase	New England Biolabs®	M0258S
p300	Active Motif	81158
Ladders		
1 kb DNA ladder	New England Biolabs®	N3232L
Precision Plus Protein all blue	BioRad	1610373
Precision Plus Protein dual color	BioRad	1610374
Proteins		
Acetyl-CoA	Sigma-Aldrich	A2056
Commercial kits		
NucleoSpin® Plasmid	Macherey-Nagel	740588.250
NucleoSnap Plasmid Midi	Macherey-Nagel	740494.50
NucleSpin® Gel and PCR Clean-up	Macherey-Nagel	740609.250
Quick Ligation Kit	NEB	M2200S
Luciferase Assay system, 10-pack	Promega	E1501
Equipment		
NanoDrop UV-Vis Spectrophotometer	Thermo Scientific	
2.5 mM dNTP Mix	Thermo Scientific	R72501
Grade 17 Chr Whatman filter paper	Whatman/VWR	588-3010
Immobilon®-FL transfer membrane	Merck Millipore	IPFL00010

Countess® Automated Cell Counter	Invitrogen	
VWR® Ultrasonic Cleaners	VWR™	142-6044
2720 Thermal Cycler PCR machine	Applied Biosystem	
Criterion XT 4-12% Bis-Tris gel	BioRad	3450123
Odyssey CLx from LI-COR®	LI-COR® Biosciences	
TD20/20 luminometer	Turner BioSystems	E4221
4% agarose beads with attached anti-flag M2 monoclonal antibody	Sigma-Aldrich	M8823
Computer software	**Version**	**Company**
NanoDrop 2000	1.6	Thermo Scientific
Excel	16.25	Microsoft
CLC Main Workbench	8.0	CLC bio

References

1. Aalfs, J. D., & Kingston, R. E. (2000). What does 'chromatin remodeling' mean? *Trends Biochem Sci, 25*(11), 548-555.
2. Aasland, R., Stewart, A. F., & Gibson, T. (1996). The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB. *Trends Biochem Sci, 21*(3), 87-88.
3. Alberts Bruce, J. D. W., Martin Raff (2002). *Molecular Biology of The Cell*
4. Alm-Kristiansen, A. H., Lorenzo, P. I., Molvaersmyr, A. K., Matre, V., Ledsaak, M., Saether, T., & Gabrielsen, O. S. (2011). PIAS1 interacts with FLASH and enhances its co-activation of c-Myb. *Mol Cancer, 10*, 21. doi:10.1186/1476-4598-10-21
5. Alm-Kristiansen, A. H., Norman, I. L., Matre, V., & Gabrielsen, O. S. (2009). SUMO modification regulates the transcriptional activity of FLASH. *Biochem Biophys Res Commun, 387*(3), 494-499. doi:10.1016/j.bbrc.2009.07.053
6. Alm-Kristiansen, A. H., Saether, T., Matre, V., Gilfillan, S., Dahle, O., & Gabrielsen, O. S. (2008). FLASH acts as a co-activator of the transcription factor c-Myb and localizes to active RNA polymerase II foci. *Oncogene, 27*(34), 4644-4656. doi:10.1038/onc.2008.105
7. Andersson, K. B., Kowenz-Leutz, E., Brendeford, E. M., Tygsett, A. H., Leutz, A., & Gabrielsen, O. S. (2003). Phosphorylation-dependent down-regulation of c-Myb DNA binding is abrogated by a point mutation in the v-myb oncogene. *J Biol Chem, 278*(6), 3816-3824. doi:10.1074/jbc.M209404200
8. Arany, Z., Sellers, W. R., Livingston, D. M., & Eckner, R. (1994). E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. *Cell, 77*(6), 799-800.
9. Arrowsmith, C. H., Bountra, C., Fish, P. V., Lee, K., & Schapira, M. (2012). Epigenetic protein families: a new frontier for drug discovery. *Nat Rev Drug Discov, 11*(5), 384-400. doi:10.1038/nrd3674
10. Aziz, N., Miglarese, M. R., Hendrickson, R. C., Shabanowitz, J., Sturgill, T. W., Hunt, D. F., & Bender, T. P. (1995). Modulation of c-Myb-induced transcription activation by a phosphorylation site near the negative regulatory domain. *Proc Natl Acad Sci U S A, 92*(14), 6429-6433.
11. Baluda, M. A., & Reddy, E. P. (1994). Anatomy of an integrated avian myeloblastosis provirus: structure and function. *Oncogene, 9*(10), 2761-2774.
12. Banani, S. F., Lee, H. O., Hyman, A. A., & Rosen, M. K. (2017). Biomolecular condensates: organizers of cellular biochemistry. *Nat Rev Mol Cell Biol, 18*(5), 285-298. doi:10.1038/nrm.2017.7
13. Bannister, A. J., & Kouzarides, T. (2011). Regulation of chromatin by histone modifications. *Cell Res, 21*(3), 381-395. doi:10.1038/cr.2011.22
14. Basta, J., & Rauchman, M. (2015). The nucleosome remodeling and deacetylase complex in development and disease. *Transl Res, 165*(1), 36-47. doi:10.1016/j.trsl.2014.05.003

15. Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., & Becker, J. (1998). Structure determination of the small ubiquitin-related modifier SUMO-1. *J Mol Biol, 280*(2), 275-286. doi:10.1006/jmbi.1998.1839
16. Bender, T. P., Thompson, C. B., & Kuehl, W. M. (1987). Differential expression of c-myb mRNA in murine B lymphomas by a block to transcription elongation. *Science, 237*(4821), 1473-1476.
17. Bengtsen, M., Klepper, K., Gundersen, S., Cuervo, I., Drablos, F., Hovig, E., . . . Eskeland, R. (2015). c-Myb Binding Sites in Haematopoietic Chromatin Landscapes. *PLoS One, 10*(7), e0133280. doi:10.1371/journal.pone.0133280
18. Bergholtz, S., Andersen, T. O., Andersson, K. B., Borrebaek, J., Luscher, B., & Gabrielsen, O. S. (2001). The highly conserved DNA-binding domains of A-, B- and c-Myb differ with respect to DNA-binding, phosphorylation and redox properties. *Nucleic Acids Res, 29*(17), 3546-3556.
19. Bernier-Villamor, V., Sampson, D. A., Matunis, M. J., & Lima, C. D. (2002). Structural basis for E2-mediated SUMO conjugation revealed by a complex between ubiquitin-conjugating enzyme Ubc9 and RanGAP1. *Cell, 108*(3), 345-356.
20. Bianchi, E., Zini, R., Salati, S., Tenedini, E., Norfo, R., Tagliafico, E., . . . Ferrari, S. (2010). c-myb supports erythropoiesis through the transactivation of KLF1 and LMO2 expression. *Blood, 116*(22), e99-110. doi:10.1182/blood-2009-08-238311
21. Bies, J., Markus, J., & Wolff, L. (2002). Covalent attachment of the SUMO-1 protein to the negative regulatory domain of the c-Myb transcription factor modifies its stability and transactivation capacity. *J Biol Chem, 277*(11), 8999-9009. doi:10.1074/jbc.M110453200
22. Bies, J., & Wolff, L. (1997). Oncogenic activation of c-Myb by carboxyl-terminal truncation leads to decreased proteolysis by the ubiquitin-26S proteasome pathway. *Oncogene, 14*(2), 203-212. doi:10.1038/sj.onc.1200828
23. Boggio, R., Colombo, R., Hay, R. T., Draetta, G. F., & Chiocca, S. (2004). A mechanism for inhibiting the SUMO pathway. *Mol Cell, 16*(4), 549-561. doi:10.1016/j.molcel.2004.11.007
24. Boyer, L. A., Langer, M. R., Crowley, K. A., Tan, S., Denu, J. M., & Peterson, C. L. (2002). Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Mol Cell, 10*(4), 935-942.
25. Brandt, S. J., & Koury, M. J. (2009). Regulation of LMO2 mRNA and protein expression in erythroid differentiation. *Haematologica, 94*(4), 447-448. doi:10.3324/haematol.2008.005140
26. Bruderer, R., Tatham, M. H., Plechanovova, A., Matic, I., Garg, A. K., & Hay, R. T. (2011). Purification and identification of endogenous polySUMO conjugates. *EMBO Rep, 12*(2), 142-148. doi:10.1038/embor.2010.206
27. Chakravarti, D., LaMorte, V. J., Nelson, M. C., Nakajima, T., Schulman, I. G., Juguilon, H., . . . Evans, R. M. (1996). Role of CBP/P300 in nuclear receptor signalling. *Nature, 383*(6595), 99-103. doi:10.1038/383099a0
28. Chymkowitch, P., Nguea, A. P., Aanes, H., Koehler, C. J., Thiede, B., Lorenz, S., . . . Enserink, J. M. (2015). Sumoylation of Rap1 mediates the

recruitment of TFIID to promote transcription of ribosomal protein genes. *Genome Res, 25*(6), 897-906. doi:10.1101/gr.185793.114
29. Chymkowitch, P., Nguea, P. A., & Enserink, J. M. (2015). SUMO-regulated transcription: challenging the dogma. *Bioessays, 37*(10), 1095-1105. doi:10.1002/bies.201500065
30. Clappier, E., Cuccuini, W., Kalota, A., Crinquette, A., Cayuela, J. M., Dik, W. A., . . . Soulier, J. (2007). The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. *Blood, 110*(4), 1251-1261. doi:10.1182/blood-2006-12-064683
31. Cossec, J. C., Theurillat, I., Chica, C., Bua Aguin, S., Gaume, X., Andrieux, A., . . . Dejean, A. (2018). SUMO Safeguards Somatic and Pluripotent Cell Identities by Enforcing Distinct Chromatin States. *Cell Stem Cell, 23*(5), 742-757 e748. doi:10.1016/j.stem.2018.10.001
32. Dahle, O., Andersen, T. O., Nordgard, O., Matre, V., Del Sal, G., & Gabrielsen, O. S. (2003). Transactivation properties of c-Myb are critically dependent on two SUMO-1 acceptor sites that are conjugated in a PIASy enhanced manner. *Eur J Biochem, 270*(6), 1338-1348.
33. Dai, P., Akimaru, H., Tanaka, Y., Hou, D. X., Yasukawa, T., Kanei-Ishii, C., . . . Ishii, S. (1996). CBP as a transcriptional coactivator of c-Myb. *Genes Dev, 10*(5), 528-540.
34. Dasgupta, P., Linnenbach, A. J., Giaccia, A. J., Stamato, T. D., & Reddy, E. P. (1989). Molecular cloning of the breakpoint region on chromosome 6 in cutaneous malignant melanoma: evidence for deletion in the c-myb locus and translocation of a segment of chromosome 12. *Oncogene, 4*(10), 1201-1205.
35. Dasgupta, P., & Reddy, E. P. (1989). Identification of alternatively spliced transcripts for human c-myb: molecular cloning and sequence analysis of human c-myb exon 9A sequences. *Oncogene, 4*(12), 1419-1423.
36. Dash, A. B., Orrico, F. C., & Ness, S. A. (1996). The EVES motif mediates both intermolecular and intramolecular regulation of c-Myb. *Genes Dev, 10*(15), 1858-1869.
37. Dawlaty, M. M., Malureanu, L., Jeganathan, K. B., Kao, E., Sustmann, C., Tahk, S., . . . van Deursen, J. M. (2008). Resolution of sister centromeres requires RanBP2-mediated SUMOylation of topoisomerase IIalpha. *Cell, 133*(1), 103-115. doi:10.1016/j.cell.2008.01.045
38. Di Stefano, B., & Hochedlinger, K. (2018). Novel Roles for SUMOylation in Cellular Plasticity. *Trends Cell Biol, 28*(12), 971-973. doi:10.1016/j.tcb.2018.10.001
39. Dini, P. W., & Lipsick, J. S. (1993). Oncogenic truncation of the first repeat of c-Myb decreases DNA binding in vitro and in vivo. *Mol Cell Biol, 13*(12), 7334-7348.
40. Dohmen, R. J. (2004). SUMO protein modification. *Biochim Biophys Acta, 1695*(1-3), 113-131. doi:10.1016/j.bbamcr.2004.09.021
41. Drabsch, Y., Hugo, H., Zhang, R., Dowhan, D. H., Miao, Y. R., Gewirtz, A. M., . . . Gonda, T. J. (2007). Mechanism of and requirement for estrogen-regulated MYB expression in estrogen-receptor-positive breast cancer cells. *Proc Natl Acad Sci U S A, 104*(34), 13762-13767. doi:10.1073/pnas.0700104104

