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Structural biology of transcriptional regulation in the c-Myc network

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Cover: HSQC spectra of Ser62 phosphorylated c-Myc₁₋₈₈.

During the course of the research underlying this thesis, Sara Helander was enrolled in Forum Scientium, a multidisciplinary doctoral program at Linköping University, Sweden.

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Just Do It

Abstract

The oncogene c-Myc is overexpressed in many types of human cancers and regulation of c-Myc expression is crucial in a normal cell. The intrinsically disordered N-terminal transactivation domain interacts with a wide range of proteins regulating c-Myc activity. The highly conserved Myc box I region includes residues Thr58 and Ser62, which are involved in the phosphorylation events that control c-Myc degradation by ubiquitination. Aggressive cell growth, leading to tumor formation, occurs if activated c-Myc is not degraded by ubiquitination. Such events may be triggered by defects in the regulated network of interactions involving Pin1 and phospho-dependent kinases.

In this thesis, the properties of the intrinsically disordered unphosphorylated c-Myc₁₋₈₈ and its interaction with Bin1 are studied by nuclear magnetic resonance (NMR) spectroscopy and surface plasmon resonance (SPR). Furthermore, the interaction of Myc₁₋₈₈ with Pin1 is analyzed in molecular detail, both for unphosphorylated and Ser62 phosphorylated c-Myc₁₋₈₈, providing a first molecular description of a disordered but specific c-Myc complex. A detailed analysis of the dynamics and structural properties of the transcriptional activator TAF in complex with TBP, both by NMR spectroscopy and crystallography, provides insight into transcriptional regulation and how c-Myc could interact with TBP. Finally, the structure of a novel N-terminal domain motif in FKBP25, which we name the Basic Tilted Helix Bundle (BTHB) domain, and its binding to YY1, which also binds c-Myc, is described. By investigating the structural and dynamic properties of c-Myc and c-Myc-interacting proteins, this thesis thus provides further insight to the molecular basis for c-Myc functionality in transcriptional regulation.

Populärvetenskaplig sammanfattning

Vår kropp är ett komplext system. Vi ska kunna röra oss, hormonsystemet ska fungera och vårt immunförsvar ska skydda oss mot bakterier och virus. Proteiner är involverade i alla dessa processer och i våra celler finns många olika typer av proteiner. Proteiner består av aminosyror och aminosyrorna sitter ihop som på ett långt pärlband. Beroende på i vilken ordning aminosyrorna sitter så kommer pärlbandet av aminosyror att veckas ihop olika mellan olika proteiner. Detta ger varje protein en speciell struktur och därmed en speciell funktion i kroppen. Proteiner är inte statiska, de är rörliga och det bidrar också till funktionen. Vissa proteiner är extremt rörliga eftersom de inte veckas ihop lika mycket som andra proteiner. Om proteinerna inte får sin rätta struktur och inte kan utföra sin uppgift så leder det ofta till sjukdomar, till exempel cancer.

I denna avhandling har vi studerat c-Myc samt proteiner som ingår i nätverket kring c-Myc. Om c-Myc inte kan brytas ner så blir mängden av proteinet för hög i kroppen, vilket i slutändan leder till för hög celltillväxt och cancertumörer. Vi har studerat en del av c-Myc som är väldigt flexibel och involverad i regleringen av andra proteiner i kroppen. Vi har med hjälp av kärnmagnetisk resonansspektroskopi (NMR) kunnat göra en molekylär karta över aminosyrorna som ingår i den flexibla delen av c-Myc och vi har studerat proteinets rörlighet och struktur. Vidare har vi studerat hur c-Myc samverkar med det tumörinhiberande proteinet Bin1. Vi har även tittat på de mekanismer som styr nedbrytningen av c-Myc genom att studera interaktion mellan c-Myc och Pin1, ett protein som är mycket viktigt för nedbrytningen av c-Myc.

Våra studier har bidragit till en ökad kunskap kring c-Myc och dess molekylära funktion, vilket i slutändan leder till en ökad förståelse för c-Mycs roll i cancer.

List of publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV).

- I** Andresen, C., **S. Helander**, A. Lemak, C. Farés, V. Csizmok, J. Carlsson, LZ. Penn., JD. Forman-Kay, CH. Arrowsmith, P. Lundström, M. Sunnerhagen (2012). *"Transient structure and dynamics in the disordered c-Myc transactivation domain affect Bin1 binding."*
Nucleic Acids Research, NAR **40**(13): 6353-6366.
- II** Anandapadamanaban, M., C. Andresen*, **S. Helander***, Y. Ohyama, MI. Siponen, P. Lundström, T. Kokubo, M. Ikura, M. Moche, M. Sunnerhagen (2013). *"High-resolution structure of TBP with TAF1 reveals anchoring patterns in transcriptional regulation."*
Nature Structural & Molecular Biology, NSMB **20**(8): 1008-1014.
*These authors contributed equally to the work.
- III** **Helander S***, Montecchio M*, Lemak A., Farès C., Almlöf J., Li Y., Yee A., Arrowsmith CH., Dhe-Paganon S., Sunnerhagen M. et al. *"Basic Tilted Helix Bundle - A new protein fold in human FKBP25/FKBP3 and HectD1."*
Biochemical and Biophysical Research Communications, BBRC, *in press*.
*These authors contributed equally to the work.
- IV** **Helander S.**, Su Y., Montecchio M., Pilstål R., Johansson M., Kuruvilla J., Cristobal S., Wallner B., Sears R., Lundström P., Sunnerhagen M. *"Pre-anchoring of Pin1 to unphosphorylated c-Myc in a dynamic complex affects c-Myc stability and activity."*
Pending submission to Nature Structure and Molecular Biology, NSMB.

Papers not included in the thesis

- V William B. Tu, **Sara Helander**, Robert Pilstål, Ashley Hickman, Corey Lourenco, Igor Jurisica, Brian Raught, Björn Wallner, Maria Sunnerhagen, Linda Z. Penn “*Myc and its interactors take shape.*” BBA Gene Regulatory Mechanisms, *submitted*.

Contribution report

Paper I: I performed the SPR experiments, interpreted data and summarized the results. I did a part of the protein purification and I actively participated in the project discussions, in particular regarding the integration of results from SPR and NMR. In the article, I wrote the SPR part.

Paper II: I performed the NMR relaxation experiments, evaluated and summarized the data. I actively participated in the discussions regarding the project and took an active part in the writing process.

Paper III: I purified protein (YY1), evaluated structural and bioinformatic data on a functional level, and experimentally performed and evaluated the FKBP25-YY1 binding. I took an active part in the writing, in putting together the different parts of the article, in communicating with co-authors and in submitting the paper.

Paper IV: From the start of this investigation, I have been highly involved in setting up the hypothesis and experimental strategies, in setting up and pursuing experiments, and in discussing with collaborators. I purified proteins and planned and performed the NMR and SPR experiments, and evaluated and summarized data. I supervised diploma students with projects connected to the study. I participated and took an active part in the discussions and I played a major role in the writing and in finalizing of the manuscript.

Abbreviations

APP	Amyloid precursor protein
ATP	Adenosine triphosphate
Bin1	Bridging integrator protein 1
c-Myc	Cellular myelocytomatosis oncogene
CBP	CREB-binding protein
CD	Circular dichroism
Cdk2/4	Cyclin-dependent kinase 2/4
CHIP	C terminus of HSC70-interacting protein
CPMG	Carr-Purcell-Meiboom-Gill
CSA	Chemical shift anisotropy
CSP	Chemical shift perturbation
Cyps	Cyclophilins
E-box	Enhancer box
ERK	Extracellular receptor kinases
Fbw7	F-box/WD repeat-containing protein 7
FID	Free induced decay
FKBPs	FK506-binding proteins
FT	Fourier transform
Gsk3 β	Glycogen synthase kinase beta
GTPase	GTPase activating proteins
HAT	Histone acetylation complex
KID	Kinase inducible domain
L-Myc	Lung carcinoma myelocytomatosis oncogene
Max	Myc-associated factor X
MBI-IV	Myc homology box I-IV
Mdm2	Mouse double minute 2 homolog
Miz-1	Myc-interacting zinc finger protein 1
Mnt	Max network transcriptional repressor
mRNA	Messenger RNA
N-Myc	Neuroblastoma myelocytomatosis oncogene
NMR	Nuclear magnetic resonance
PI3K	Phosphatidylinositol 3-kinase
PIC	Preinitiation complex
Pin1	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1

PP2A	Protein phosphatase
PPIs	Peptidyl-proline isomerases
rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
siRNA	Small interfering RNA
Skp2	S-phase kinase-associated protein 2
SPR	Surface plasmon resonance
TAFs	TBP-associated factors
TBP	TATA-binding protein
TGF- β	Transforming growth factor beta
tRNA	Transfer RNA
TRRAP	Transactivation/transformation-associated protein
v-Myc	Myelocytomatosis viral oncogene
WW	Trp-Trp binding module
YY1	Yin yang 1

Amino acids

Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid
Cys, C	Cysteine
Glu, E	Glutamic acid
Gln, Q	Glutamine
Gly, G	Glycine
His, H	Histidine
Ile, I	Isoleucine
Leu, L	Leucine
Lys, K	Lysine
Met, M	Methionine
Phe, F	Phenylalanine
Pro, P	Proline
Ser, S	Serine
Thr, T	Threonine
Trp, W	Tryptophan

Tyr, Y
Val, V

Tyrosine
Valine

Contents

PREFACE	1
1. INTRODUCTION	3
1.1 PROTEIN STRUCTURE	3
1.2 INTRINSICALLY DISORDERED PROTEINS.....	5
1.2.1 <i>Function of intrinsically disordered proteins</i>	7
2. THE C-MYC ONCOPROTEIN	9
2.1 THE MYC FAMILY AND THE ROLE IN HUMAN CANCERS.....	9
2.2 CONSERVED REGIONS AND THE INTERACTION WITH COFACTORS.....	10
2.2.1 <i>The Myc transactivation domain</i>	12
2.3 TRANSCRIPTIONAL ACTIVATION AND REPRESSION	13
2.3.1 <i>Transcriptional activation</i>	14
2.3.2 <i>Transcriptional repression</i>	16
2.4 BIOLOGICAL ACTIVITIES OF C-MYC	17
2.4.1 <i>Cell cycle</i>	18
2.4.2 <i>Cell growth, differentiation, apoptosis and cellular transformation</i>	18
2.5 REGULATION OF C-MYC STABILITY AND ACTIVITY	19
2.5.1 <i>Phosphorylation sites</i>	20
2.5.2 <i>Phosphorylation at Ser62 and Thr58</i>	20
2.5.2 <i>Ubiquitination and degradation</i>	21
2.5 C-MYC AS A THERAPEUTIC TARGET.....	22
3. PEPTIDYL-PROLYL ISOMERASES.....	25
3.1 PEPTIDYL-PROLYL <i>CIS-TRANS</i> ISOMERASES	25
3.1 PIN1.....	26
3.1.1 <i>Structure</i>	26
3.1.1 <i>Pin1 and cellular regulation</i>	27
3.2 FK506 BINDING PROTEINS.....	29
3.2.1 <i>FKBP25</i>	29
3.2.2 <i>Role in chromatin modification and human cancer</i>	30
4. METHODOLOGY	33
4.1 CIRCULAR DICHROISM SPECTROSCOPY	33

