Post-transcriptional control of C/EBPα and C/EBPβ proteins

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CHAPTER VI

Discussion and Perspectives

C/EBPα acetylation

CCAAT/enhancer binding proteins (C/EBPs) are a family of transcription factors important for the function of several organs like liver, skin, lung and adipose tissue. C/EBPα and C/EBPβ are the founding and most studied members of this family. They contain a highly conserved basic leucine zipper domain at the C-terminus that is involved in dimerization and DNA binding as well as activation domains in the N-terminus. C/EBPα and C/EBPβ are involved in the regulation of several cellular processes like gene expression, proliferation, differentiation, energy metabolism, innate immunity, inflammation and senescence. To fulfill these many functions, C/EBPα and C/EBPβ undergo several post-transcriptional alterations downstream of signaling events regulating their specific cellular activities.

Currently, lysine acetylation emulates phosphorylation in frequency of occurrence. It is a reversible process and it is regulated by two groups of enzymes, lysine acetyltransferases (KATs) transferring the acetyl groups to lysine residues of the substrate proteins and lysine de-acetylases (KDACs) catalyzing the reverse reaction. It is apparent that acetylation of non-histone proteins is important for regulating the cellular energy metabolism. For example, a large number of mitochondrial proteins[1], as well as many metabolic enzymes [2,3] are found to be acetylated. Furthermore, many transcription factors are subjected to lysine acetylation affecting their intracellular localization, stability, activity and interaction with other proteins[4,5]. In **chapter III** of this thesis I focused on the acetylation of C/EBPα and investigated whether C/EBPα is acetylated and how that can regulate its activity with emphasis on its metabolic function.

C/EBPα is acetylated at multiple lysines by p300 and deacetylated by SIRT1

Specific acetylation of the transcription factors associated with regulation of metabolic genes and its relevance to metabolic homeostasis like Forkhead box O1 (FOXO1), cAMP-responsive element-binding (CREB) protein and C/EBPβ have been reported[5]. We examined C/EBPα acetylation in liver cells and demonstrated that acetylation of endogenous and ectopically expressed C/EBPα is detectable. We demonstrate that C/EBPα is acetylated by p300 and deacetylated by SIRT1 and that the acetylation status of C/EBPα regulates its transcriptional activity. Our analysis

show that C/EBPα is acetylated by p300 at multiple lysines and the residue K298 primes for p300-catalyzed acetylation at various additional lysines. It was previously reported that p300 interacts with multiple conserved regions of C/EBPα transactivation domains. It was suggested that p300 is recruited to the promoters of C/EBPα target genes through this interaction to increase chromatin accessibility by acetylating the histones nearby and induce gene expression[6]. Here we show that C/EBPα itself is acetylated by p300 which increases its transcriptional activity on a C/EBPα responsive promoter. However, it has still to be investigated whether p300 is stimulating the transcriptional activity by only acetylating C/EBPα or by acetylating other transcription factors located in the same transcriptional complex. We show that KQ-C/EBPα mutants have higher binding affinity to p300. Therefore the higher reporter activity observed with this mutant could be the result of increased recruitment of p300 when C/EBPα is acetylated and thereby due to the acetylation-induced stabilization of the transcription factor/co-activator complex. It has been shown that p300 contains a bromo-domain which interacts with acetylated lysine residues and thereby plays an important role in protein binding selectivity of p300[8]. Since the Q mutation of C/EBPα K298 alone strongly induced the acetylation of other C/EBPα lysine residues and on its own induces the activity of the C/EBPα dependent reporter it is probably the acetyl-lysine recognition site mediating tight interaction with the bromodomain of p300.

Our transcriptome analysis revealed 110 upregulated and 122 downregulated genes in cells expressing the K159/298R mutant mimicking deacetylated C/EBPα when compared with cells expressing the K159/298Q-acetylation mimicking C/EBPα mutant. The non-acetylated K159/298R mutant of C/EBPα induces the expression of genes involved in the function of mitochondria and oxidation-reduction processes and reduces the expression of glycoprotein genes. So far we do not know whether the acetylation state of C/EBPα regulates the transcription of those genes by direct promoter association or indirectly through affecting other regulators of gene expression. Because of its tumor suppression function, C/EBPα-associated DNA fragments (chromatin immunoprecipitation DNA sequencing-ChIP-Seq) database is not available. However, C/EBPα and C/EBPβ bind to the same recognition sequence and by using ENCODE database (http://genome.ucsc.edu/ENCODE/) we found that most of both up- and downregulated genes show C/EBPβ-association with their