42. Dupont, C., Armant, D. R., & Brenner, C. A. (2009). Epigenetics: definition, mechanisms and clinical perspective. *Semin Reprod Med, 27*(5), 351-357. doi:10.1055/s-0029-1237423
43. Eifler, K., & Vertegaal, A. C. (2015). Mapping the SUMOylated landscape. *FEBS J, 282*(19), 3669-3680. doi:10.1111/febs.13378
44. Fernandez, K. S., & de Alarcon, P. A. (2013). Development of the hematopoietic system and disorders of hematopoiesis that present during infancy and early childhood. *Pediatr Clin North Am, 60*(6), 1273-1289. doi:10.1016/j.pcl.2013.08.002
45. Fu, S. L., & Lipsick, J. S. (1996). FAETL motif required for leukemic transformation by v-Myb. *J Virol, 70*(8), 5600-5610.
46. Fuglerud, B. M., Ledsaak, M., Rogne, M., Eskeland, R., & Gabrielsen, O. S. (2018). The pioneer factor activity of c-Myb involves recruitment of p300 and induction of histone acetylation followed by acetylation-induced chromatin dissociation. *Epigenetics Chromatin, 11*(1), 35. doi:10.1186/s13072-018-0208-y
47. Fuglerud, B. M., Lemma, R. B., Wanichawan, P., Sundaram, A. Y. M., Eskeland, R., & Gabrielsen, O. S. (2017). A c-Myb mutant causes deregulated differentiation due to impaired histone binding and abrogated pioneer factor function. *Nucleic Acids Res, 45*(13), 7681-7696. doi:10.1093/nar/gkx364
48. Gabrielsen, O. S., Sentenac, A., & Fromageot, P. (1991). Specific DNA binding by c-Myb: evidence for a double helix-turn-helix-related motif. *Science, 253*(5024), 1140-1143.
49. Galanty, Y., Belotserkovskaya, R., Coates, J., Polo, S., Miller, K. M., & Jackson, S. P. (2009). Mammalian SUMO E3-ligases PIAS1 and PIAS4 promote responses to DNA double-strand breaks. *Nature, 462*(7275), 935-939. doi:10.1038/nature08657
50. Ganter, B., & Lipsick, J. S. (1999). Myb and oncogenesis. *Adv Cancer Res, 76*, 21-60.
51. Gareau, J. R., Reverter, D., & Lima, C. D. (2012). Determinants of small ubiquitin-like modifier 1 (SUMO1) protein specificity, E3 ligase, and SUMO-RanGAP1 binding activities of nucleoporin RanBP2. *J Biol Chem, 287*(7), 4740-4751. doi:10.1074/jbc.M111.321141
52. Gerondakis, S., & Bishop, J. M. (1986). Structure of the protein encoded by the chicken proto-oncogene c-myb. *Mol Cell Biol, 6*(11), 3677-3684.
53. Gill, G. (2004). SUMO and ubiquitin in the nucleus: different functions, similar mechanisms? *Genes Dev, 18*(17), 2046-2059. doi:10.1101/gad.1214604
54. Girdwood, D., Bumpass, D., Vaughan, O. A., Thain, A., Anderson, L. A., Snowden, A. W., . . . Hay, R. T. (2003). P300 transcriptional repression is mediated by SUMO modification. *Mol Cell, 11*(4), 1043-1054.
55. Goodman, R. H., & Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. *Genes Dev, 14*(13), 1553-1577.
56. Graf, T., McNagny, K., Brady, G., & Frampton, J. (1992). Chicken "erythroid" cells transformed by the Gag-Myb-Ets-encoding E26 leukemia virus are multipotent. *Cell, 70*(2), 201-213.

57. Greig, K. T., Carotta, S., & Nutt, S. L. (2008). Critical roles for c-Myb in hematopoietic progenitor cells. *Semin Immunol, 20*(4), 247-256. doi:10.1016/j.smim.2008.05.003
58. Guerra, J., Withers, D. A., & Boxer, L. M. (1995). Myb binding sites mediate negative regulation of c-myb expression in T-cell lines. *Blood, 86*(5), 1873-1880.
59. Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., . . . Brown, M. (1996). p300 is a component of an estrogen receptor coactivator complex. *Proc Natl Acad Sci U S A, 93*(21), 11540-11545.
60. Hardeland, U., Steinacher, R., Jiricny, J., & Schar, P. (2002). Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *EMBO J, 21*(6), 1456-1464. doi:10.1093/emboj/21.6.1456
61. Hay, R. T. (2005). SUMO: a history of modification. *Mol Cell, 18*(1), 1-12. doi:10.1016/j.molcel.2005.03.012
62. Hecker, C. M., Rabiller, M., Haglund, K., Bayer, P., & Dikic, I. (2006). Specification of SUMO1- and SUMO2-interacting motifs. *J Biol Chem, 281*(23), 16117-16127. doi:10.1074/jbc.M512757200
63. Hegvold, A. B., & Gabrielsen, O. S. (1996). The importance of the linker connecting the repeats of the c-Myb oncoprotein may be due to a positioning function. *Nucleic Acids Res, 24*(20), 3990-3995.
64. Hickey, C. M., Wilson, N. R., & Hochstrasser, M. (2012). Function and regulation of SUMO proteases. *Nat Rev Mol Cell Biol, 13*(12), 755-766. doi:10.1038/nrm3478
65. Hilgarth, R. S., Murphy, L. A., Skaggs, H. S., Wilkerson, D. C., Xing, H., & Sarge, K. D. (2004). Regulation and function of SUMO modification. *J Biol Chem, 279*(52), 53899-53902. doi:10.1074/jbc.R400021200
66. Hsieh, Y. L., Kuo, H. Y., Chang, C. C., Naik, M. T., Liao, P. H., Ho, C. C., . . . Shih, H. M. (2013). Ubc9 acetylation modulates distinct SUMO target modification and hypoxia response. *EMBO J, 32*(6), 791-804. doi:10.1038/emboj.2013.5
67. Hu, Y. L., Ramsay, R. G., Kanei-Ishii, C., Ishii, S., & Gonda, T. J. (1991). Transformation by carboxyl-deleted Myb reflects increased transactivating capacity and disruption of a negative regulatory domain. *Oncogene, 6*(9), 1549-1553.
68. Hugo, H., Cures, A., Suraweera, N., Drabsch, Y., Purcell, D., Mantamadiotis, T., . . . Ramsay, R. G. (2006). Mutations in the MYB intron I regulatory sequence increase transcription in colon cancers. *Genes Chromosomes Cancer, 45*(12), 1143-1154. doi:10.1002/gcc.20378
69. Introna, M., Golay, J., Frampton, J., Nakano, T., Ness, S. A., & Graf, T. (1990). Mutations in v-myb alter the differentiation of myelomonocytic cells transformed by the oncogene. *Cell, 63*(6), 1289-1297.
70. Jamin, N., Gabrielsen, O. S., Gilles, N., Lirsac, P. N., & Toma, F. (1993). Secondary structure of the DNA-binding domain of the c-Myb oncoprotein in solution. A multidimensional double and triple heteronuclear NMR study. *Eur J Biochem, 216*(1), 147-154.
71. Jin, S., Zhao, H., Yi, Y., Nakata, Y., Kalota, A., & Gewirtz, A. M. (2010). c-Myb binds MLL through menin in human leukemia cells and is an important