4.1.1 Secondary structure evaluation.....	35
4.1.2 Thermal stability evaluation.....	36
4.2 SURFACE PLASMON RESONANCE.....	36
4.3 NUCLEAR MAGNETIC RESONANCE	38
4.3.1 Theory.....	38
4.3.2 Resonance assignment.....	41
4.3.3 Dynamics.....	43
4.3.4 Interaction analysis using NMR.....	47
5. SUMMARY OF PAPERS	49
6. CONCLUSIONS.....	53
7. FUTURE PERSPECTIVES	55
ACKNOWLEDGMENTS	57
REFERENCES.....	61

Preface

Last year, during a lecture for teenagers visiting the chemistry department, I got the question: "Did you already decide to be a PhD and do research when you were our age (13-14 years old)?" My answer was: "No, at that age I had never heard about it!" Today, I know a lot more and during the years as a PhD student I have been fortunate to work with great scientists, both in national and international collaborations. Science never stops and successful, as well as unsuccessful, experiments increase our knowledge but in addition they usually lead to more curiosity and even more questions. This is a part of the deal and pushes the research more and more forward towards the goal.

This thesis summarizes the results obtained during my journey as a PhD student. Chapters 1 to 4 are intended to give the reader an introductory background and literature overview to the appended papers. During the years, the research has been focused on structural biology studies on the c-Myc protein along with studies on proteins associated with the c-Myc protein. A brief summary of the findings and conclusions can be found in Chapter 5 and 6, as well as in more detail in the appended papers. Chapter 7 discusses future perspectives and unsolved questions related to the c-Myc protein. I hope you will enjoy reading the thesis!

Linköping, April 2014

1. Introduction

1.1 Protein structure

In year 1838, the well-known Swedish chemist Jöns Jacob Berzelius, who originated from Linköping, suggested the word “protein” in a letter addressed to his Dutch colleague Gerardus Johannes Mulder (Vickery 1950). Proteins are essential for life and crucial for vital processes in our body. Amino acids are the building blocks of proteins and their amino acid composition, together with the fold of the protein, is essential for protein function. The 20 different amino acids are small molecules composed of nitrogen, carbon, oxygen, and hydrogen. In addition, cysteine and methionine also contain sulfur. When joined together, forming a peptide bond with the carboxyl group from one amino acid and the amine group from the second amino acid, the protein backbone is formed. The side chain of each amino acid protrudes out from the backbone (**Figure 1**). Each protein has a unique order of the amino acids, referred as the primary structure of a protein (Creighton 1993; Williamson 2012).

Figure 1, The protein backbone. R₁ and R₂ represent the side chain for each amino acid.

The next level of protein structure is the secondary structure. The two main secondary structure elements are the α -helix and the β -sheet (**Figure 2**). The planarity of the peptide bond restricts the conformational space and thereby the packing of the polypeptide. Furthermore, backbone hydrogen bonds are formed between the carbonyl oxygen and the amide group, thus stabilizing the secondary structure elements. For α -helices, hydrogen bonding is formed between oxygen of residue i to the amine nitrogen of residue $i+4$. The amino acid side chain protrudes out from the helix and each turn in the helix consists of 3.6 residues/turn. The α -helix has a dipole moment due to the polarization of the amide and carbonyl bonds, and since the amide NH group points towards the N-terminal end and the carbonyl group towards the C-terminal end this results in a positive N-terminal and negative C-terminal. The second type of secondary structure, β -sheets, is made up of several parallel or antiparallel β -strands. Antiparallel β -strands are most common and here, the stabilizing hydrogen bonds are perpendicular to the direction of the β -strands, while they are more asymmetrical in parallel β -sheets (Creighton 1993; Williamson 2012).

Figure 2, Secondary structure elements, α -helix to the left and antiparallel β -sheet in the middle. The tertiary structure of human Pin1 is shown to the right (PDB ID: 1PIN).

The arrangement of the secondary structure elements in space forms the tertiary structure of a protein (**Figure 2**). The secondary and tertiary structure is important for protein function, although an increasing number of intrinsically disordered proteins have been found (discussed in section 1.2).

Proteins are not rigid bodies and protein dynamics are essential for protein function. As discussed in section 4.3.3, proteins display dynamics on different time-scales ranging from fast picosecond motions (bond vector vibrations) to slow motions on the microsecond time-scale (conformational rearrangements). Protein dynamics are important for protein folding, protein-protein interactions and enzyme catalysis (Henzler-Wildman and Kern 2007; Mittag, Kay et al. 2010; Williamson 2012).

1.2 Intrinsically disordered proteins

During the last 15 years a growing class of proteins have been studied: the intrinsically disordered proteins (IDPs) (Dunker, Lawson et al. 2001). Contrary to classically folded proteins, IDPs are partially disordered or fully disordered in the functional state and the lack of a stable tertiary structure is required for correct function of the protein (Dyson and Wright 2005).

IDP sequences have a low frequency of hydrophobic amino acids but a high proportion of Ser, Gly, Pro, Asn and Gln or charged amino acids, Lys, Arg, Glu and Asp (Dyson 2011). Usually, hydrophobic residues such as Trp, Tyr,

Phe and Leu are found within motifs that recognize binding partners (Fuxreiter, Tompa et al. 2007; Brown, Johnson et al. 2010). Furthermore, the sequences commonly contain motifs that can be recognized by enzymes, for instance kinases, responsible for posttranslational modifications (Iakoucheva, Radivojac et al. 2004).

Disordered proteins or regions of proteins do not display a single, well-structured tertiary conformation. Instead they can adopt several stable conformations, referred to as static disorder, or they can be described as a structural ensemble of interconverting conformations, referred to as dynamic disorder (Tompa and Fuxreiter 2008).

Many IDPs fold into various structures upon binding with different interacting partners, a process named as “folding upon binding” or “coupled folding and binding”. Mechanistically, two possibilities appear; induced folding or conformational selection. For induced folding, the disordered protein interacts with its binding partner in a fully disordered state and the association with the target protein induces folding. For conformational selection, the association partner ‘selects’ the most favorable conformation in the conformational ensemble of the disordered protein. Binding induces a population shift towards the ‘selected’ state, resulting in a redistribution of the population ensemble. This shift is necessary for retaining the equilibrium and continues the binding reaction towards the binding state (Nussinov, Ma et al. 2014).

Even if many IDPs have been shown to fold upon binding, there are examples of IDPs that are disordered even in the bound state and form so called ‘fuzzy’ complexes with their binding partners. Disorder in the bound state can be both static and dynamic leading to different categories of disorder, ‘fuzziness’, in the partner-bound state (Tompa and Fuxreiter 2008). In the ‘polymorphic model’, the fuzziness is described as a static fuzziness where the disordered protein adopts several stable conformations, referred as static disorder in the previous section. Moreover, Tompa et al. further categorize the second type of model, the dynamic disorder into: ‘clamp’, ‘flanking’ and ‘random’ models. The clamp model

consists of proteins with two bound and folded regions that are connected by a disordered linker. Upon binding, the linker remains disordered and favors binding by limiting the conformational freedom for the two folded domains (Tompa and Fuxreiter 2008). The importance of this type of fuzziness is shown in studies where absence of the linker or shortening the linker abolishes binding or decreases the binding affinity (van Leeuwen, Strating et al. 1997; Rock, Ramamurthy et al. 2005). In the flanking model, disordered segments that maintain disorder in the bound state flank short binding elements, which become ordered upon binding. Deleting the flanking regions may reduce binding affinity. For instance, deletion of the flanking segments in the disordered kinase inducible domain, KID, reduces binding to the KIX domain of the CREB-binding protein, CBP (Zor, Mayr et al. 2002). In a couple of cases, the whole protein remains disordered in the bound state. Tompa and coworkers refer to this type of fuzziness as the 'random' model (Tompa and Fuxreiter 2008). This kind of fuzziness has been shown for the disordered protein Sic1 in the complex with Cdc4 and for the regulatory R region of the CFTR protein, associated with cystic fibrosis (Mittag, Orlicky et al. 2008; Bozoky, Krzeminski et al. 2013).

1.2.1 Function of intrinsically disordered proteins

Along with the discoveries of intrinsic disorder for a large number of proteins, the classical view, connecting protein fold and function has been extended towards a broader picture of protein fold and function. The intrinsic disorder can be a part of the function and IDPs have been related to a range of functions such as transcriptional regulation, cellular signal transduction and protein phosphorylation. The ability to bind a multitude of structurally diverse partners is an advantage in interaction networks, further emphasizing the role of IDPs in transcription and cellular signaling (Dunker, Cortese et al. 2005; Dyson 2011). In addition, many IDPs contain multiple binding motifs, allowing them to act as 'hubs' in interaction networks (Forman-Kay and Mittag 2013). Furthermore, intrinsic disorder has been suggested to correlate with chaperone function and disordered segments are found in a wide range of chaperones (Tompa 2012).

A large number of IDPs have been correlated with diseases. In addition to the proto-oncogene c-Myc, which is discussed later in this thesis, the tumor suppressor p53 comprises an intrinsically disordered N-terminal (Ayed, Mulder et al. 2001; Bell, Klein et al. 2002; Wells, Tidow et al. 2008). Moreover, the regulatory R region of the cystic fibrosis protein CFTR, remains disordered in the bound state (Bozoky, Krzeminski et al. 2013). Misfolding of IDPs can also occur, where the protein forms insoluble aggregates or amyloids, as exemplified by α -synuclein, Tau and A β that have been associated with Parkinson's and Alzheimer's disease (Uversky 2009).