promoters suggesting that they might also be direct targets of C/EBPα. A ChIP analysis of the promoter region of the regulated genes can be applied using a C/EBPα specific antibody to check the binding of acetylated and deacetylated C/EBPα to these promoters. The finding that genes upregulated in response to K159/298Q-C/EBPα overexpression and those upregulated in response to K159/298R-C/EBPα overexpression fall in different GO-term categories suggests that the acetylation of C/EBPα regulates its transcriptional activity by determining the interaction partners and that depending on the interaction partner a different group of target genes gets activated. This type of regulation is not easy to be investigated by a reporter that contains only C/EBP binding sites and might explain why K159R- and K298R-C/EBPα mutants had no effect on the reporter activity.

It has been shown in leukemic cell lines expressing endogenous C/EBPα as well as in primary leukemic samples that C/EBPα is acetylated by GCN5 at K298, K302 and K326, all located in the C-terminal DNA binding or dimerization domains. Simultaneous acetylation at these residues inhibited C/EBPα granulopoietic function by reducing its DNA binding and thereby its transcriptional activity[7]. Although all three lysines K298, K302 and K326 were not covered in our MS analysis, only K298 was predicted by bioinformatics software to be acetylated by p300 and deacetylated by SIRT1 and because of that it was included in our experiments. Our results show no difference in subcellular localization or DNA binding ability of C/EBPα when it was acetylated by p300 or deacetylated by SIRT1 as well as when C/EBPα was mutated to C/EBPα-K159/298Q or C/EBPα-K159/298R. However, we analyzed the double K159/298Q and K159/298R mutations where K159 resides in the N-terminus transactivation domain and only one residue, K298 resides in the C-terminal DNA binding domain. The difference between the data from Bararia et al and our results thus might be originating from the partially different lysine residues addressed: it is possible that the acetylation of lysine K298 alone in the DNA binding domain is not enough to alter C/EBPα DNA binding but that the acetylation of more lysines in this region is required to loose DNA binding. Interestingly, Bararia et al also showed that single acetylation mimicking mutation in one of the three lysines had no effect on DNA binding and transactivation[7]. Thus, it might be possible that different cell systems (leukemia cell lines versus hepatoma cell lines) and/ or different acetyltransferases (GCN5 versus p300) stimulate the acetylation of different lysine

residues, thereby differently affecting C/EBPα activity. However, Bararia et al found that GCN5 represses the activity of a C/EBPα driven luciferase reporter while we found that although GCN5 binds to C/EBPα it doesn't alter the reporter activity even with increased expression levels, suggesting that the type of acetyltransferase involved cannot explain all of the different results between our and the Bararia et al study. Interestingly, it has been shown that C/EBPε is acetylated by p300 and deacetylated by SIRT1 at lysines K121 and K198 and this acetylation is important for terminal neutrophil differentiation. Similar to our finding for C/EBPα, the cellular localization of C/EBPε was not affected and its acetylation by p300 increased its activity on a C/EBP-binding site containing M-CSFR-promoter reporter. However it was found that deacetylated C/EBPε mutants have less DNA binding opposite to our finding for C/EBPα and also to what Bararia et al have found[60].

Together, the findings of our and other studies indicate that the regulation of C/EBPα function by acetylation might be complex and suggest that several KATs regulated by different stimuli can acetylate C/EBPα at multiple residues in different cellular settings resulting in different outcomes on its DNA binding and transactivation.

The crosstalk between acetylation and other posttranslational modification of C/EBPα

Both acetylation and phosphorylation can regulate key cellular processes in response to extracellular signals and different posttranslational modifications of nuclear proteins can be interdependent[9]. For example, FOXO1 acetylation increases its phosphorylation and decreases its DNA binding ability[10] as well as the phosphorylation of p53 increases its association with p300 acetyltransferase and by that increases its acetylation[11,12]. Here we show that C/EBPα is acetylated at multiple lysines and it has been shown before that C/EBPα is sumoylated, phosphorylated and methylated as well which brings up the question about a potential crosstalk between the these different modifications.