driver of MLL-associated leukemogenesis. *J Clin Invest, 120*(2), 593-606. doi:10.1172/JCI38030
72. Joazeiro, C. A., & Weissman, A. M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell, 102*(5), 549-552.
73. Kagey, M. H., Melhuish, T. A., Powers, S. E., & Wotton, D. (2005). Multiple activities contribute to Pc2 E3 function. *EMBO J, 24*(1), 108-119. doi:10.1038/sj.emboj.7600506
74. Kalkbrenner, F., Guehmann, S., & Moelling, K. (1990). Transcriptional activation by human c-myb and v-myb genes. *Oncogene, 5*(5), 657-661.
75. Kamieniarz, K., & Schneider, R. (2009). Tools to tackle protein acetylation. *Chem Biol, 16*(10), 1027-1029. doi:10.1016/j.chembiol.2009.10.002
76. Kamitani, T., Nguyen, H. P., & Yeh, E. T. (1997). Preferential modification of nuclear proteins by a novel ubiquitin-like molecule. *J Biol Chem, 272*(22), 14001-14004.
77. Kanei-Ishii, C., MacMillan, E. M., Nomura, T., Sarai, A., Ramsay, R. G., Aimoto, S., . . . Gonda, T. J. (1992). Transactivation and transformation by Myb are negatively regulated by a leucine-zipper structure. *Proc Natl Acad Sci U S A, 89*(7), 3088-3092.
78. Kanei-Ishii, C., Ninomiya-Tsuji, J., Tanikawa, J., Nomura, T., Ishitani, T., Kishida, S., . . . Ishii, S. (2004). Wnt-1 signal induces phosphorylation and degradation of c-Myb protein via TAK1, HIPK2, and NLK. *Genes Dev, 18*(7), 816-829. doi:10.1101/gad.1170604
79. Kanei-Ishii, C., Sarai, A., Sawazaki, T., Nakagoshi, H., He, D. N., Ogata, K., . . . Ishii, S. (1990). The tryptophan cluster: a hypothetical structure of the DNA-binding domain of the myb protooncogene product. *J Biol Chem, 265*(32), 19990-19995.
80. Kasper, L. H., Boussouar, F., Ney, P. A., Jackson, C. W., Rehg, J., van Deursen, J. M., & Brindle, P. K. (2002). A transcription-factor-binding surface of coactivator p300 is required for haematopoiesis. *Nature, 419*(6908), 738-743. doi:10.1038/nature01062
81. Kaur, K., Park, H., Pandey, N., Azuma, Y., & De Guzman, R. N. (2017). Identification of a new small ubiquitin-like modifier (SUMO)-interacting motif in the E3 ligase PIASy. *J Biol Chem, 292*(24), 10230-10238. doi:10.1074/jbc.M117.789982
82. Kauraniemi, P., Hedenfalk, I., Persson, K., Duggan, D. J., Tanner, M., Johannsson, O., . . . Borg, A. (2000). MYB oncogene amplification in hereditary BRCA1 breast cancer. *Cancer Res, 60*(19), 5323-5328.
83. Kerscher, O. (2007). SUMO junction-what's your function? New insights through SUMO-interacting motifs. *EMBO Rep, 8*(6), 550-555. doi:10.1038/sj.embor.7400980
84. Kitagawa, K., Hiramatsu, Y., Uchida, C., Isobe, T., Hattori, T., Oda, T., . . . Kitagawa, M. (2009). Fbw7 promotes ubiquitin-dependent degradation of c-Myb: involvement of GSK3-mediated phosphorylation of Thr-572 in mouse c-Myb. *Oncogene, 28*(25), 2393-2405. doi:10.1038/onc.2009.111
85. Klug, H., Xaver, M., Chaugule, V. K., Koidl, S., Mittler, G., Klein, F., & Pichler, A. (2013). Ubc9 sumoylation controls SUMO chain formation and meiotic synapsis in Saccharomyces cerevisiae. *Mol Cell, 50*(5), 625-636. doi:10.1016/j.molcel.2013.03.027

86. Knipscheer, P., Flotho, A., Klug, H., Olsen, J. V., van Dijk, W. J., Fish, A., . . . Pichler, A. (2008). Ubc9 sumoylation regulates SUMO target discrimination. *Mol Cell, 31*(3), 371-382. doi:10.1016/j.molcel.2008.05.022
87. Ko, E. R., Ko, D., Chen, C., & Lipsick, J. S. (2008). A conserved acidic patch in the Myb domain is required for activation of an endogenous target gene and for chromatin binding. *Mol Cancer, 7*, 77. doi:10.1186/1476-4598-7-77
88. Kotaja, N., Karvonen, U., Janne, O. A., & Palvimo, J. J. (2002). PIAS proteins modulate transcription factors by functioning as SUMO-1 ligases. *Mol Cell Biol, 22*(14), 5222-5234.
89. Lahortiga, I., De Keersmaecker, K., Van Vlierberghe, P., Graux, C., Cauwelier, B., Lambert, F., . . . Cools, J. (2007). Duplication of the MYB oncogene in T cell acute lymphoblastic leukemia. *Nat Genet, 39*(5), 593-595. doi:10.1038/ng2025
90. Lallemand-Breitenbach, V., & de The, H. (2010). PML nuclear bodies. *Cold Spring Harb Perspect Biol, 2*(5), a000661. doi:10.1101/cshperspect.a000661
91. Lallemand-Breitenbach, V., Jeanne, M., Benhenda, S., Nasr, R., Lei, M., Peres, L., . . . de The, H. (2008). Arsenic degrades PML or PML-RARalpha through a SUMO-triggered RNF4/ubiquitin-mediated pathway. *Nat Cell Biol, 10*(5), 547-555. doi:10.1038/ncb1717
92. Ledsaak, M., Bengtsen, M., Molvaersmyr, A. K., Fuglerud, B. M., Matre, V., Eskeland, R., & Gabrielsen, O. S. (2016). PIAS1 binds p300 and behaves as a coactivator or corepressor of the transcription factor c-Myb dependent on SUMO-status. *Biochim Biophys Acta, 1859*(5), 705-718. doi:10.1016/j.bbagrm.2016.03.011
93. Lei, W., Rushton, J. J., Davis, L. M., Liu, F., & Ness, S. A. (2004). Positive and negative determinants of target gene specificity in myb transcription factors. *J Biol Chem, 279*(28), 29519-29527. doi:10.1074/jbc.M403133200
94. Lima, C. D., & Reverter, D. (2008). Structure of the human SENP7 catalytic domain and poly-SUMO deconjugation activities for SENP6 and SENP7. *J Biol Chem, 283*(46), 32045-32055. doi:10.1074/jbc.M805655200
95. Lipsick, J. S. (1996). One billion years of Myb. *Oncogene, 13*(2), 223-235.
96. Lipsick, J. S. (2010). The C-MYB story--is it definitive? *Proc Natl Acad Sci U S A, 107*(40), 17067-17068. doi:10.1073/pnas.1012402107
97. Lipsick, J. S., & Wang, D. M. (1999). Transformation by v-Myb. *Oncogene, 18*(19), 3047-3055. doi:10.1038/sj.onc.1202745
98. Liu, F., Lei, W., O'Rourke, J. P., & Ness, S. A. (2006). Oncogenic mutations cause dramatic, qualitative changes in the transcriptional activity of c-Myb. *Oncogene, 25*(5), 795-805. doi:10.1038/sj.onc.1209105
99. Liu, H. W., Banerjee, T., Guan, X., Freitas, M. A., & Parvin, J. D. (2015). The chromatin scaffold protein SAFB1 localizes SUMO-1 to the promoters of ribosomal protein genes to facilitate transcription initiation and splicing. *Nucleic Acids Res, 43*(7), 3605-3613. doi:10.1093/nar/gkv246
100. Liu, H. W., Zhang, J., Heine, G. F., Arora, M., Gulcin Ozer, H., Onti-Srinivasan, R., . . . Parvin, J. D. (2012). Chromatin modification by SUMO-1 stimulates the promoters of translation machinery genes. *Nucleic Acids Res, 40*(20), 10172-10186. doi:10.1093/nar/gks819