2. The c-Myc oncoprotein

2.1 The myc family and the role in human cancers

One of the most studied groups of genes is the Myc oncogene family, comprising c-Myc, N-Myc, L-Myc, B-Myc and S-Myc. c-Myc, N-Myc and L-Myc have transforming activity and N-Myc and L-Myc were first found in neuroblastoma and lung cancer, respectively (Oster, Ho et al. 2002; Meyer and Penn 2008). Despite the fact that the c-Myc protein has been studied for more than 30 years, many questions remain regarding c-Myc and its role in human cancer.

The human c-Myc was discovered in the beginning of the 1980s and the protein was originally discovered as the homolog v-gag-myc, present in myelocytomatosis virus (Lee and Reddy 1999; Meyer and Penn 2008). Since then, c-Myc has been shown to be overexpressed in many types of human cancers. Recent tumor sequencing results shows that c-Myc is one of the most amplified genes in many cancer types, and tumors from breast cancers show a high degree of c-Myc driven cell proliferation (Ciriello, Miller et al. 2013). Regulation of c-Myc expression is crucial for obtaining normal cell functions and since it regulates the transcription of a wide range of genes; even small changes may influence the cell growth, proliferation, apoptosis, differentiation and transformation (Meyer and Penn 2008; Levens 2010).

2.2 Conserved regions and the interaction with cofactors

The C-terminal part of c-Myc contains a basic helix-loop-helix-leucine zipper (bHLH-LZ) motif (**Figure 3**), which upon interaction with the bHLH-LZ motif of Max, forms a c-Myc/Max heterodimer (**Figure 4**). N-terminal to the HLH-LZ motif is the basic region (BR) (a.a. 355-369), which is involved in the c-Myc/Max binding to DNA but also necessary for full transformation of primary immortal cells (Meyer and Penn 2008). The c-Myc/Max heterodimer binds to specific DNA sequences (5'-CACGTG-3') named enhancer boxes (E-box) (**Figure 4**) (Blackwood and Eisenman 1991; Nair and Burley 2003).

Heterodimerization with Max is necessary for c-Myc DNA binding and c-Myc is not able to form homodimers (Lavigne, Crump et al. 1998). As opposed to c-Myc, Max is able to homodimerize and bind DNA E-boxes. The biological role of Max/Max homodimers are unclear, but they are suggested to have a role in transcriptional repression (Kretzner, Blackwood et al. 1992) although other studies show that Max/Mad heterodimers promotes transcriptional repression, while the effect cannot be achieved by Max homodimers (Yin, Grove et al. 1998).

The expression levels of c-Myc, Max and Mad regulate the transcription of their targets genes. The expression of Max seems to be constant, and the c-Myc/Max heterodimer favors the transcription of many genes involved in cell proliferation, while the Max/Mad heterodimer is found in growth-arrested cells that lack c-Myc expression. In addition to the Max interaction, the HLH-LZ motif has been shown to mediate c-Myc gene repression through the interaction with Miz-1 (Peukert, Staller et al. 1997). Furthermore, TRPC4AP/TRUSS complex suppresses c-Myc transactivation and transformation by binding to the C-terminal domain (Choi, Wright et al. 2010).

Figure 3, Schematic illustration of c-Myc showing the conserved regions and the interaction with co-factors discussed in the main text. The N-terminal transactivation domain (TAD) includes NC1, MBI and MBII. The central region contains MBIIIa and MBIIIb followed by the C-terminal domain comprising MBIV, BR and HLH-LZ. Adapted from Tu et al. 2014, submitted.

In addition to the HLH-LZ and BR motif, c-Myc is composed of four Myc homology boxes, named Myc Box I-IV (MBI-IV) (**Figure 3**). The regions are highly conserved between c-Myc, N-Myc and L-Myc and across species (Cowling and Cole 2006).

The homology boxes MBIV (a.a. 304-324), MBIIIa (a.a. 188-199) and MBIIIb (a.a. 259-270) are part of the central domain, which is followed by the transactivation domain (TAD) comprising MBI and MBII (see section 2.2.1) (**Figure 3**). Most of the interactions have been mapped to MBI and MBII. But some interactions have been mapped to MBIV and the two MBIII boxes. For example YY1 interacts with c-Myc bHLH-LZ, MBIV and MBIIIb and inhibits transformation (Austen, Cerni et al. 1998).

Figure 4, Crystal structure of the c-Myc/Max heterodimer and the interaction with DNA. c-Myc is shown in blue, Max in grey and DNA in light orange. The zipper, helix-loop-helix and basic region are indicated with dashed circles (PDB ID; 1NKP).

2.2.1 The Myc transactivation domain

The N-terminal transactivation domain (TAD) (a.a. 1-143) interacts with a wide range of proteins, thereby regulating c-Myc activity (Kato, Barrett et al. 1990). Two Myc boxes are found within the TAD domain, MBI (a.a. 44-63) and MBII (a.a. 128-143). MBII is essential for c-Myc transforming activity and transcriptional activation and repression, since it interacts with a wide range of co-factors (**Figure 3**). Among those, the large protein complex TRRAP interacts with MBII in c-Myc, thereby facilitating c-Myc recruitment of histone acetylation complex (HAT) to chromatin (McMahon, Wood et al. 2000). Furthermore, the interaction with TRRAP is essential for c-Myc transformation (McMahon, Van Buskirk et al. 1998). Our study (paper I) identifies transient structure N-terminal to MBI, in a region that has earlier been named NC1 and which is conserved between several members of the Myc family (DePinho, Legouy et al. 1986; Sugiyama, Kume et al. 1989). Interestingly, this region is essential for TRRAP binding (McMahon, Van Buskirk et al. 1998) and in addition we show that this region interacts with Pin1 (paper IV).

Structural characterization of the N-terminal part has been a challenge. Even if the structure for the C-terminal part of c-Myc is known, structural

details for full-length c-Myc are still missing. Our studies (paper I), show that the c-Myc TAD function as an intrinsically disordered protein, comprising transient structure in both NC1 and MBI (**Figure 3**) (Fladvad, Zhou et al. 2005; Andresen, Helander et al. 2012). Previous circular dichroism (CD) studies of a c-Myc construct, covering MBII, shows a partly helical fold where the structural content is increased upon interaction with the co-factors TBP and MM1 (McEwan, Dahlman-Wright et al. 1996; Fladvad, Zhou et al. 2005). Contrary to the MBII constructs, the MBI-containing construct c-Myc₁₋₈₈ shows an overall random coil structure (Fladvad, Zhou et al. 2005). Our recent studies, discussed in detail in paper I, reveal a dynamic transient structure around amino acid 22-33 as well as for MBI (Andresen, Helander et al. 2012). Two phosphorylation sites, Thr58 and Ser62 are found within MBI and co-factors interacting with this region are most often found to be sensitive to phosphorylation. For example, phosphorylation at Thr58 and/or Ser62 mediates c-Myc degradation by Pin1, and Fbw7 (for details see section 2.5) (Yada, Hatakeyama et al. 2004; Yeh, Cunningham et al. 2004) and MBI have been shown to be important for transformation as well as c-Myc stability and activity (Hann 2006; Vervoorts, Luscher-Firzlaff et al. 2006). Additionally, the tumor suppressor Bin1 is able to bind a short peptide, comprising unphosphorylated MBI, but phosphorylation of Ser62 inhibits Bin1 binding (Pineda-Lucena, Ho et al. 2005). Our recent studies using unphosphorylated c-Myc₁₋₈₈ addresses this interaction further, showing Bin1 binding to c-Myc MBI as well as to a second low affinity binding site N-terminal to MBI (Andresen, Helander et al. 2012).

2.3 Transcriptional activation and repression

Taken together, the c-Myc TAD domain and the interaction and interplay with various co-factors are crucial and important for the regulation of c-Myc biological activity. c-Myc can interact with a wide range of proteins and directly or indirectly activate or repress transcription of target genes. The different domains (discussed in section 2.2) play an important role in the activation/repression machinery and over the years, both point mutations and deletion mutants of c-Myc have been designed and studied, in order to

answer questions related to the transcriptional machinery and the activity of c-Myc.

2.3.1 Transcriptional activation

DNA unwinding and chromatin remodeling is essential for the access to gene promoter regions by transcription factors. Chromatin remodeling, which opens up the chromatin, is crucial for transcription and c-Myc is associated with two types of chromatin remodeling: histone acetylation and ATP-dependent remodeling (Oster, Ho et al. 2002).

c-Myc can increase histone acetylation, by recruitment of histone acetylation complexes (HAT) to chromatin. TRRAP and GCN5 are a part of the HAT complex STAGA and the TAD domain of c-Myc is shown to bind TRRAP, which in turn binds GCN5 and acetylates histones (McMahon, Wood et al. 2000). Three regions of TRRAP (a.a. 1261-1579, 1899-2026, 3402-3828) have been shown to interact with c-Myc TAD and activate transcription (McMahon, Van Buskirk et al. 1998). Moreover, other chromatin remodeling protein complexes, such as TIP60, interact with c-Myc and TRRAP and recruit the TIP60 complex subunits TIP48, TIP49 and p400 to chromatin (Frank, Parisi et al. 2003).

RNA polymerase I, II and III (Pol I, Pol II, Pol III) play an important role in the cell cycle and are involved in the transcription of ribosomal protein genes and synthesis of transfer RNA (tRNA) and 5S ribosomal RNA (5S rRNA). Both tRNA and 5S rRNA need to be synthesized in excess in order to favor protein expression in a growing cell. Pol III transcription is necessary for cell growth and it has been shown that c-Myc bind to the Pol III-specific transcription factor TFIIIB and thereby activates Pol III transcription. The c-Myc TAD domain seems to be important for the interaction, since deletion of residues 106-143 prevents activation of tRNA genes (Gomez-Roman, Grandori et al. 2003). Furthermore, c-Myc inhibits the tumor suppressors p53 and retinoblastoma (Rb) protein through binding to TFIIIB, thereby repressing p53 and Rb regulation of TFIIIB (Felton-Edkins, Kenneth et al. 2003). The interaction between c-Myc and TFIIIB is likely favored by the c-

Myc/TRRAP/GCN5 interaction, promoting c-Myc activated Pol III transcription (Kenneth, Ramsbottom et al. 2007).