The phosphorylation of C/EBPα at serine 193 by cyclin dependent kinase-4 (cdk-4) stimulates the C/EBPα/p300 complex formation[13,14] and thereby increases steatosis in mice[15,16]. Thus, it is possible (likely) that phosphorylation of C/EBPα at serine 193 stimulates acetylation of C/EBPα which might even play a role in the development of

steatosis in response to serine 193 phosphorylation. Therefore, it would be interesting to analyze the C/EBPα acetylation state in serine 193 mutant expressing cells.

Sumoylation of C/EBPα at K159 prevents its interaction with BRG1 (a core subunit of the SWI/SNF chromatin remodeling complex) and by that the C/EBPα induced proliferation arrest and the transactivation of the liver specific albumin gene[17]. The finding that K159 in C/EBPα is both sumoylated and acetylated suggests a switch mechanism regulating the transcriptional and anti-proliferative activity of C/EBPα. The increased activity of the luciferase reporter by the K159Q mutation could be explained by the prevention of sumoylation at this site which might increase the interaction with BRG1 or other cofactors of C/EBPα. However, the K159R mutation didn't increase the transcriptional activity although it similarly prevents sumoylation suggesting other mechanism that mediate the reporter stimulating activity of acetylated C/EBPα. To exclude genes in our transcriptome that were affected by sumoylation of C/EBPα we limited our analysis to the differentially expressed genes (DEGs) whose median expression in the wt condition was between the median expression of KQ and KR conditions. By that we insured that the DEGs are results of acetylation/deacetylation regulation and avoided the effects which might result from the prevention of sumoylation at K159.

Relationship between C/EBPα Deacetylation and SIRT1

In addition to the known function of C/EBPα in glucose metabolism, hepatic glycogen storage and gluconeogenesis[18], it has been reported that C/EBPα plays an important role in mitochondrial biogenesis and the transcription of both nuclear and mitochondrial encoded genes for mitochondrial proteins in brown adipose tissue (BAT)[19].

SIRT1 is a NAD^+ dependent lysine deacetylase that has been implicated in a long list of biological functions, including control of lipolytic rates in white adipose tissue[20], regulation of insulin secretion from pancreatic β-cells[21], modulation of cytoplasmic and mitochondrial acetyl-CoA synthetase activity[22]. SIRT1 activation has beneficial effects on mammalian metabolism including improvements in glucose tolerance and decreased hepatic glucose production and induction of β-oxidation[23,24]. It is believed that the effect of SIRT1 is mediated in a large part through deacetylating the transcriptional regulators FOXO1 and PGC1a[23]. Our data link C/EBPα to SIRT1

and suggest deacetylated C/EBPα as a new mediator of the mitochondrial biogenesis function of SIRT1. The finding that deacetylated C/EBPα expressing cells have upregulated mitochondrial and oxidation reduction process related genes and increased mitochondrial mass supports this suggestion. It is known that SIRT1 facilitates the conversion of changes in the nutritional status, which it senses via NAD+ levels, into modulation of cellular metabolism. The link between C/EBPα and SIRT1 is further verified by the finding that cells expressing wt C/EBPα have a respiration rate similar to acetylated C/EBPα expressing cells at high glucose (low NAD+ and low SIRT1 activity) while it was similar to deacetylated C/EBPα expressing cells at low glucose (high NAD+ and high SIRT1 activity). The last phenotype was reverted upon SIRT1 inhibitor treatment. In addition, It has been shown that mice treated with resveratrol (SIRT1 activator) display increased oxygen consumption[25] and improved glucose tolerance explained by high levels of adiponectin[25,26]. SIRT1 regulates adiponectin gene expression through FOXO1-C/EBPα complex[27]. It was suggested that SIRT1 increases adiponectin transcription in adipocytes by deacetylating FOXO1 and enhancing its interaction with C/EBPα. However, $Foxo1^{KR/KR}$ mice, having lysine to arginine mutations, and SirBACO mice, having moderate over expression of SIRT1, shared limited metabolic features[28]. Thus other transcription factors might be involved in the downstream effects of SIRT1. Our finding together with the widespread role of C/EBPα in mammalian metabolism provides a testable hypothesis that part of the downstream effects of SIRT1 are mediated by the deacetylated C/EBPα. We don't know whether deacetylated C/EBPα induces mitochondrial genes independently or in collaboration with PGC1α or FOXO1 and this question is important to be investigated in the future. We raise the question whether C/EBPα deacetylation by SIRT1 also regulates the FOXO1-C/EBPα complex formation and by that adiponectin expression. This question can be answered by expressing C/EBPα acetylation or deacetylation mutants with FOXO1 and investigating their complex formation by co-immunoprecipitation and the resulting transcriptional effects on an adiponectin promoter and on endogenous adiponectin gene expression.