101. Liu, Q., Jin, C., Liao, X., Shen, Z., Chen, D. J., & Chen, Y. (1999). The binding interface between an E2 (UBC9) and a ubiquitin homologue (UBL1). *J Biol Chem, 274*(24), 16979-16987.
102. Liu, S., Fan, Z., Geng, Z., Zhang, H., Ye, Q., Jiao, S., & Xu, X. (2013). PIAS3 promotes homology-directed repair and distal non-homologous end joining. *Oncol Lett, 6*(4), 1045-1048. doi:10.3892/ol.2013.1472
103. Lorenzo, P. I., Brendeford, E. M., Gilfillan, S., Gavrilov, A. A., Leedsak, M., Razin, S. V., . . . Gabrielsen, O. S. (2011). Identification of c-Myb Target Genes in K562 Cells Reveals a Role for c-Myb as a Master Regulator. *Genes Cancer, 2*(8), 805-817. doi:10.1177/1947601911428224
104. Luscher, B., Christenson, E., Litchfield, D. W., Krebs, E. G., & Eisenman, R. N. (1990). Myb DNA binding inhibited by phosphorylation at a site deleted during oncogenic activation. *Nature, 344*(6266), 517-522. doi:10.1038/344517a0
105. Lyst, M. J., & Stancheva, I. (2007). A role for SUMO modification in transcriptional repression and activation. *Biochem Soc Trans, 35*(Pt 6), 1389-1392. doi:10.1042/BST0351389
106. Maison, C., Bailly, D., Roche, D., Montes de Oca, R., Probst, A. V., Vassias, I., . . . Almouzni, G. (2011). SUMOylation promotes de novo targeting of HP1alpha to pericentric heterochromatin. *Nat Genet, 43*(3), 220-227. doi:10.1038/ng.765
107. Malaterre, J., Mantamadiotis, T., Dworkin, S., Lightowler, S., Yang, Q., Ransome, M. I., . . . Ramsay, R. G. (2008). c-Myb is required for neural progenitor cell proliferation and maintenance of the neural stem cell niche in adult brain. *Stem Cells, 26*(1), 173-181. doi:10.1634/stemcells.2007-0293
108. Maroui, M. A., Kheddache-Atmane, S., El Asmi, F., Dianoux, L., Aubry, M., & Chelbi-Alix, M. K. (2012). Requirement of PML SUMO interacting motif for RNF4- or arsenic trioxide-induced degradation of nuclear PML isoforms. *PLoS One, 7*(9), e44949. doi:10.1371/journal.pone.0044949
109. Matre, V., Nordgard, O., Alm-Kristiansen, A. H., Ledsaak, M., & Gabrielsen, O. S. (2009). HIPK1 interacts with c-Myb and modulates its activity through phosphorylation. *Biochem Biophys Res Commun, 388*(1), 150-154. doi:10.1016/j.bbrc.2009.07.139
110. Matunis, M. J., Coutavas, E., & Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol, 135*(6 Pt 1), 1457-1470.
111. Mayes, K., Qiu, Z., Alhazmi, A., & Landry, J. W. (2014). ATP-dependent chromatin remodeling complexes as novel targets for cancer therapy. *Adv Cancer Res, 121*, 183-233. doi:10.1016/B978-0-12-800249-0.00005-6
112. McCann, S., Sullivan, J., Guerra, J., Arcinas, M., & Boxer, L. M. (1995). Repression of the c-myb gene by WT1 protein in T and B cell lines. *J Biol Chem, 270*(40), 23785-23789.
113. McCormack, M. P., & Rabbitts, T. H. (2004). Activation of the T-cell oncogene LMO2 after gene therapy for X-linked severe combined

immunodeficiency. *N Engl J Med, 350*(9), 913-922. doi:10.1056/NEJMra032207
114. Meulmeester, E., & Melchior, F. (2008). Cell biology: SUMO. *Nature, 452*(7188), 709-711. doi:10.1038/452709a
115. Miglarese, M. R., Richardson, A. F., Aziz, N., & Bender, T. P. (1996). Differential regulation of c-Myb-induced transcription activation by a phosphorylation site in the negative regulatory domain. *J Biol Chem, 271*(37), 22697-22705.
116. Minty, A., Dumont, X., Kaghad, M., & Caput, D. (2000). Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *J Biol Chem, 275*(46), 36316-36323. doi:10.1074/jbc.M004293200
117. Mo, X., Kowenz-Leutz, E., Laumonnier, Y., Xu, H., & Leutz, A. (2005). Histone H3 tail positioning and acetylation by the c-Myb but not the v-Myb DNA-binding SANT domain. *Genes Dev, 19*(20), 2447-2457. doi:10.1101/gad.355405
118. Molvaersmyr, A. K., Saether, T., Gilfillan, S., Lorenzo, P. I., Kvaloy, H., Matre, V., & Gabrielsen, O. S. (2010). A SUMO-regulated activation function controls synergy of c-Myb through a repressor-activator switch leading to differential p300 recruitment. *Nucleic Acids Res, 38*(15), 4970-4984. doi:10.1093/nar/gkq245
119. Mucenski, M. L., McLain, K., Kier, A. B., Swerdlow, S. H., Schreiner, C. M., Miller, T. A., . . . Potter, S. S. (1991). A functional c-myb gene is required for normal murine fetal hepatic hematopoiesis. *Cell, 65*(4), 677-689.
120. Muller, S., Ledl, A., & Schmidt, D. (2004). SUMO: a regulator of gene expression and genome integrity. *Oncogene, 23*(11), 1998-2008. doi:10.1038/sj.onc.1207415
121. Myrset, A. H., Bostad, A., Jamin, N., Lirsac, P. N., Toma, F., & Gabrielsen, O. S. (1993). DNA and redox state induced conformational changes in the DNA-binding domain of the Myb oncoprotein. *EMBO J, 12*(12), 4625-4633.
122. Namanja, A. T., Li, Y. J., Su, Y., Wong, S., Lu, J., Colson, L. T., . . . Chen, Y. (2012). Insights into high affinity small ubiquitin-like modifier (SUMO) recognition by SUMO-interacting motifs (SIMs) revealed by a combination of NMR and peptide array analysis. *J Biol Chem, 287*(5), 3231-3240. doi:10.1074/jbc.M111.293118
123. Ness, S. A. (1996). The Myb oncoprotein: regulating a regulator. *Biochim Biophys Acta, 1288*(3), F123-139.
124. Ness, S. A., Marknell, A., & Graf, T. (1989). The v-myb oncogene product binds to and activates the promyelocyte-specific mim-1 gene. *Cell, 59*(6), 1115-1125.
125. Nicolaides, N. C., Correa, I., Casadevall, C., Travali, S., Soprano, K. J., & Calabretta, B. (1992). The Jun family members, c-Jun and JunD, transactivate the human c-myb promoter via an Ap1-like element. *J Biol Chem, 267*(27), 19665-19672.
126. Nicolaides, N. C., Gualdi, R., Casadevall, C., Manzella, L., & Calabretta, B. (1991). Positive autoregulation of c-myb expression via Myb binding sites in the 5' flanking region of the human c-myb gene. *Mol Cell Biol, 11*(12), 6166-6176.