Cells overexpressing c-Myc show altered expression of Pol II target genes. The c-Myc TAD domain is shown to induce Pol II phosphorylation through the interaction with C-terminal domain (CTD) kinases, phosphorylating the CTD domain of Pol II (Eberhardy and Farnham 2001; Eberhardy and Farnham 2002). Moreover, c-Myc interacts with ribosomal DNA (rDNA) and activates Pol I-directed transcription by recruiting HAT complexes, thereby increasing the histone acetylation at the rDNA (Arabi, Wu et al. 2005).

The TATA-binding protein (TBP) is together with RNA Pol I, II or III part of the preinitiation complex (PIC) that together with specific co-activators initiates transcription. In the RNA Pol II transcription complex, TBP associates with TBP-associated factors (TAFs) forming the multiprotein complex TFIID (Bieniossek, Papai et al. 2013). TAFs regulate transcription through various interactions, many which favor transcription, acting as positive co-factors (Martel, Brown et al. 2002) or by interaction with negative co-factors, lowering transcriptional activity (Kokubo, Swanson et al. 1998; Chitikila, Huisinga et al. 2002).

c-Myc TAD binds TBP and it has been reported that TBP increases c-Myc transactivation (Hateboer, Timmers et al. 1993; Barrett, Lee et al. 2005; Fladvad, Zhou et al. 2005). However, so far no studies have elucidated the location of the c-Myc binding region on TBP. Our group has studied the binding pattern between c-Myc and yeast TBP (yTBP), using a c-Myc construct comprising MBII. The preliminary results (unpublished) show that c-Myc interacts with the DNA binding groove of TBP. Further, residues in yeast TAF1 (yTAF1) are also affected by the interaction with c-Myc. c-Myc₉₅₋₁₅₈ binding resulted in reduced HNCO intensities (**Figure 5**). Continued studies of this together with previous knowledge of TBP regulatory interactions are bound to gain insight into how c-Myc may influence and regulate the transcription machinery.

Figure 5 Spheres show the α -carbon in residues that show dramatically reduced ($> 90\%$: dark gold, $>80\%$: light gold) HNCN intensities as a result of Myc₉₅₋₁₅₈ binding in the yTBP (grey) - yTAF1 (green) fusion protein (Anandapadamanaban M., Helander S., unpublished results)

2.3.2 Transcriptional repression

In addition to transcriptional activation, c-Myc is able to repress specific target genes. So far, the repressive mechanisms are not as elucidated as the transcription activation mechanisms, but c-Myc seems to repress at least as many targets as it activates (Meyer and Penn 2008). While the C-terminal part of c-Myc is important for repression of target genes the role of the c-Myc/Max heterodimer in repression needs to be investigated further, since Max appears essential for c-Myc repression (Oster, Ho et al. 2002; Mao, Watson et al. 2003).

c-Myc can recruit Max and interact with Miz-1, forming a ternary complex that represses transcription. Moreover, c-Myc directly interacts with Miz-1 and the binding inhibits co-activator recruitment by Miz-1 and interferes with the formation of a Miz-1-p300 complex, thereby inhibiting transcriptional activation by Miz-1 (Staller, Peukert et al. 2001).

The Bin-1 protein functions as a tumor suppressor and interacts through its SH3 domain in the C-terminal, to c-Myc MBI, thereby controlling cell

proliferation and apoptosis (Elliott, Sakamuro et al. 1999; DuHadaway, Sakamuro et al. 2001; Pineda-Lucena, Ho et al. 2005; Andresen, Helander et al. 2012). The binding between c-Myc and Bin-1 can be inhibited by phosphorylation of Ser62, leading to increased c-Myc activity (Pineda-Lucena, Ho et al. 2005). The role of Bin-1 as a tumor suppressor is further emphasized by the fact that Bin-1 inhibits c-Myc transformation as well as tumor growth and it has been found that tumor cells lack Bin-1 expression (Sakamuro, Elliott et al. 1996).

2.4 Biological activities of c-Myc

c-Myc can regulate a wide range of biological activities and through its function as a transcription factor, c-Myc affects cell proliferation, cell growth, differentiation, transformation and apoptosis (**Figure 6**) (Ponzielli, Katz et al. 2005). The role of c-Myc expression in cell cycle progression is complex and will only be discussed briefly in the sections below.

Figure 6, The cellular effects of c-Myc regulation. The target genes regulated by c-Myc control crucial biological activities, including apoptosis, cell growth, cellular transformation, differentiation and proliferation.

2.4.1 Cell cycle

The eukaryotic cell cycle is divided into four phases. During the first phase, named G_1 , cells make important decisions and go through tightly controlled checkpoint controls. Cells prepare for DNA synthesis by increasing protein and organelle synthesis and grow in size. Behind the restriction point, in late G_1 phase, cells must complete cell division and enter the next step, the S phase. In the absence of growth factors or if the conditions are unfavorable for replication, cells may enter a resting state called G_0 . DNA synthesis occurs during S phase and cells can proceed directly from S phase to mitosis (M phase), but commonly they delay their entrance and proceed into a gap phase called G_2 . This gap phase is poorly understood but cells prepare for entering M phase and cell division (Alberts 2008).

Cells with abnormal expression of c-Myc gene will express high levels of proteins controlling cell cycle. Progress through early G_1 can be promoted by stimulation of growth-promoting genes, including cyclin D1/D2 and Cdk4, by the c-Myc/Max complex. Another possibility for c-Myc to push cells through the G_1 phase is to associate with the transcription factor Miz-1 (see section 2.3.2) and function as a transcription repressor. The c-Myc/Miz-1 complex can inhibit Cdk inhibitor genes, such as p21 and p15, which inhibit the kinase activity of Cdk2 and Cdk4/6 complexes (Gartel and Shchors 2003). By blocking the expression of cell cycle inhibitor genes, c-Myc will be resistant to actions from the growth-inhibitory signal TGF- β . In summary, cancer cells with abnormal level of c-Myc can continue to proliferate under conditions that normally would prevent proliferation and still advance into S phase (Alberts 2008).

2.4.2 Cell growth, differentiation, apoptosis and cellular transformation

The regulation of cell proliferation and cell growth needs to be tightly controlled. Studies in murine B cells demonstrate that c-Myc is involved in the regulation of growth-promoting signals as cells with constitutive expression of c-Myc show increased protein synthesis and increased cell growth, even in the absence of cell division (Iritani and Eisenman 1999; Schuhmacher, Staeger et al. 1999).

The c-Myc/Max heterodimer favors the transcription of many genes involved in cell proliferation. The activities of the c-Myc/Max heterodimer are controlled in part by different mitogenic signals. The level of c-Myc is influenced by the signals while the level of Max is almost constant. In addition, Max can interact with Mxd family of proteins, such as Mad1 and Mnt, and this interaction is suggested to be tumor suppressive. The Max/Mxd complex recognizes the same E-box sequence as the c-Myc/Max complex. Mxd protein levels are increased during growth arrest conditions and differentiation and compete with c-Myc for Max binding to mediate growth inhibitory functions (Larsson, Pettersson et al. 1994; Larsson, Bahram et al. 1997; Grandori, Cowley et al. 2000). Moreover, c-Myc can influence apoptosis by acting on pro- and anti-apoptotic factors. In particular, many c-Myc-repressed target genes are linked to apoptosis (Meyer, Kim et al. 2006; Meyer and Penn 2008).

In oncology cellular transformation is defined as the change a normal cell undergoes to become a malignant cancer cell. The MBII region is important for c-Myc's ability to transform cells, but the MBI region seems to be less important. Although, the Burkitt's Lymphoma mutant Thr58A shows increased transformation ability compared to wild-type c-Myc, while Ser62A inhibits transformation (Pulverer, Fisher et al. 1994; Thibodeaux, Liu et al. 2009).

2.5 Regulation of c-Myc stability and activity

The cellular half-life of the c-Myc protein is very short, approximately 20-30 minutes (Hann and Eisenman 1984) before it targeted for degradation by the proteasome. The role of post-translational modifications on c-Myc stability and activity has been studied extensively during the years, and several different modifications, for example phosphorylation, ubiquitination, glycosylation and acetylation have been found. Up to this date, studies on phosphorylated c-Myc clearly show an ability to regulate c-Myc biological activities, whereas the biological effects for the other modifications seem to be more ambiguous (Hann 2006).

2.5.1 Phosphorylation sites

There are four known phosphorylation sites in c-Myc TAD: Thr58, Ser62, Ser71 and Ser81. In addition, clusters of phosphorylation sites (a.a. 247-252 and a.a. 343-348) have been found in both the central and C-terminal domain. Both the phosphorylation clusters and the Ser71 and Ser81 seem to be important in regulating transformation and alanine mutants show increased transformation compared to wild-type c-Myc. Ser71, Ser81 and the cluster at a.a. 343-348 shows equal protein stability as wild type, showing that the increased transformation is independent of c-Myc protein stability (Wasylishen, Chan-Seng-Yue et al. 2013). As discussed previously, MBII is important for c-Myc function, although there are no known phosphorylation sites in MBII.

2.5.2 Phosphorylation at Ser62 and Thr58

The phosphorylation at Ser62 and Thr58 in MBI is sequential (**Figure 7**) and initial phosphorylation of Ser62 is required for Thr58 phosphorylation (Lutterbach and Hann 1994). GSK3 β is shown to phosphorylate Thr58 (Pulverer, Fisher et al. 1994; Gregory, Qi et al. 2003). The activity of GSK3 β is regulated by the PI3K pathway and phosphorylation of GSK3 β by PI3K induced Akt kinase inhibits GSK3 β . Upstream of Akt is Ras, which functions as a proto-oncogene encoding a GTPase protein. GTPase triggers Ras, which is active when bound to guanosine triphosphate (GTP) (Alberts 2008). *In-vivo* phosphorylation of Ser62 stabilizes c-Myc (Sears, Nuckolls et al. 2000) and phosphorylation of Ser62 is mediated through MEK activated ERK and Cdk2 (Hydbring, Bahram et al. 2010).

The tumor suppressor Axin1 has been proposed to facilitate a degradation complex for c-Myc, favoring the interaction of c-Myc with GSK3 β , PP2A and Pin1 (Arnold, Zhang et al. 2009). The pSer/Thr-Pro motif in MBI acts as a target for Pin1, where Pin1 is suggested to alter the *cis-trans* isomerization of Pro63 and thereby triggers c-Myc dephosphorylation by PP2A. Dephosphorylation at Ser62 by PP2A destabilizes c-Myc and acts as a signal for ubiquitination by Fbw7, followed by degradation (**Figure 7**) (Yeh, Cunningham et al. 2004; Arnold, Zhang et al. 2009). The current view,

presented above, is based on cellular assays. Studies on a molecular level are needed in order to evaluate the mechanisms in further detail.