One of the most important questions that still need to be solved is the physiological *in vivo* consequences of C/EBPα acetylation/deacetylation. The answer to this question can be achieved by generating knock-in mouse models expressing

either a constitutively acetylated or deacetylated mutant of C/EBPα. With these mouse models we can investigate whether C/EBPα acetylation controls whole body energy homeostasis. If SIRT1-regulated C/EBPα links the metabolic state of the mice to cellular gene expression depending on the nutrient availability or deprivation, mice expressing the deacetylated C/EBPα mutant might have a similar phenotype to SIRT1 overexpression which mimics some effects of calorie restriction (CR) and promotes insulin sensitivity[29]. In addition, the C/EBPα acetylation mutant mice could be combined with (tissue-specific) Sirt1 knock-out or overexpressing mice to analyze a potential rescue of SIRT1 dependent phenotypes. These findings could have important implications to design specific C/EBPα/SIRT1 based therapeutic interventions for disorders such as obesity, type II diabetes, and metabolic syndromes.

Taking the above mentioned findings together and knowing that C/EBPα is an important regulator of lipid and glucose metabolism we suggest that the acetylation state of C/EBPα might link the metabolic state of the organism to cellular gene expression, allowing target tissues to adjust their metabolism to nutrient availability (acetylated C/EBPα) or deprivation (deacetylated C/EBPα) by recruiting different complexes of activators or repressors to affect the hepatic expression of genes involved in gluconeogenesis, glycogen storage, and lipid metabolism[18,30,31]

A screening strategy to reduce C/EBPβ-LIP translation

Previously, we have shown that mTORC1 via phosphorylation of 4E-BPs specifically controls the translation of the C/EBPβ-LIP protein isoform and that mTORC1 inhibition reduces LIP expression[32,33]. Furthermore, C/EBPβ$^{\Delta uORF}$ knockin mice that have reduced C/EBPβ-LIP expression and thus reflect reduced mTORC1-signaling at the C/EBPβ level, displayed CR-like metabolic improvements without reducing calorie intake[33]. In **chapter IV** we developed a reporter system that acts as a surrogate for C/EBPβ-mRNA translation. We used this reporter in a high-throughput screening to discover new drugs that reduce C/EBPβ-LIP translation and exhibit potential CR-mimetic properties.

C/EBPβ-LIP targeting

C/EBPβ plays an important role in regulating genes related to different cellular processes including glucose and fat metabolism. Its activity is regulated by a special mechanism characterized by the competition of two isoforms LAP and LIP which are expressed from one mRNA by the usage of different translation start sites. The translation of C/EBPβ mRNA into LAP isoform occurs via normal translation initiation at LAP-AUG codon while the translation into LIP isoform requires an initial translation of the uORF in the mRNA leader sequence, followed by translation re-initiation from the downstream LIP-AUG codon. C/EBPβ-LIP lacks the transactivation domains but it retains the DNA binding domain and by that it competes with C/EBPβ activator isoform (LAP) and inhibits its function. Thus, the ratio between LIP and LAP is the key factor determining the biological function of C/EBPβ. We know that the C/EBPβ-LIP/LAP ratio is decreased upon CR and increased upon high fat diet and ageing[33 and unpublished data]. Furthermore, C/EBPβ$^{\Delta uORF}$ mice lacking the cis-regulatory upstream open reading frame (uORF) in the C/EBPβ-mRNA, which is required for mTORC1-stimulated translation into C/EBPβ-LIP were produced. They show a strong reduction of LIP expression in different tissues and have reduced body weight and display an improved metabolic phenotype, including reduced fat accumulation and increased β-oxidation, improved insulin sensitivity and glucose tolerance as well as enhanced activity[33].The idea of finding drugs that modify the LIP/LAP ratio got matured when we observed that the CR-like healthy phenotype in the C/EBPβ$^{\Delta uORF}$ knockin mice developed without a reduction in calorie intake[33]. We believed that decreasing the LIP/LAP ratio might be therapeutically valuable to gain metabolic improvements similar to CR.