127. O'Neil, J., Tchinda, J., Gutierrez, A., Moreau, L., Maser, R. S., Wong, K. K., . . . Look, A. T. (2007). Alu elements mediate MYB gene tandem duplication in human T-ALL. *J Exp Med, 204*(13), 3059-3066. doi:10.1084/jem.20071637
128. O'Rourke, J. P., & Ness, S. A. (2008). Alternative RNA splicing produces multiple forms of c-Myb with unique transcriptional activities. *Mol Cell Biol, 28*(6), 2091-2101. doi:10.1128/MCB.01870-07
129. Ogata, K., Hojo, H., Aimoto, S., Nakai, T., Nakamura, H., Sarai, A., . . . Nishimura, Y. (1992). Solution structure of a DNA-binding unit of Myb: a helix-turn-helix-related motif with conserved tryptophans forming a hydrophobic core. *Proc Natl Acad Sci U S A, 89*(14), 6428-6432.
130. Oh, I. H., & Reddy, E. P. (1999). The myb gene family in cell growth, differentiation and apoptosis. *Oncogene, 18*(19), 3017-3033. doi:10.1038/sj.onc.1202839
131. Ording, E., Bergholtz, S., Brendeford, E. M., Jamin, N., & Gabrielsen, O. S. (1996). Flexibility in the second half-site sequence recognised by the c-Myb R2 domain--in vitro and in vivo analysis. *Oncogene, 13*(5), 1043-1051.
132. Ording, E., Kvavik, W., Bostad, A., & Gabrielsen, O. S. (1994). Two functionally distinct half sites in the DNA-recognition sequence of the Myb oncoprotein. *Eur J Biochem, 222*(1), 113-120.
133. Pani, E., & Ferrari, S. (2008). p38MAPK delta controls c-Myb degradation in response to stress. *Blood Cells Mol Dis, 40*(3), 388-394. doi:10.1016/j.bcmd.2007.09.010
134. Pani, E., Menigatti, M., Schubert, S., Hess, D., Gerrits, B., Klempnauer, K. H., & Ferrari, S. (2008). Pin1 interacts with c-Myb in a phosphorylation-dependent manner and regulates its transactivation activity. *Biochim Biophys Acta, 1783*(6), 1121-1128. doi:10.1016/j.bbamcr.2008.02.020
135. Pattabiraman, D. R., McGirr, C., Shakhbazov, K., Barbier, V., Krishnan, K., Mukhopadhyay, P., . . . Gonda, T. J. (2014). Interaction of c-Myb with p300 is required for the induction of acute myeloid leukemia (AML) by human AML oncogenes. *Blood, 123*(17), 2682-2690. doi:10.1182/blood-2012-02-413187
136. Pattabiraman, D. R., Sun, J., Dowhan, D. H., Ishii, S., & Gonda, T. J. (2009). Mutations in multiple domains of c-Myb disrupt interaction with CBP/p300 and abrogate myeloid transforming ability. *Mol Cancer Res, 7*(9), 1477-1486. doi:10.1158/1541-7786.MCR-09-0070
137. Pelicci, P. G., Lanfrancone, L., Brathwaite, M. D., Wolman, S. R., & Dalla-Favera, R. (1984). Amplification of the c-myb oncogene in a case of human acute myelogenous leukemia. *Science, 224*(4653), 1117-1121.
138. Pereira, L. A., Hugo, H. J., Malaterre, J., Huiling, X., Sonza, S., Cures, A., . . . Ramsay, R. G. (2015). MYB elongation is regulated by the nucleic acid binding of NFkappaB p50 to the intronic stem-loop region. *PLoS One, 10*(4), e0122919. doi:10.1371/journal.pone.0122919
139. Persson, M., Andren, Y., Mark, J., Horlings, H. M., Persson, F., & Stenman, G. (2009). Recurrent fusion of MYB and NFIB transcription factor genes in carcinomas of the breast and head and neck. *Proc Natl Acad Sci U S A, 106*(44), 18740-18744. doi:10.1073/pnas.0909114106