Figure 7, The sequential phosphorylation of c-Myc and the degradation pathway.

2.5.2 Ubiquitination and degradation

Compared to the phosphorylation of c-Myc, less is known about the ubiquitination pattern. Ubiquitination is important for degradation and due to the short half-life, c-Myc is continuously ubiquitinated and degraded by the proteasome. There are a total of 26 lysines in c-Myc and four are found within c-Myc TAD (a.a. 1-143) and two are situated close to the TAD domain.

The ubiquitination ligases Fbw7, Skp2, TRPC4AP/TRUSS, PirH2 and CHIP promote ubiquitination and degradation. For many of the ligases, less is known about the molecular mechanism behind, however Skp2 has been found to bind c-Myc *in vivo* and ubiquitinate MBII (von der Lehr, Johansson et al. 2003; Yada, Hatakeyama et al. 2004; Choi, Wright et al. 2010; Hakem, Bohgaki et al. 2011).

As described previously (section 2.5.2) phosphorylation is important for c-Myc degradation and dephosphorylation at Ser62 by PP2A acts as a signal for ubiquitination by Fbw7. Fbw7 targets MBI and in addition Fbw7 can act as a tumor suppressor, and loss of Fbw7 increase the levels of c-Myc, thereby stimulating tumorigenesis (Kim, Herbst et al. 2003; Yada, Hatakeyama et al. 2004; Muller and Eilers 2009).

HectH9 polyubiquitinates c-Myc at Lys63, but in opposite to Skp2 and Fbw7, ubiquitination of MBI by HectH9 is not correlated to protein degradation. Instead, it seems to alter the transcriptional activation of c-Myc target genes.

Moreover, HectH9 has been shown to promote N-Myc degradation by ubiquitination of Lys48 (Zhao, Heng et al. 2008).

2.5 c-Myc as a therapeutic target

Since the c-Myc gene is deregulated in a variety of human cancers, targeting c-Myc at both the gene and protein level may have an impact in the treatment of human cancers. Small molecules are useful and can be used to target c-Myc at several levels. One possibility is to inhibit the c-Myc/Max dimerization. Four charged amino acids (Glu410, Glu417, Arg423, Arg424) in the c-Myc leucine zipper are necessary for dimerization. Mutating these residues resulted in a protein that interfered with the heterodimer formation, altering DNA binding efficiency as well as inhibited cell proliferation (Soucek, Helmer-Citterich et al. 1998). Further, three binding sites for small-molecules have been found within the bHLH-LZ in c-Myc, all targeted with micro molar affinities by small molecules (Hammoudeh, Follis et al. 2009).

As discussed previously, the conserved regions in c-Myc TAD, MBI and MBII, are essential for the biological functions of c-Myc (Cowling and Cole 2006). Thus, targeting these important regions by small molecules may be a way to therapeutically target c-Myc. Structural details for the c-Myc TAD has been missing, but the studies in this thesis (paper I and IV) provide further insight and offer a platform for characterizing the interaction between c-Myc TAD and small molecules in detail. The challenge associated with this is the conformational properties of c-Myc TAD and the lack of a well-structured state. A limited number of studies have evaluated the binding of small molecules to intrinsically disordered proteins (Zhu, De Simone et al. 2013; Toth, Gardai et al. 2014). In a recent study using small molecules targeting the structural ensemble of α -synuclein, eight binding pockets were identified to be present in the ensemble of monomer conformations (Toth, Gardai et al. 2014). Even though α -synuclein is intrinsically disordered, local transiently structured states populate the conformational ensemble. The binding sites were identified by applying a computational screen on the ensemble of structure, identified in previous studies (Bertoncini, Jung et al. 2005; Dedmon, Lindorff-Larsen et al. 2005).

Targeting c-Myc for cancer treatment is challenging. Still, many questions remain regarding the c-Myc gene and the overexpression in human cancers. Detailed knowledge around the molecular mechanisms regulating c-Myc is important and increased understanding of the regulating interactions will provide insight into the role of c-Myc in human cancers.

3. Peptidyl-prolyl isomerases

3.1 Peptidyl-prolyl *cis-trans* isomerases

One possibility to regulate cellular processes is through phosphorylation of serine and threonine residues that precede a proline. Various enzymes phosphorylate such motifs, such as CDKs, ERKs and GSK3 β , which all belong to the large superfamily of proline-directed protein kinases.

The two isomers of proline, *cis* and *trans*, act as a switch that can regulate protein function. The intrinsic isomerization reaction is a slow process, but peptidyl-prolyl *cis-trans* isomerases catalyze this reaction, which may be a rate-limiting step in protein folding and refolding. By catalyzing the *cis* and *trans* isomerization, the peptidyl-proline isomerases (PPIs) act as folding chaperones (Dilworth, Gudavicius et al. 2012).

Peptidyl-propyl isomerases can be divided up into different families: parvulins, cyclophilins (Cyps) and FK506-binding proteins (FKBPs) (Dilworth, Gudavicius et al. 2012). The following sections cover a brief discussion of two members in the parvulin and FKBP family, Pin1 and FKBP25.

3.1 Pin1

Pin1 is a member of the parvulin family and two parvulin proteins are found in humans, Pin1 and Pin14 (together with the Pin14 isoform Pin17) (Mueller, Kessler et al. 2006; Mueller and Bayer 2008). Pin1 is the most studied protein in the parvulin family. Over the years many Pin1 associated transcription factors have been discovered and the associated mechanisms include events such as protein stability, protein-protein interactions, translocation and dephosphorylation (Liou, Zhou et al. 2011; Dilworth, Gudavicius et al. 2012).

3.1.1 Structure

Pin1 consists of an N-terminal WW domain and a catalytically active C-terminal PPIase domain (**Figure 8**) (Ranganathan, Lu et al. 1997; Bayer, Goettsch et al. 2003). The WW domain is known to act as a protein interaction domain. Both domains have been shown to recognize phosphorylated serine or threonine followed by a proline (pSer/Thr-Pro motifs) (Lu, Zhou et al. 1999; Verdecia, Bowman et al. 2000), although the WW domain displays a higher affinity for pSer/Thr-Pro motifs than the PPIase domain (Lu, Zhou et al. 1999). However, even in the presence of the pSer/Thr-Pro motif, the WW domain seems to bind with relatively low affinity, with a K_D of 100 μ M for the highest affinity peptide derived from Tau (Lippens, Landrieu et al. 2007).

The active site for the catalytically active PPIase domain involves a cysteine residue that interacts with the pSer/Thr residue, thereby initiating the *cis-trans* rotation around the prolyl bond in the substrate (Labeikovsky, Eisenmesser et al. 2007). The binding site for the substrate proline is composed of three hydrophobic residues (Phe134, Met130 and Leu122), whereas Cys113, His59, His157 and Ser154 are responsible for the catalytic activity and the *cis-trans* isomerization (**Figure 8**) (Ranganathan, Lu et al. 1997).

The WW and PPIase domains are connected by a flexible linker, which may confer dynamic properties in substrate binding (Jacobs, Saxena et al. 2003; Labeikovsky, Eisenmesser et al. 2007). When comparing the NMR

spectroscopy structure for Pin1 with the X-ray structure (**Figure 8**), differences in the orientation between the two domains can be seen. The WW domain is closer to the PPIase domain in the X-ray structure, while the NMR structure shows a more dynamic linker, meaning that the Pin1 structure is more flexible with the WW domain more distant from the PPIase domain (Ranganathan, Lu et al. 1997; Bayer, Goettsch et al. 2003). The dynamics observed in the NMR spectroscopy structure indicate that the flexible linker and the conformational flexibility are of importance for the function of Pin1 catalysis, and recent studies show that domain interactions increase the affinity of Pin1 for peptide ligands (Labeikovsky, Eisenmesser et al. 2007; Matena, Sinnen et al. 2013).

Figure 8, The X-ray structure (left) (PDB ID: 1PIN) and the NMR structure (PDB ID: 1NMV) (right) for the human Pin1. The linker connecting the PPIase (light grey) and the WW domain (dark grey) is shown in the NMR structure. Only one structure from the conformational ensemble is shown. The binding pocket for substrate proline, with hydrophobic residues (Phe134, Met130 and Leu122) is shown in blue. The catalytic site (orange) is composed of Cys113, His59, His157 and Ser154.

3.1.1 Pin1 and cellular regulation

Pin1 can both stabilize and destabilize proteins. Many Pin1 substrates, including c-Myc, fall into the class of intrinsically disordered proteins (IDPs), and this property together with the pSer/Thr-Pro motif is thought to be important for Pin1 recognizing different substrates *in vitro* (Lippens, Landrieu et al. 2007). On the other hand, phosphorylation on Pin1, as on Ser16 in the binding pocket of the WW domain, inhibits Pin1 substrate

binding, showing that phosphorylation can regulate Pin1 function (Lu, Zhou et al. 2002). Thus, both substrate and Pin1 phosphorylation are important for both Pin1 function and regulation.

Pin1 is overexpressed in 38 of 60 tumor types examined (Ayala, Wang et al. 2003; Bao, Kimzey et al. 2004) and Cyclin D1 is overexpressed in many breast cancer tumors (Bartkova, Lukas et al. 1994). Pin1 binds to Thr286-phosphorylated cyclin D1 and catalyzes isomerization. Upon binding, the stability of cyclin D1 is increased, leading to accumulation of cyclin D1 in the nucleus (Liou, Ryo et al. 2002). As discussed previously, Pin1 isomerizes c-Myc, thereby promoting its ubiquitination and degradation (Sears 2004), which in the end leads to decreased tumorigenesis. However, Pin1 can also increase tumorigenesis by destabilizing tumor suppressors and growth inhibitors (Liou, Ryo et al. 2002).