The obvious way to modify the function of C/EBPβ isoforms toward less LIP and more LAP is to target C/EBPβ-LIP and reduce its inhibitory effects. However, this is very challenging because both C/EBPβ-LIP and LAP are expressed from the same mRNA and LIP sequences are 100% included in LAP and this makes it not possible to target LIP without affecting LAP. Therefore, we decided to reduce the C/EBPβ-LIP/LAP ratio by interfering at the translational level and based our reporter construct on the translation initiation and uORF-dependent re-initiation mechanism that regulates the translation of the C/EBPβ-mRNA into different protein isoforms. It is known that this translation mechanism can be targeted pharmacologically by

mTORC1 inhibitors[33]. Thus, We established a cellular reporter system using firelfly and renilla luciferases as readouts for LAP and LIP expression that is applicable for high throughput screening.

Screening for C/EBPβ-LIP reducing drugs

We constructed a dual reporter plasmid that expresses a renilla luciferase transcript for the simulation of translation initiation into LAP and a firefly luciferase transcript for the simulation of translation re-initiation into LIP, both under control of the C/EBPβ-5′-leader sequence. In the initiation cassette of this reporter plasmid, only the ribosomes that skip the uORF in the C/EBPβ-5′-leader sequence and initiate translation at LAP-AUG are able to produce a renilla luciferase signal representing LAP translation. While in the re-initiation cassette, the firefly luciferase signal representing LIP translation can only be achieved by ribosomes that translate the uORF in the C/EBPβ 5′leader sequence first and then re-initiate at the firefly luciferase-AUG that is placed at the position of the LIP AUG. However, those ribosomes that skip the uORF in the re-initiation cassette and initiate translation at LAP-AUG reading frame can't produce any firefly signal due to the introduction of a +1 shift mutation which brings the LAP-AUG and the firefly luciferase-AUG in different reading frames. The usage of this bidirectional reporter enables the expression of Firefly and Renilla from the same cell and prevents any effects due to different transfection efficiencies or integration sites in stable expression systems.

Before setting up the drug screening we validated our reporter system and insured that it acts correctly as a surrogate of C/EBPβ-mRNA translation. The system was validated using pharmacological inhibition and genetic mutations in the mTORC1 pathway, overexpression of wt and mutated eIFs (eIF2α, eIF4E), and a specific regulator of translation re-initiation (DENR) and compared with the expression of endogenous C/EBPβ-LIP and LAP isoforms. The system properly imitated the translation initiation and re-initiation events of the C/EBPβ-mRNA. Then, we screened a library of 780 FDA approved drugs in an automated high throughput cellular assay looking for the hits that reduce translation re-initiation under the control of the C/EBPβ-uORF. This type of screen is categorized as phenotypic screen leading to the identification of molecules that alter the phenotype of the cell or organism in a desirable manner acting on one or more unknown targets.

In our case, identifying drugs that reduce translation re-initiation by this type of screen and afterwards investigating their molecular mechanisms of action might not only lead to the development of novel CR mimetics but could also result in the identification of new pathways and factors that are playing roles in mRNA translation control. Another advantage of our drugs screen is the use of the FDA approved drugs library which offers the potential for drug repurposing. Since the pharmacokinetic and pharmacodynamic parameters are established for these approved drugs, a repurposing strategy reduces the cost and time scale for their evaluation against alternative diseases and accelerates their novel clinical uses. Therefore, a repurposing screen is a very promising strategy for developing novel therapies and it has already been applied to identify approved drugs with novel anti-cancer[34], anti-microbial[35] and anti-viral activity[36].