140. Pichler, A., Gast, A., Seeler, J. S., Dejean, A., & Melchior, F. (2002). The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell, 108*(1), 109-120.
141. Prouse, M. B., & Campbell, M. M. (2012). The interaction between MYB proteins and their target DNA binding sites. *Biochim Biophys Acta, 1819*(1), 67-77. doi:10.1016/j.bbagrm.2011.10.010
142. Ramsay, R. G. (2005). c-Myb a stem-progenitor cell regulator in multiple tissue compartments. *Growth Factors, 23*(4), 253-261. doi:10.1080/08977190500233730
143. Ramsay, R. G., & Gonda, T. J. (2008). MYB function in normal and cancer cells. *Nat Rev Cancer, 8*(7), 523-534. doi:10.1038/nrc2439
144. Rodriguez, M. S., Dargemont, C., & Hay, R. T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem, 276*(16), 12654-12659. doi:10.1074/jbc.M009476200
145. Rodriguez-Castaneda, F., Lemma, R. B., Cuervo, I., Bengtsen, M., Moen, L. M., Ledsaak, M., . . . Gabrielsen, O. S. (2018). The SUMO protease SENP1 and the chromatin remodeler CHD3 interact and jointly affect chromatin accessibility and gene expression. *J Biol Chem, 293*(40), 15439-15454. doi:10.1074/jbc.RA118.002844
146. Rosonina, E., Akhter, A., Dou, Y., Babu, J., & Sri Theivakadadcham, V. S. (2017). Regulation of transcription factors by sumoylation. *Transcription, 8*(4), 220-231. doi:10.1080/21541264.2017.1311829
147. Saether, T., Berge, T., Ledsaak, M., Matre, V., Alm-Kristiansen, A. H., Dahle, O., . . . Gabrielsen, O. S. (2007). The chromatin remodeling factor Mi-2alpha acts as a novel co-activator for human c-Myb. *J Biol Chem, 282*(19), 13994-14005. doi:10.1074/jbc.M700755200
148. Saether, T., Pattabiraman, D. R., Alm-Kristiansen, A. H., Vogt-Kielland, L. T., Gonda, T. J., & Gabrielsen, O. S. (2011). A functional SUMO-interacting motif in the transactivation domain of c-Myb regulates its myeloid transforming ability. *Oncogene, 30*(2), 212-222. doi:10.1038/onc.2010.397
149. Sahin, U., de The, H., & Lallemand-Breitenbach, V. (2014). PML nuclear bodies: assembly and oxidative stress-sensitive sumoylation. *Nucleus, 5*(6), 499-507. doi:10.4161/19491034.2014.970104
150. Saikumar, P., Murali, R., & Reddy, E. P. (1990). Role of tryptophan repeats and flanking amino acids in Myb-DNA interactions. *Proc Natl Acad Sci U S A, 87*(21), 8452-8456.
151. Sakura, H., Kanei-Ishii, C., Nagase, T., Nakagoshi, H., Gonda, T. J., & Ishii, S. (1989). Delineation of three functional domains of the transcriptional activator encoded by the c-myb protooncogene. *Proc Natl Acad Sci U S A, 86*(15), 5758-5762.
152. Sampson, D. A., Wang, M., & Matunis, M. J. (2001). The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J Biol Chem, 276*(24), 21664-21669. doi:10.1074/jbc.M100006200
153. Sano, Y., & Ishii, S. (2001). Increased affinity of c-Myb for CREB-binding protein (CBP) after CBP-induced acetylation. *J Biol Chem, 276*(5), 3674-3682. doi:10.1074/jbc.M006896200

154. Schmidt, D., & Muller, S. (2003). PIAS/SUMO: new partners in transcriptional regulation. *Cell Mol Life Sci, 60*(12), 2561-2574. doi:10.1007/s00018-003-3129-1
155. Seeber, A., & Gasser, S. M. (2017). Chromatin organization and dynamics in double-strand break repair. *Curr Opin Genet Dev, 43*, 9-16. doi:10.1016/j.gde.2016.10.005
156. Sharma, P., Yamada, S., Lualdi, M., Dasso, M., & Kuehn, M. R. (2013). Senp1 is essential for desumoylating Sumo1-modified proteins but dispensable for Sumo2 and Sumo3 deconjugation in the mouse embryo. *Cell Rep, 3*(5), 1640-1650. doi:10.1016/j.celrep.2013.04.016
157. Sharrocks, A. D. (2006). PIAS proteins and transcriptional regulation--more than just SUMO E3 ligases? *Genes Dev, 20*(7), 754-758. doi:10.1101/gad.1421006
158. Shen, L. N., Dong, C., Liu, H., Naismith, J. H., & Hay, R. T. (2006). The structure of SENP1-SUMO-2 complex suggests a structural basis for discrimination between SUMO paralogues during processing. *Biochem J, 397*(2), 279-288. doi:10.1042/BJ20052030
159. Song, J., Durrin, L. K., Wilkinson, T. A., Krontiris, T. G., & Chen, Y. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A, 101*(40), 14373-14378. doi:10.1073/pnas.0403498101
160. Sramko, M., Markus, J., Kabat, J., Wolff, L., & Bies, J. (2006). Stress-induced inactivation of the c-Myb transcription factor through conjugation of SUMO-2/3 proteins. *J Biol Chem, 281*(52), 40065-40075. doi:10.1074/jbc.M609404200
161. Sriramachandran, A. M., & Dohmen, R. J. (2014). SUMO-targeted ubiquitin ligases. *Biochim Biophys Acta, 1843*(1), 75-85. doi:10.1016/j.bbamcr.2013.08.022
162. Stadhouders, R., Thongjuea, S., Andrieu-Soler, C., Palstra, R. J., Bryne, J. C., van den Heuvel, A., . . . Soler, E. (2012). Dynamic long-range chromatin interactions control Myb proto-oncogene transcription during erythroid development. *EMBO J, 31*(4), 986-999. doi:10.1038/emboj.2011.450
163. Stehmeier, P., & Muller, S. (2009). Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. *Mol Cell, 33*(3), 400-409. doi:10.1016/j.molcel.2009.01.013
164. Su, H. L., & Li, S. S. (2002). Molecular features of human ubiquitin-like SUMO genes and their encoded proteins. *Gene, 296*(1-2), 65-73.
165. Su, Y. F., Yang, T., Huang, H., Liu, L. F., & Hwang, J. (2012). Phosphorylation of Ubc9 by Cdk1 enhances SUMOylation activity. *PLoS One, 7*(4), e34250. doi:10.1371/journal.pone.0034250
166. Sullivan, J., Feeley, B., Guerra, J., & Boxer, L. M. (1997). Identification of the major positive regulators of c-myb expression in hematopoietic cells of different lineages. *J Biol Chem, 272*(3), 1943-1949.
167. Sun, H., Leverson, J. D., & Hunter, T. (2007). Conserved function of RNF4 family proteins in eukaryotes: targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J, 26*(18), 4102-4112. doi:10.1038/sj.emboj.7601839