Although the molecular role of phosphorylation in the Pin1-c-Myc interaction is not yet described in detail, more is known about how Pin1 affects two proteins involved in amyloid pathogenesis: Tau and APP. Hyperphosphorylation of Tau leads to self-association and phosphorylation of Thr231 in Tau is an early event that triggers misfolding and aggregation (Luna-Munoz, Chavez-Macias et al. 2007). Pin1 is shown to bind phosphorylated Thr231 and promote Thr231 dephosphorylation by altering the *cis-trans* isomerization at Pro232 (Lu, Wulf et al. 1999), thereby preventing Tau self-association. Moreover, Pin1 is unable to bind unphosphorylated Tau, mutated at Thr231, indicating that phosphorylated Thr231 is the primary binding site for Pin1 in Tau (Lu, Wulf et al. 1999). The APP protein can be cleaved by α -secretase or β - and γ -secretases. Cleavage of APP, by β - and γ -secretases, results in formation of the plaque forming β -amyloid peptide (A β). Phosphorylation of APP, by Cdk5 and Gsk3 β influence the cleavage process and leads to a more pathogenic form of A β (Phiel, Wilson et al. 2003; Cruz, Kim et al. 2006). Pin1 is able to bind APP, phosphorylated at Thr668. The binding regulates APP conformation and favors isomerization at Pro669. Thus, Pastorino et al. hypothesize that the *trans* conformation of APP would favor non-amyloidogenic APP processing of A β (Pastorino, Sun et al. 2006).

3.2 FK506 binding proteins

The family of FK506 binding proteins (FKBPs) is found to bind immunosuppressant drugs, such as FK506 and rapamycin (Galat 2013). As mentioned previously, they belong to the group of peptidyl-propyl isomerases and the PPIase of FKBPs are involved in the folding process. Moreover, many FKBPs have chaperon activity, thereby preventing non-native interactions (Suzuki, Nagata et al. 2003; Monaghan and Bell 2005). FKBPs have been found at various cellular locations and the human genome encodes for a wide range of FKBPs, spanning from multidomain proteins to small single domain FKBPs (Galat 2013).

3.2.1 FKBP25

The human FKBP25, with a molecular weight of 25.1 kDa, has a positively charged N-terminal domain followed by the C-terminal domain, comprising the PPIase activity (Galat, Lane et al. 1992). The highly hydrophilic N-terminal of FKBP25 (discussed in Paper III) is unique among the FKBPs, moreover, most other FKBPs are found in the cytoplasm, while FKBP25 is found mainly in the nucleus (Galat 2013).

The C-terminal secondary structure consists of five antiparallel β -strands, with a short α -helix present against the β -sheet. One tryptophan in the α -helix contributes, together with strand β_4 and β_5 and three loops (40s, 50s and 80s), to the drug binding pocket (**Figure 9**) (Liang, Hung et al. 1996).

Figure 9, Crystal structure of FKBP25, C-terminal part (PDB ID: 1PBK), with the structures that contributes to the binding pocket of rapamycin colored. β 4 (green), β 5 (blue), W (red), loop 40s (yellow), loop 50s (light brown), loop 80s (pale cyan).

Rapamycin binds FKBP25 with a $K_D \leq 1$ nM (Galat, Lane et al. 1992) and drug binding inhibits the interaction with various intracellular proteins, where many of these are involved in chromatin remodeling (Andersen, Wilkinson et al. 2003; Galat 2013).

Moreover, FKBP25 associates with the transcription factor YY1 and the interaction is unaffected by rapamycin binding (Yang, Yao et al. 2001). The recent study from our group describes the structure of the FKBP25 N-terminal domain (discussed in paper III) and shows that the binding patch for YY1 is located distant from a highly positively charged surface, which is suggested to bind DNA (Helander, Montecchio et al. 2014).

3.2.2 Role in chromatin modification and human cancer

As mentioned in the previous section, FKBP25 interacts with YY1. Moreover, the interaction increases the DNA binding activity of YY1 and this together with the finding that FKBP25 is able to bind HDAC1 and HDAC2 and interfere with histone deacetylase activity shows that FKBP25 may have a role in regulating chromatin structure (Yang, Yao et al. 2001).

As discussed in section 2.4.2, Mdm2 is able to interact with the tumor suppressor p53, leading to ubiquitination and degradation of p53. FKBP25 has been shown to associate with Mdm2, enhancing ubiquitination and degradation of Mdm2 and increased expression of FKBP25 leads to decreased Mdm2 levels and at the same time increased p53 levels (**Figure 10**) (Ochocka, Kampanis et al. 2009). The full length FKBP25 is required for the interaction with Mdm2 and deleting either the C-terminal or the N-terminal led to loss of interaction. Interestingly, the interaction was not inhibited by rapamycin, suggesting that FKBP25 affect Mdm2 in a PPIase independent manner. Further, knockdown of FKBP25, using siRNA, leads to increased levels of Mdm2 and as a consequence, reduced levels of p53 (Ochocka, Kampanis et al. 2009).

Figure 10, Schematic illustration of the FKBP25-Mdm2 interaction and the regulation of p53 by Mdm2. Binding of FKBP25 to Mdm2 enhances ubiquitination and degradation of Mdm2, which leads to increasing levels of p53 and activation of genes controlling cell cycle.

4. Methodology

A wide range of methods are used within the field of structural biology. Nuclear magnetic resonance (NMR) and X-ray crystallography can be used to gain structural information at the amino acid level while fluorescence and circular dichroism (CD) are more low-resolution methods, mostly used for protein stability and secondary structure analysis. Other techniques, such as small angle X-ray scattering (SAXS), give information about low-resolution 3D structure and protein domain orientations.

In addition to the experimental methods, computational methods have developed and in combination with the experimental data, these can be powerful to model for example the structural ensembles of IDPs (Schneider, Huang et al. 2012). The following section covers a brief description of the methods that I have predominantly used during my PhD studies.

4.1 Circular dichroism spectroscopy

One of the most common and easily assessable methods used in structural biology is circular dichroism (CD), which can be used for studies of secondary structure, low-resolution tertiary structure studies as well as stability measurements of the protein of interest.

Circular dichroism is based on the different absorption between left and right handed polarized light and a CD signal will be observed for molecules that are optically active and display a chiral center. Proteins have intrinsically optical properties due to the chirality in the polypeptide backbone and the 3D structure asymmetric environment. The difference in absorption between the left and right-handed circularly polarized light, is given by the difference in absorbance according to Lambert-Beers law (Eq. 1), where $\Delta\varepsilon$ is the difference in extinction coefficients for the absorption, c is the sample concentration and l is the path length. The polarized light becomes elliptical polarized when passed through the sample. The difference in absorption by the sample is commonly reported as ellipticity (θ) in degrees (Eq. 2), where b and a are the major and minor axes of the resulting ellipse (Kelly, Jess et al. 2005):

$$\Delta A = A_L - A_R = (\varepsilon_L - \varepsilon_H) \cdot c \cdot l = \Delta\varepsilon \cdot c \cdot l \quad (1)$$

$$\theta = \tan^{-1}(b/a) \quad (2)$$

ΔA can be converted to θ through the numerical relationship (Eq. 3):

$$\theta = 32.98\Delta A \quad (3)$$

The ellipticity measured as a function of wavelength generates a CD spectrum (Greenfield and Fasman 1969).

In proteins, the peptide bond has absorption between 190 to 240 nm and the aromatic amino acid side chains absorb between 260 to 320 nm. The difference in absorption in the far-UV spectra (190-240 nm) can be used to study the secondary structure of the protein, while the different absorption from aromatic amino acid residues in the near-UV region (260-320 nm) can be used to study the tertiary structure and changes in the tertiary structure (Kelly, Jess et al. 2005). In addition to this, folding and protein stability can be studied by observing changes in secondary structure as function of temperature or concentration of denaturants. In the following section secondary structure and protein stability studies will be explained in more detail.

4.1.1 Secondary structure evaluation

The ellipticity in the far-UV region (190-240 nm), arising from the differential absorbance of the peptide bond and gives rise to characteristic CD spectra for the secondary structural elements found in proteins (**Figure 11**).

Figure 11, Schematic illustration of secondary structure profiles in CD spectra.

A positive peak at 195 nm can be observed for α -helices and together with two minima at 222 and 208 nm, while β -sheets show a positive peak at 198 nm and a minimum at 216 nm. Random coils typically display minima at 198-200 nm (Greenfield and Fasman 1969; Greenfield 2006). The overall secondary structure for a protein can be analyzed by measuring the CD in the far-UV region (190-240 nm) and further evaluating the data by using existing algorithms which use data from far-UV spectra from well-known proteins. The online server DICHROWEB can be used to analyze data with various algorithms and databases and thereby calculate the estimated content of secondary structure elements (Lobley, Whitmore et al. 2002; Whitmore and Wallace 2004).

4.1.2 Thermal stability evaluation

The stability and unfolding of proteins can be studied by following the change of secondary structure when the protein is exposed to heat or chemical denaturants such as urea or guanidinium chloride. The thermal stability of the protein can be investigated by following the change in CD signal as a function of temperature. This information can be used to evaluate the protein stability, by examining the thermal melting midpoint (T_m). In addition to this, loss of CD signal can provide information about the unfolding process, where unfolding of α -helices and β -sheets can be monitored by changes in ellipticity at 222 nm and 216 nm, respectively (Kelly and Price 1997).

4.2 Surface plasmon resonance

Surface plasmon resonance (SPR) is one of the methods which, can be used to study affinity, kinetics and thermodynamics of protein-protein interactions. The technology takes advantage of the SPR phenomenon and was first described for sensing applications in 1983 (Liedberg et al 1983). The technique has been further developed and is now a well spread biosensing method with the label free monitoring of biomolecular interactions as a major advantage.

In general, SPR sensors measure changes in refractive index at the surface of a metal film (Homola 2008). SPR occurs when incoming light excites free electrons in a thin metal film creating a so-called surface plasmon (SP) that propagates along the interface between a metal and the external medium. In SPR sensors based on the Kretschmann configuration (**Figure 12**), incoming light passes through a glass prism at an angle of incidence (θ) where total internal reflection occurs and part of the light penetrates the thin metal film and excites the surface plasmon. This resonance condition occurs when the energy of the incoming light matches the energy of the surface plasmon, which can be achieved by passing the incoming light through a glass prism. A sharp decrease in the intensity of the reflected light can be detected when the surface plasmon resonance condition is fulfilled which hence, occurs at a specific angle of incidence known as the SPR angle (θ_{sp}). Since the SPR angle is dependent on the refractive index at the metal

film interface, any change in the refractive index, for instance, binding of biomolecules causes a shift in the SPR angle ($\Delta\theta_{sp}$), which forms the basis of the SPR biosensing technique (Homola 2008). In this work, a Biacore system (GE Healthcare, Uppsala, Sweden) has been used, where $\Delta\theta_{sp}$ is measured in response units (RU) and plotted as a function of time. A change of $\Delta\theta_{sp} = 0.0001^\circ$ corresponds to 1 RU.