In our screening we identified drugs that affect the translation re-initiation/initiation ratio. The focus of this project was to identify drugs that decrease the LIP/LAP ratio and among the 162 identified drugs that altered the ratio between Renilla (initiation) and Firefly (re-initiation) 45 were ratio decreasing drugs. In addition to rapamycin which we used in the screen as an external positive control, the compounds library contained everolimus and sirolimus (mTORC1 inhibitors derived from rapamycin) and both of them were found in the 45 hits that decreased the re-initiation/initiation ratio. This shows that our screening approach is indeed functional. Four drugs which had the maximum effect in reducing the ratio were tested on three different cell lines to reproduce their effects on the reporter system and to examine their effects on endogenous C/EBPβ-LIP/LAP expression. Among these four drugs, adefovir dipivoxil (ADV) passed all the counter assays and gave results similar to rapamycin in all tested cell lines without any cell type dependency. ADV is a nucleotide analogue of adenosine monophosphate prescribed to treat chronic infections with hepatitis B virus. ADV is administered orally and after its absorption it is cleaved to adefovir by esterases and undergoes intracellular phosphorylation to its active metabolite, adefovir diphosphate[37]. The mechanism of action of ADV in mRNA translation initiation is still unclear and it is beyond the focus of this project but it is one of the most important investigations that has to be done in the future. The methods to test the effect of ADV as a CR mimetic via the C/EBPβ-LIP pathway in cell culture are quite limited. It has been shown that LIP downregulation

results in a metabolic shift towards more fatty acid β-oxidation (FAO) in cell culture experiments and in C/EBPβ$^{\Delta uORF}$ mice .Similarly, CR of mice results in reduced C/EBPβ-LIP expression and increased β-oxidation although the direct connection between these two events have not yet been proven under CR conditions. Furthermore, increased expression of lipogenesis and lipolysis genes in WAT and increased expression of β-oxidation genes in liver are also observed in both C/EBPβ$^{\Delta uORF}$ and calorie restricted mice[33,38-41]. In our study, we were able to show that short term ADV treatment increases fatty acid oxidation (FAO) in a liver cell line, which could be reversed by overexpression of LIP which demonstrates the involvement of LIP regulation in response to ADV treatment. In addition, ADV treatment resulted in the upregulation of β-oxidation genes (*SCAD, MCAD, VLCAD* and *AOX*) and the concomitant downregulation of the lipogenesis genes, *SCD1* and *SREBP1,* in the same manner as it has been detected in livers extracts from C/EBPβ$^{\Delta uORF}$ mice. These experiments showed that ADV treatment through down-regulation of LIP is able to induce a metabolic change that is seen both in C/EBPβ$^{\Delta uORF}$ mice and in mice under CR and which has been suggested to play a major role in the life and health span extending effects of CR[38]. Since it is not possible to investigate other metabolic parameters in cell culture an important future experiment will be the ADV treatment of mice to investigate whether the drug alters the systemic metabolism similar to the effects found in the C/EBPβ$^{\Delta uORF}$ mice.

In conclusion, our results in this project support the suitability of the developed translation re-initiation reporter system for high throughput screening of CR-mimetics that act via the C/EBPβ-LIP pathway. Further investigations of the identified drugs that increase or decrease the LIP/LAP ratio and their targets and mechanisms of action might open many new doors to the discovery of translation control mechanisms or pathways that are important for metabolic diseases and cancer.

SBDS regulates translation re-initiation from C/EBPα and C/EBPβ mRNAs

Currently, it is proven that genetic alterations in numerous components of the translational machinery cause an entire class of inherited syndromes known as "ribosomopathies" associated with increased cancer susceptibility. In **chapter V** we focused on Shwachman Diamond Syndrome (SDS) which is an autosomal recessive disorder caused by Shwachman Bodian Diamond Syndrome (SBDS) protein loss of function. SBDS is an important protein for ribosomal maturation and here we discuss our finding that SBDS plays a role in the translational regulation of C/EBPα and C/EBPβ mRNAs and that SBDS deficiency indirectly suppresses the expression of MYC by decreasing the C/EBPα-p30/p42 isoform ratio.

SBDS promotes translation re-initiation downstream of uORFs in C/EBPα and C/EBPβ mRNAs

SDS is characterized by exocrine pancreatic dysfunction, skeletal abnormalities and bone marrow failure with neutropenia and is associated with a high risk of acute myeloid leukemia (AML) in older patients[42]. SBDS is necessary for the release of eIF6 from the mature 60S subunit which is required for ribosomal subunit association during translation initiation[43-45]. However, the connection between mRNA translation and the SDS phenotype is still lacking. It is not known if processes for 60S subunit maturation other than eIF6 release are affected by SBDS loss of function and general analysis of translated mRNAs depending on SBDS has not been performed yet. It is known that C/EBPα is an essential factor for neutrophil differentiation and C/EBPα mutations in AML patients result in a differentiation block[46]. Furthermore, our group has shown before that the truncated isoforms (C/EBPα-p30 and C/EBPβ-LIP) are translated by translation re-initiation mechanism controlled by uORF cis-regulatory elements present in C/EBPα and C/EBPβ mRNAs and this mechanism is highly sensitive to changes of activity/expression of translation regulators[32]. This prompted us to test the hypothesis that SBDS controls the translation of C/EBPα and C/EBPβ mRNAs and that the potential deregulation of C/EBP mRNA translation upon SBDS mutation might be involved in the development of the SDS phenotypes.