168. Tahirov, T. H., Sato, K., Ichikawa-Iwata, E., Sasaki, M., Inoue-Bungo, T., Shiina, M., . . . Ogata, K. (2002). Mechanism of c-Myb-C/EBP beta cooperation from separated sites on a promoter. *Cell, 108*(1), 57-70.
169. Tanikawa, J., Yasukawa, T., Enari, M., Ogata, K., Nishimura, Y., Ishii, S., & Sarai, A. (1993). Recognition of specific DNA sequences by the c-myb protooncogene product: role of three repeat units in the DNA-binding domain. *Proc Natl Acad Sci U S A, 90*(20), 9320-9324.
170. Tatham, M. H., Geoffroy, M. C., Shen, L., Plechanovova, A., Hattersley, N., Jaffray, E. G., . . . Hay, R. T. (2008). RNF4 is a poly-SUMO-specific E3 ubiquitin ligase required for arsenic-induced PML degradation. *Nat Cell Biol, 10*(5), 538-546. doi:10.1038/ncb1716
171. Tatham, M. H., Jaffray, E., Vaughan, O. A., Desterro, J. M., Botting, C. H., Naismith, J. H., & Hay, R. T. (2001). Polymeric chains of SUMO-2 and SUMO-3 are conjugated to protein substrates by SAE1/SAE2 and Ubc9. *J Biol Chem, 276*(38), 35368-35374. doi:10.1074/jbc.M104214200
172. Tatham, M. H., Kim, S., Jaffray, E., Song, J., Chen, Y., & Hay, R. T. (2005). Unique binding interactions among Ubc9, SUMO and RanBP2 reveal a mechanism for SUMO paralog selection. *Nat Struct Mol Biol, 12*(1), 67-74. doi:10.1038/nsmb878
173. Tatham, M. H., Kim, S., Yu, B., Jaffray, E., Song, J., Zheng, J., . . . Chen, Y. (2003). Role of an N-terminal site of Ubc9 in SUMO-1, -2, and -3 binding and conjugation. *Biochemistry, 42*(33), 9959-9969. doi:10.1021/bi0345283
174. Thompson, M. A., Flegg, R., Westin, E. H., & Ramsay, R. G. (1997). Microsatellite deletions in the c-myb transcriptional attenuator region associated with over-expression in colon tumour cell lines. *Oncogene, 14*(14), 1715-1723. doi:10.1038/sj.onc.1201007
175. Thompson, M. A., Rosenthal, M. A., Ellis, S. L., Friend, A. J., Zorbas, M. I., Whitehead, R. H., & Ramsay, R. G. (1998). c-Myb down-regulation is associated with human colon cell differentiation, apoptosis, and decreased Bcl-2 expression. *Cancer Res, 58*(22), 5168-5175.
176. Tober, J., McGrath, K. E., & Palis, J. (2008). Primitive erythropoiesis and megakaryopoiesis in the yolk sac are independent of c-myb. *Blood, 111*(5), 2636-2639. doi:10.1182/blood-2007-11-124685
177. Tomita, A., Towatari, M., Tsuzuki, S., Hayakawa, F., Kosugi, H., Tamai, K., . . . Saito, H. (2000). c-Myb acetylation at the carboxyl-terminal conserved domain by transcriptional co-activator p300. *Oncogene, 19*(3), 444-451. doi:10.1038/sj.onc.1203329
178. Torelli, G., Venturelli, D., Colo, A., Zanni, C., Selleri, L., Moretti, L., . . . Torelli, U. (1987). Expression of c-myb protooncogene and other cell cycle-related genes in normal and neoplastic human colonic mucosa. *Cancer Res, 47*(20), 5266-5269.
179. Ullmann, R., Chien, C. D., Avantaggiati, M. L., & Muller, S. (2012). An acetylation switch regulates SUMO-dependent protein interaction networks. *Mol Cell, 46*(6), 759-770. doi:10.1016/j.molcel.2012.04.006
180. van Driel, R., Fransz, P. F., & Verschure, P. J. (2003). The eukaryotic genome: a system regulated at different hierarchical levels. *J Cell Sci, 116*(Pt 20), 4067-4075. doi:10.1242/jcs.00779

181. Walker, M. J., Silliman, E., Dayton, M. A., & Lang, J. C. (1998). The expression of C-myb in human metastatic melanoma cell lines and specimens. *Anticancer Res, 18*(2A), 1129-1135.
182. Wallrapp, C., Muller-Pillasch, F., Solinas-Toldo, S., Lichter, P., Friess, H., Buchler, M., . . . Gress, T. M. (1997). Characterization of a high copy number amplification at 6q24 in pancreatic cancer identifies c-myb as a candidate oncogene. *Cancer Res, 57*(15), 3135-3139.
183. Wan, J., Subramonian, D., & Zhang, X. D. (2012). SUMOylation in control of accurate chromosome segregation during mitosis. *Curr Protein Pept Sci, 13*(5), 467-481.
184. Watson, J. D., & Crick, F. H. (1953). Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature, 171*(4356), 737-738.
185. Westman, B. J., Verheggen, C., Hutten, S., Lam, Y. W., Bertrand, E., & Lamond, A. I. (2010). A proteomic screen for nucleolar SUMO targets shows SUMOylation modulates the function of Nop5/Nop58. *Mol Cell, 39*(4), 618-631. doi:10.1016/j.molcel.2010.07.025
186. Weston, K. (1998). Myb proteins in life, death and differentiation. *Curr Opin Genet Dev, 8*(1), 76-81.
187. Weston, K., & Bishop, J. M. (1989). Transcriptional activation by the v-myb oncogene and its cellular progenitor, c-myb. *Cell, 58*(1), 85-93.
188. Winn, L. M., Lei, W., & Ness, S. A. (2003). Pim-1 phosphorylates the DNA binding domain of c-Myb. *Cell Cycle, 2*(3), 258-262.
189. Woo, C. H., Sopchak, L., & Lipsick, J. S. (1998). Overexpression of an alternatively spliced form of c-Myb results in increases in transactivation and transforms avian myelomonoblasts. *J Virol, 72*(8), 6813-6821.
190. Wu, C. S., Ouyang, J., Mori, E., Nguyen, H. D., Marechal, A., Hallet, A., . . . Zou, L. (2014). SUMOylation of ATRIP potentiates DNA damage signaling by boosting multiple protein interactions in the ATR pathway. *Genes Dev, 28*(13), 1472-1484. doi:10.1101/gad.238535.114
191. Xiao, C., Calado, D. P., Galler, G., Thai, T. H., Patterson, H. C., Wang, J., . . . Rajewsky, K. (2007). MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. *Cell, 131*(1), 146-159. doi:10.1016/j.cell.2007.07.021
192. Zhang, B., Wang, Q., & Pan, X. (2007). MicroRNAs and their regulatory roles in animals and plants. *J Cell Physiol, 210*(2), 279-289. doi:10.1002/jcp.20869
193. Zhao, H., Jin, S., & Gewirtz, A. M. (2012). The histone acetyltransferase TIP60 interacts with c-Myb and inactivates its transcriptional activity in human leukemia. *J Biol Chem, 287*(2), 925-934. doi:10.1074/jbc.M111.279950
194. Zhao, H., Kalota, A., Jin, S., & Gewirtz, A. M. (2009). The c-myb proto-oncogene and microRNA-15a comprise an active autoregulatory feedback loop in human hematopoietic cells. *Blood, 113*(3), 505-516. doi:10.1182/blood-2008-01-136218
195. Zhao, L., Glazov, E. A., Pattabiraman, D. R., Al-Owaidi, F., Zhang, P., Brown, M. A., . . . Gonda, T. J. (2011). Integrated genome-wide chromatin occupancy and expression analyses identify key myeloid pro-

differentiation transcription factors repressed by Myb. *Nucleic Acids Res, 39*(11), 4664-4679. doi:10.1093/nar/gkr024
196. Zhao, X. (2018). SUMO-Mediated Regulation of Nuclear Functions and Signaling Processes. *Mol Cell, 71*(3), 409-418. doi:10.1016/j.molcel.2018.07.027
197. Zhou, Y., & Ness, S. A. (2011). Myb proteins: angels and demons in normal and transformed cells. *Front Biosci (Landmark Ed), 16*, 1109-1131.