Figure 12, Schematic illustration of a SPR setup based on the Kretschmann configuration.

The sensor surface is functionalized with a biorecognition element (ligand) using a coupling method, for instance through amine coupling. The interaction partner (analyte) is injected over the surface and the binding causes a change in refractive index, which enables the interaction to be monitored in real time. This means that association and dissociation between the immobilized ligand and the analyte can be detected. In the resulting sensorgram, the analyte-ligand association increases the response signal, while the dissociation phase is observed as a decreased signal (**Figure 13**). If needed, the surface can be regenerated, which enables an additional injection of the analyte. For kinetic and binding affinity studies,

which require repeated injections of the analyte, regeneration of the surface without harming the ligand is crucial for obtaining a reliable dataset.

Figure 13, Schematic illustration of a sensorgram, showing the association and dissociation phases followed by a regeneration pulse.

4.3 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy is one of the most used methods in the field of organic chemistry and structural biology, since it can be used for structural determination of both organic molecules and larger macromolecules, such as proteins. The methods used for studying three-dimensional structures of proteins were developed by Kurt Wüthrich in the beginning of the 1980s (Wuthrich, Wider et al. 1982) and in 2002 he received the Nobel Prize in chemistry for his contribution to the field of structural biology. In addition to structure determination, NMR spectroscopy is used to study protein-protein interactions as well as protein folding and dynamics.

4.3.1 Theory

The following theoretical section covers the most fundamental parts of the protein NMR method described according to the descriptions in (Hore 1995; Rule and Hitchens 2006; Teng 2012).

NMR takes advantage of the magnetic properties of the ^1H , ^{13}C and ^{15}N nuclei in biological samples. The magnetic property of the nucleus is

dependent of the nuclear spin quantum number (I). A nucleus has magnetic properties when;

$$I \neq 0 \quad (4)$$

^1H , ^{13}C and ^{15}N have $I = \frac{1}{2}$, while ^2H has $I = 1$, when placed into an external magnetic field, (the NMR spectrometer) the nuclear spin dipoles line up and take two possible orientations, with the magnetic quantum number, m , if $I = \frac{1}{2}$: opposite ($m = -\frac{1}{2}$) to or aligned ($m = +\frac{1}{2}$) with the external magnetic field (B_0). Nuclei with spin quantum number $I > 0$ have a nuclear magnetic moment, $\mu = \gamma I$ which arises from the spin of unpaired protons and/or neutrons. Each nucleus has a gyromagnetic ratio, γ , and the energy of the nuclei is dependent on the nucleus gyromagnetic ratio and the interaction with the external magnetic field. In absence of a magnetic field the spins are evenly distributed and no energy difference arises. In an external magnetic field, the magnetic moments interact with the magnetic field and the energy for the two spin states (E_β for $m = +\frac{1}{2}$ spins and E_α for $m = -\frac{1}{2}$ spins) can be calculated and the energy difference is given by (Eq. 5)

$$\Delta E = E_\beta - E_\alpha = \gamma h B_0 / 2\pi \quad (5)$$

where h is Planck's constant, B_0 is the magnetic field strength and E_α and E_β are the energies for the spin states ($m = -\frac{1}{2}$ and $m = +\frac{1}{2}$, respectively). Absorption of electromagnetic radiation by the nucleus cause transitions between the two energy states and resonance occurs when the energy of radiation matches the energy difference between the two states. The resonance frequency is calculated as (Eq. 6)

$$\Delta E = h\nu \quad (6)$$

The precession frequency of the nucleus, is calculated according to the Larmor equation (Eq. 7)

$$\nu_0 = \gamma B_0 / 2\pi \quad (7)$$

The Larmor equation gives the frequency for a nucleus that precesses in a fixed field, but the local environment of the nucleus also affects the frequency. The surrounding electron distribution will affect the frequency for the nucleus, and as a result, a small frequency difference between nuclei in different parts of the protein is observed. This effect is the basis for the different chemical shifts observed in NMR spectra. For protein NMR, each amino acid shows characteristic chemical shifts, δ , which can be used for the backbone assignment of proteins (discussed further in section 4.3.2). Since the chemical shifts are independent of the magnetic field, they are commonly used instead of resonance frequencies. The relationship between chemical shifts and resonance frequencies (in Hertz) is shown below (Eq. 8).

$$\delta = (\nu - \nu_{\text{ref}} / \nu_{\text{ref}}) \cdot 10^6 \quad (8)$$

The unit is parts-per-million, ppm and ν_{ref} is known as the reference frequency.

When performing an NMR experiment, a radio frequency pulse, RF pulse, is applied to the sample under study. The RF pulse sequence, which is a set of RF pulses and delays, will excite the nuclei in the sample and the nuclei will transfer the absorbed energy to the neighboring nuclei. At equilibrium, the net magnetization of the sample, $\mathbf{M}_0$, is aligned with the z-axis (**Figure 14**). By applying a RF pulse (90°) along the y-axis, magnetization is turned to the x-axis and precesses with frequency, ω , about the z-axis. The radiofrequency coil can now detect the resonance frequency. The precession and the relaxation back to equilibrium are called free induction decay (FID). Since different nuclei have different surrounding and properties, this will affect the FID. The FID is Fourier transformed (FT) into frequency. In the end, this results in an NMR spectrum, which contains information about the chemical shifts of the nuclei in the sample.

Figure 14, Magnetization (green) is transferred to the x-axis by the RF pulse; precesses about the z-axis and the resonance frequency can be detected.

4.3.2 Resonance assignment

In order to solve the solution structure for a protein, 2D and 3D spectra needs to be recorded and each amino acid residue in the sample needs to be assigned. Each nucleus has certain chemical shifts in different dimensions in the 2D or 3D spectra and by combining a set of experiments, which detect the transfer of magnetization between nuclei in different ways, the observable nuclei from each residue can be assigned. The assigned spectra can be used for various applications such as interaction studies and protein structure calculations. For example, HSQC and HNCOC spectra can be used for the study of protein-protein interactions. The 2D ^{15}N HSQC spectrum contains information about the proton attached to the nitrogen in the protein backbone and each amide corresponds to a peak in the spectrum (**Figure 15**). Although ^{15}N HSQC is most commonly used, ^{13}C HSQC can be used to obtain information about the protons connected to carbon.

Figure 15, ^{15}N HSQC spectra, showing the well-folded Pin1 to the left and the intrinsically disordered c-Myc₁₋₈₈ to the right.

Common for all the 3D experiments are that they provide information about the ^1H , ^{15}N and ^{13}C nuclei in the protein, but the transfer of magnetization differs between different experiments. A common set of 3D experiments include; HNCA, HN(CO)CA, HN(CA)CB, HN(CA)CO, HNC(O) and CBCA(CO)NH. All of the experiments above, except CBCA(CO)NH, start with a transfer of the magnetization from the amide proton to the amide nitrogen. Further, depending on experiment, magnetization is transferred to or through C_α , C_β or CO in the protein backbone. For example, C_α from both the internal (i) and sequential ($i-1$) amino acid can be detected with an HNCA experiment, while an HNC(O) experiment detects the carbonyl carbon (CO) in the preceding residue. Other experiments, such as the HNCACB gives the intra- and inter-residue C_α and C_β shifts, while the CBCA(CO)NH experiments gives C_α and C_β for the sequential inter-residue (Rule and Hitchens 2006). The magnetization pathway and the recorded chemical shifts are illustrated in figure 16.

Figure 16, The magnetization transfer pathway for HNCA, HNCO, HNCACB and CBCA(CO)NH experiments. Chemical shifts are recorded for the boxed nuclei. Arrows indicate the magnetization transfer and i and $i-1$ are the internal and the preceding residue, respectively.

While automated assignment is possible, using various types of computer software, for example MARS, PINE and ABACUS (Jung and Zweckstetter 2004; Bahrami, Assadi et al. 2009; Lemak, Gutmanas et al. 2011), manual assignments are still used. The manual assignment is time consuming, but can be required especially for assignment of intrinsically disordered proteins or other proteins with a high degree of spectral overlap. Applying a “step-by-step” approach, where combined sets of spectra are used, will give information about the internal and/or the preceding amino acid and in the end, the peaks can be assigned to specific amino acids in the protein sequence.

4.3.3 Dynamics

Proteins are not rigid structures; they are flexible and display dynamics on different time-scales ranging from fast picosecond motions (bond vector vibrations) to slow motions on the second time scale (conformational rearrangements) and they tumble in solution on the nanosecond timescale. The dynamic behavior of a protein can be evaluated at atomic

resolution using NMR relaxation experiments, sensitive to a range of time-scales (Mittermaier and Kay 2009).

Commonly used backbone relaxation experiments measure the ^{15}N R_1 longitudinal relaxation rate ($1/T_1$), the ^{15}N R_2 transverse relaxation rate ($1/T_2$), the heteronuclear Overhauser effect (^{15}N -NOE) and Carr-Purcell-Meiboom-Gill (CPMG) relaxation dispersion. The various experiments record the process in which non-equilibrium magnetization returns to the equilibrium state. The experiments mentioned above are measured as two-dimensional experiments, in which the relaxation delay is varied between spectra and the peak intensities are monitored as a function of time (**Figure 17**) (Teng 2012).

Figure 17, Peak intensity monitored as a function of relaxation delay.

For backbone experiments, measuring relaxation on the picosecond to nanosecond time-scale (R_1 , R_2 and NOE), two types of processes influence the relaxation decay, dipole-dipole interactions and chemical shift anisotropy (CSA).

Dipole-dipole interactions induce relaxation of the ^{15}N nucleus when molecular tumbling or internal structural motions, cause fluctuations in the internal magnetic field, due to re-orientation of the ^1H - ^{15}N bond vector to the external magnetic field. Relaxation of the ^{15}N nucleus occurs when the

molecular motions have the appropriate frequency, oscillating close to the linear combinations of Larmor frequency for the coupled nuclei (Eq. 7). (Jarymowycz and Stone 2006; Teng 2012).

In proteins, CSA relaxation of the ^{15}N nucleus occurs when the protein tumbles in the solution, resulting in a fluctuating magnetic field caused by variations in shielding from the external magnetic field (Jarymowycz and Stone 2006; Teng 2012).