In this project we discovered C/EBPα and C/EBPβ mRNAs as the first specific mRNAs to be affected by SBDS deficiency. Interestingly, we found that only the truncated isoforms (C/EBPα-p30 and C/EBPβ-LIP) are affected by SBDS loss of function while the full length isoforms (C/EBPα-p42 and C/EBPβ-LAP) were not affected. Currently it is proposed that about 49% of the human transcriptome contains uORFs[47]. Usually, uORFs inhibit the downstream expression of the main ORF but this is not the case for C/EBPα-p30 and C/EBPβ-LIP whose expression by translation re-initiation requires the translation of the uORFs. We believe that uORF mediated translation re-initiation is controlled by additional regulatory mechanisms compared to the normal translation initiation and our findings nominate SBDS as one of these regulators. Studies investigating the regulatory mechanisms of uORF mediated translation re-initiation are still very few. Schleich et al have shown in Drosophila that the DENR-MCT1 complex specially promotes translation re-initiation downstream of uORFs to control cell proliferation and tissue growth[48]. However, the mechanistic details of this process are not clarified yet. The mechanism underlying the effect of SBDS on C/EBPα-p30 and C/EBPβ-LIP translation re-initiation is still to be identified. It could be that SBDS affects the recognition of the uORF start codon or of the downstream re-initiation start codon. It might also affect mRNA scanning after uORF translation termination, the termination event itself or the loading of the new initiator tRNA. It is also important to investigate whether SBDS affects other mRNAs with uORFs in their 5'UTR and if it requires specific uORF sequences for interaction. Brina et al observed that downregulation of eIF6, the antagonistic factor of SBDS, resulted in reduced expression of C/EBPβ LIP, whereas LAP expression was not affected[49]. This suggests that SBDS might affect translation re-initiation by increasing the amounts of free eIF6 which might facilitate uORF translation termination or scanning after uORF termination. Combined overexpression or knock down of SBDS and eIF6 and checking C/EBPα and C/EBPβ isoform ratios will provide a hint whether SBDS regulates uORF mediated translation re-initiation in an eIF6 dependent manner or whether it acts in an eIF6 independent way.

SBDS, C/EBPα-p30 and neutropenia

One of the main symptoms of SDS is bone marrow failure associated with neutropenia which is an abnormal low neutrophil level in the blood. In the current

SBDS literature it is debated if the SDS neutropenia results from failure of progenitor cells to differentiate or to proliferate[50-52]. While, C/EBPα-p42 is known as tumor suppressor and induces genes that are essential for neutrophil differentiation[53,54], it is believed that C/EBPα-p30 functions as a negative inhibitor of C/EBPα-p42 and by that stimulates cell proliferation and inhibits the expression of differentiation related genes. Furthermore, C/EBPα is found to be mutated in around 9% of the AML patients and some of these mutations result in abrogation of C/EBPα-p42 expression without affecting expression of the C/EBPα-p30 isoform and thereby in a strong shift in the C/EBPα isoform ratio towards p30. Similarly, knockin mice expressing only C/EBPα-p30 developed AML demonstrating that the C/EBPα isoform ratio has a strong influence on haematopoietic cell proliferation and neutrophil differentiation. MYC is an important regulator of haematopoietic stem cell self-renewal and differentiation. It is known that C/EBPα-p42 inhibits MYC expression by interacting with the E2F transcription factor complex at its promoter which is required for driving proliferating myeloid precursor cells to undergo cell cycle arrest. Interestingly, C/EBPα-p30 opposes this function[55]. Treatment of HL60 myelomonocytic leukemia cells with the drug CDDO decreases the C/EBPα-p30/p42 ratio and thereby resulted in strong reduction of Myc expression and reduced proliferation[56] indicating that changes in the C/EBPα-p30/p42 ratio affect cell proliferation through transcriptional control of the Myc gene. Our data show that the decrease of the C/EBPα-p30/p42 ratio by the mTOR catalytic inhibitor PP242 reduced the mRNA levels of Myc and inhibited cellular proliferation supporting the above mentioned findings by others that Myc is transcriptionally controlled by the C/EBPα isoform ratio. Furthermore, our data indicate that SBDS function stimulates proliferation through inducing C/EBPα-p30 expression and thereby relieving the C/EBPα-p42 mediated inhibition of Myc expression. Upon SBDS knockdown in HL60 we observed reduction in both Myc RNA and protein expression and cell proliferation indicating that SBDS downregulation might have an inhibitory effect on Myc transcription and cell proliferation by reducing C/EBPα-p30 and thus shifting the C/EBPα isoform ratio towards p42. The involvement of the C/EBPα isoform expression in the regulation of Myc expression was strengthened by the finding that C/EBPα-p30 ectopic expression prevented the downregulation of Myc and attenuated the inhibition of cell proliferation in SBDS knockdown HL60 cells. Furthermore

during the differentiation of HL60 by ATRA treatment, we observed high correlation between SBDS, C/EBPα-p30 and Myc expression. All three proteins were highly expressed at the early stages of the differentiation process when the cells were still proliferating while their expression was concomitantly downregulated during later differentiation stages when proliferation stopped. Our finding is in agreement with other studies showing that SBDS loss of function resulted in proliferation defects in 32Dcl3 cells and SDS-patient bone marrow $CD34^+$ hematopoietic progenitor cells[50,58]. Further research has to be done to investigate whether SBDS in addition also affects neutrophils differentiation as it has been proposed by others[51,52]. The origin of the AML phenotype in SDS patients has to be further investigated. AML development in SDS patients looks contradictory to the shift toward less C/EBPα-p30 expression. Our data show that SBDS loss of function reduces proliferation via downregulating C/EBPα-p30 and Myc and this doesn't match with the block of differentiation and uncontrolled proliferation of AML cells. However, it is well established that severe congenital neutropenia is associated with the development of AML. Dong et al found that AML patients having mutations in the gene for G-CSF receptor also suffered from severe congenital neutropenia[59]. This suggests that the AML phenotype in SDS patients might not be directly linked to C/EBPα-Myc pathway but might select for additional mutations that drive AML development. The fact that SDS is a rare disease and only a small percent of SDS patients develop AML makes it hard to screen enough patients and investigate the mechanism behind the AML development. With our findings, we are one step forward toward better understanding of the SDS etiology. Our results suggests C/EBPα translation deregulation as one potential cause of the SDS neutropenia phenotype. To further prove this, we need to analyze whether $C/EBP\alpha^{\Delta uORF}$ mice that similarly to $C/EBP\beta^{\Delta uORF}$ mice have a mutation in the uORF and thus potentially show reduced expression of the p30 isoform, develop neutropenia. However, these mice are not available so far. Alternatively, SBDS mutant mice can be crossed with C/EBPα-p30 over expressing mice to examine whether the SBDS-related neutropenia phenotype can be rescued. In addition, there are still many open questions that are important to be answered. One of these questions addresses the mechanism of translational regulation of C/EBP isoform expression by SBDS and asks whether SBDS regulate the uORF-mediated translation by its known function in releasing eIF6 from the 60s

ribosomal subunit or by a different mechanism? It is also important to investigate whether the C/EBPα or C/EBPβ isoform ratio in response to mutated SBDS plays a role for the other non-hematopoietic SDS phenotypes or whether those phenotypes are caused by translational deregulation of other mRNAs.

Conclusion

From the work presented in this thesis, we have gained better insight into the regulation of the metabolic function of C/EBPα and found that it potentially plays a role in SIRT1-controlled cellular metabolic homeostasis. We further engineered a C/EBPβ-based translation control reporter system which is suitable for high throughput drug screening and with this system we identified novel drugs that decrease C/EBPβ-LIP translation and potentially have calorie restriction mimetic properties. Finally, We have shown that the ribosomal maturation protein SBDS is important for the translation re-initiation of C/EBPα-p30 and C/EBPβ-LIP and that their impaired translation is contributing to the hematological phenotype of SDS.

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