R_1 and R_2 experiments are sensitive to dynamics at different time scales. R_1 is sensitive to picosecond to nanosecond motions, whereas R_2 is sensitive to both picosecond-to-nanosecond and microsecond-to-millisecond motions (Jarymowycz and Stone 2006; Teng 2012). The latter motions often correspond to large-scale dynamics like protein folding or ligand binding. A second difference is the dependence on the molecular tumbling time called rotational correlation time (τ_c). The fluctuating magnetic field caused by the molecular tumbling influences the R_1 and R_2 relaxation in different ways. In R_1 relaxation, which measures the longitudinal relaxation rate, the transverse components (x-y plane) of the fluctuating field cause relaxation, while the longitudinal component (z-axis) not cause any relaxation by the molecular motions. In R_2 relaxation, which measures the relaxation in the x-y plane, the relaxation rate will also be affected by the longitudinal component of fluctuating field. In the end, this results in the fact that the R_1 relaxation is less dependent on molecular tumbling times, compared to R_2 relaxation that is approximately proportional to the rotational correlation times (**Figure 18**).

Figure 18, The relation between R_1 , R_2 relaxation rates and molecular tumbling times.

The Nuclear Overhauser Effect (NOE) can occur between nuclei not connected by scalar couplings. If the nuclei are close enough in space, the two nuclei undergo cross relaxation and a part of the magnetization is transferred from one nucleus to the other. In heteronuclear ^{15}N NOE relaxation experiments, the cross relaxation gives information about the magnetization transferred from ^{15}N to ^1H . The cross relaxation rate will depend on the N-H bond vector movement, resulting in low or negative NOE values for flexible parts of the proteins, which display higher local dynamics compared to the overall tumbling of the protein. More rigid parts of the protein display higher NOE values (Rule and Hitchens 2006; Teng 2012).

In addition to the relaxation experiments discussed above, CPMG relaxation dispersion experiments can be used to probe millisecond dynamics, which can be valuable both when studying intermolecular and intramolecular exchange. Intermediate exchange processes will lead to line broadening in the NMR spectra, and the lower populated conformer is usually not observed in the spectra. The CPMG relaxation dispersion experiment is composed of a set of variable number of spin echoes (refocusing pulses) during a constant time delay and each pulse will refocus the magnetization, resulting in less line broadening so that millisecond exchange can be detected (Mittermaier and Kay 2006).

4.3.4 Interaction analysis using NMR

The classical approach to evaluate protein-protein and protein-ligand interactions by NMR is to record a set of HSQC spectra and evaluate the chemical shift perturbations (CSP). If the assignment is known, chemical shift differences for the N-H cross peak in the labeled protein can be followed upon addition of a second unlabeled protein or ligand. The amino acids involved in the binding, will display perturbed chemical shifts. The magnitude of the ^{15}N and ^1H shifts can be expressed as CSPs (Eq. 9), where the scaling factor, R_{scale} is set to 6.5 (Mulder, Schipper et al. 1999).

$$\Delta\delta = \sqrt{\Delta\delta_{\text{H}}^2 + (\Delta\delta_{\text{N}}/R_{\text{scale}})^2} \quad (9)$$

A second approach is to record HNCQ spectra with and without the ligand and evaluate the peak intensities (Mittag, Orlicky et al. 2008; Bozoky, Krzeminski et al. 2013; Lukhele, Bah et al. 2013). This approach is valuable for highly flexible proteins, such as IDPs, that usually display a major overlap between peaks in the HSQC spectra. For flexible proteins CSPs can be very small, due to the fact the interaction sites in flexible proteins most commonly only have transient restricted motion and the CSPs are small. The perturbations are hard to detect due to the rapid exchange between states, which results in line broadening, and it is therefore more valuable to investigate the peak intensity ratios in the presence and absence of a binding partner. If no interaction occurs, the ratio should be one or close to one, while amino acids participating in the interaction show decreased ratios. Still, CSPs are seen for IDPs that undergo a more disorder-to-order transition upon interaction with the binding partner.

5. Summary of papers

A short summary of the appended papers is presented below.

Paper I

"Transient structure and dynamics in the disordered c-Myc transactivation domain affects Bin1 binding" Nucleic Acids Research (NAR) **40**(13): 6353-6366.

In this paper we describe the intrinsically disordered details in c-Myc₁₋₈₈, a construct comprising the well-conserved Myc box I. In addition, the interaction with the tumor suppressor Bin1 is studied by SPR and NMR spectroscopy. The results from this paper show that c-Myc₁₋₈₈ is intrinsically disordered, but as showed by NOEs, relaxation parameters and secondary structure propensities (SSP) profiles, c-Myc displays transiently structured regions. The transiently structured regions are situated within MBI as well as N-terminal to MBI, residues 22-33. Bin1 primarily binds to Ser62 in the MBI region. c-Myc₁₋₈₈ maintains its intrinsic disorder upon binding, revealing a dynamic disorder, 'fuzzy' complex.

Paper II

"High-resolution structure of TBP with TAF1 reveals anchoring patterns in transcriptional regulation." Nature Structural & Molecular Biology (NSMB) **20**(8): 1008-1014.

In this paper we present the crystal structure (1.97 Å) and NMR spectroscopy analysis of the yeast TBP bound to the N-terminal domains TAND1 and TAND2 of TAF1. The work describes the molecular details of the transcriptional activating and repressing regions in TAF1 bound to TBP. We show that TAND1 binds to the hydrophobic concave surface of TBP, occupying the same structural space as the TATA box in the TBP-DNA structure. Furthermore, TAND2 binds a conserved TBP surface through electrostatic and hydrophobic anchoring of TAND2 to TBP. Growth phenotype of yeast strains containing TAND mutations further assays the important anchoring points and the effects of TAND2 mutations. In summary, this study highlights TBP anchoring residues, which can easily disrupted or enhanced, thus providing insight to the transcription machinery and transcriptional regulation.

Paper III

"Basic Tilted Helix Bundle - A new protein fold in human FKBP25/FKBP3 and HectD1." Biochemical and Biophysical Research Biochemical Communications (BBRC), *in press*.

In this paper, we describe the structure of a novel N-terminal domain motif in FKBP25₁₋₇₃, together with the structure of a sequence-related subdomain of the E3 ligase HectD1 that we show belongs to the same fold. We name this novel fold Basic Tilted Helix Bundle (BTHB) domain. The motif adopts a compact 5-helix bundle and contains a positive charged surface around helix H4 that we suggest have a conserved functional role and possible involved in the DNA binding of FKBP25. Further, the interaction between FKBP25₁₋₇₃ and YY1 is described. HSQC titration experiments using ¹⁵N labeled FKBP25₁₋₇₃ and unlabeled YY1₂₉₃₋₃₅₀ shows CSPs on FKBP25 distant

from the positively charged surface. YY1 shows enhance DNA binding activity of upon binding to FKBP25 (Yang, Yao et al. 2001) and this article provides structural insight to the FKBP25/YY1 interaction.

Paper IV

"Pre-anchoring of Pin1 to unphosphorylated c-Myc in a dynamic complex affects c-Myc stability and activity." Pending submission to Nature Structure and Molecular Biology (NSMB).

In this paper the interaction of c-Myc₁₋₈₈ with Pin1 is analyzed in molecular detail, both for unphosphorylated and Ser62 phosphorylated c-Myc₁₋₈₈. We have been able to specifically phosphorylate c-Myc₁₋₈₈ at Ser62 and we confirm this by NMR assignment of the phosphorylated spectra together with mass spectroscopy analysis. The interactions between unphosphorylated and Ser62 phosphorylated c-Myc₁₋₈₈ and full-length Pin1 as well as the different domains, Pin1_{WW} and Pin1_{PP1ase} are studied using SPR and NMR spectroscopy. We show that Pin1 is able to bind unphosphorylated c-Myc₁₋₈₈ at the transiently structured region (a.a. 22-33) N-terminal to MBI (described in detail in paper I). Upon Ser62 phosphorylation, Pin1 binds to this site as well as to the phosphorylated Ser62 and the surrounding residues in MBI. Further, cellular assays using c-Myc mutants, mutated at a.a. 21 to 24, show decreased binding to Pin1. The decreased binding is further supported by SPR studies. Computational simulations, using experimental constrains, adds valuable information to the present study by providing a model of the disordered Myc-Pin1 complex. Taken together, this study provides a first molecular description of a disordered but specific c-Myc complex.

6. Conclusions

Based on the studies in this thesis (paper I-IV) the following conclusions can be drawn:

- The thesis provides molecular insight to the intrinsically disordered c-Myc TAD and highlights the importance of intrinsic disordered and dynamic complexes in transcriptional regulation.
- The biophysical properties of Pin1 interactions with a longer substrate are investigated. This has provided further insight to the molecular regulation of the c-Myc degradation pathway.
- By studying the structure and dynamics of the TBP-TAND12 complex, this thesis identifies TBP anchoring residues and provides molecular insight to the transcription and the transcription machinery. Furthermore, the preliminary results included in the introduction to this thesis give a first insight to the behavior of TBP in complex with the transcriptional activator c-Myc.
- A new fold has been identified and described, which we named the “Basic Tilted Helix Bundle”, BTHB.

7. Future perspectives

Nevertheless, many questions remain regarding c-Myc regulation and degradation. So far, most studies on c-Myc have been carried out using cellular assays and low resolution *in vitro* methods. Although a large number of interaction partners have been mapped to c-Myc, molecular details are missing. This thesis provides insight to the biophysical properties of the c-Myc TAD. Detailed information of the interactions controlling c-Myc stability and activity is important since it will increase our understanding for of cancer.

We show that Pin1 is able to bind to both unphosphorylated and Ser62-phosphorylated c-Myc. This is a first step towards a more detailed view of c-Myc degradation. To further evaluate this binding, it would be interesting to specifically look at the *cis-trans* isomerization in c-Myc upon Pin1 binding. Moreover, in order to study the sequential phosphorylation events that are suggested to regulate c-Myc stability and activity and evaluate the binding to Pin1, it would be valuable to study c-Myc phosphorylated at Thr58 and phosphorylated at both Thr58 and Ser62.

So far, we have studied the structural details for c-Myc₁₋₈₈. We have constructs that cover MBII as well, but due to solubility reasons we have only been able to use these constructs for circular dichroism and surface plasmon resonance studies. We have convincingly shown that we can

increase our knowledge regarding c-Myc by studying the region covering MBI in detail. It is now important to go further and study the MBII region, since this region is important for transcriptional repression and activation as well as transformation. Detailed studies of this region and its interactions could answer questions regarding possible transient structure in MBII and its role in interactions with c-Myc MBII binding partners.

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