6. Summary, discussion and future perspectives
Summary

The prognosis for patients with acute myeloid leukemia (AML) is currently still rather poor, mainly due to disease relapse. Whereas current standard chemotherapy strategies lead to an initial reduction of leukemic blasts in the majority of patients, a small cell population persists and is apparently not efficiently targeted, leading to recurrence of the disease with variable latency. In order to improve the effectiveness of AML therapy, further characterization of those disease-initiating cells is warranted, which is the scope of this thesis.

In AML, various mutations and genetic alterations are found in the leukemic cells and lead to the characteristic clinical picture of the disease, which is defined by an accumulation of leukemic blasts in the bone marrow and peripheral blood. Despite the genetic heterogeneity, AMLs commonly follow a particular pattern of disease evolution which is characterized by a stepwise acquisition of mutations. Early events in AML evolution occur in normal hematopoietic stem and progenitor cells (HSPCs) leading to the formation of preleukemic HSPCs, which still have the capacity to differentiate normally, however, have altered properties regarding self-renewal and maintenance. Later events in those cells cause differentiation- and proliferation abnormalities resulting in the formation of leukemia stem cells (LSCs), which eventually give rise to full-blown leukemia. In contrast to the majority of leukemic cells, LSCs have self-renewal properties and are capable of initiating and maintaining the disease. Importantly, LSCs are thought to be chemoresistant and to drive disease relapse. Thus, studying the molecular mechanisms that underlie those LSC-specific properties might help to identify novel targets for AML therapy.

The transcriptional co-regulator CITED2 has important roles in promoting self-renewal and maintenance of both HSCs and LSCs. It was previously shown that knockdown of CITED2 in AML cells interferes with their long-term expansion, however, molecular mechanisms behind this observation remained unclear. In chapter 2, we aimed to study the in vivo role of CITED2 in AML maintenance and to investigate downstream alterations in signaling pathways upon CITED2 reduction. For that purpose, AML cells were transduced with a lentiviral construct for RNAi-mediated knockdown of CITED2. We observed that mice transplanted with CITED2-knockdown AML cells had a significant longer survival compared to mice transplanted with control AML cells, confirming a crucial role of CITED2 in disease maintenance. In vitro studies revealed that reduction of CITED2 leads to increased p53-mediated apoptosis, most likely resulting from an inhibition of the AKT signaling pathway. Reduction of CITED2 led to decreased levels of phosphorylated AKT, increased expression of the AKT-pathway inhibitor PHLDA3, as well as to a decreased expression of the AKT-activator SOX4 and the AKT-downstream target BCL2. The apoptotic phenotype of CITED2-knockdown cells could be rescued by simulations downregulation of PHLDA3 or upregulation of BCL2. We further observed that reduction of CITED2 led to a decreased interaction of p53 with its inhibitor MDM2, resulting in increased amounts of total p53 protein. Together
the data from chapter 2 indicate that CITED2 translates its important role in AML maintenance in part by regulating genes- and pathways that interfere with p53 activation.

The transcription factor PU.1 is a key regulator of hematopoiesis and has important functions for both maintaining HSPCs and differentiation of the myeloid lineage. Even though mutations of PU.1 itself are rarely found in AML, its expression or activation is frequently disturbed by AML-associated alterations. PU.1-knockdown mice, which resemble such a condition of impaired PU.1 activity, develop AML after a pre-leukemic phase of several months, indicating that additional alterations are required for the formation of disease-initiating cells. Potential candidates that facilitate transformation of preleukemic PU.1-knockdown cells might be factors that increase their maintenance or accelerate their proliferation properties. It was recently shown that CITED2, which has crucial roles in stem cell maintenance, is negatively regulated by PU.1, suggesting that both factors act in the same signaling network. In chapter 3, we aimed to investigate a potential synergistic effect of simultaneous PU.1 downregulation and CITED2 upregulation on stem cell biology and AML pathogenesis. Using lentiviral constructs for either overexpression of CITED2 or RNAi-mediated knockdown of PU.1, we found that simultaneous PU.1/CITED2 deregulation in human CD34⁺ cord blood (CB) cells, as well as CITED2 upregulation in preleukemic murine PU.1-knockdown (PU.1KD/ KD) bone marrow cells, significantly increased the maintenance of HSPCs compared to either factor alone.

Increased replating capacity of PU.1KD/KD/CITED2 cells in in vitro assays eventually resulted in outgrowth of transformed cells, while upregulation of CITED2 in PU.1KD/KD cells enhanced their engraftment in in vivo transplantation studies, however, without signs of leukemic transformation. Altered self-renewal and maintenance of HSPCs without a block in differentiation has been described as an early event in AML evolution, suggesting that the combined PU.1/CITED2 deregulation programs a preleukemic state. This was supported by gene expression studies showing that transcriptional changes induced by combined PU.1/CITED2 deregulation are also found in patient AML cells compared to normal CD34⁺ cells. In summary, our findings from chapter 3 suggest that simultaneous downregulation of PU.1 and upregulation of CITED2 has a synergistic effect on self-renewal pathways that are linked to clonal hematopoiesis, which is known to precede leukemic transformation.

Loss of stemness potential in Cited2-deleted murine HSCs was previously shown to be accompanied by signs of increased mitochondrial activity such as elevated mitochondrial membrane potential, elongation of mitochondrial shape and elevated ROS production. In the second part of this thesis, we focused on investigating metabolic properties of AML-initiating cells. Recent studies highlighted that LSCs characteristically have low levels of reactive oxygen species (ROS), which result from a combination of low mitochondrial activity and high activity of ROS-removing pathways such as autophagy. In chapter 4, ROS-regulating pathways and their relevance for the functionality of both normal- and leukemic stem cells were described in
Chapter 6

activity and showed a significantly lower number and activity of mitochondria. By performing RNA-seq studies we could identify gene signatures that are shared among ROS-low or ROS-high CD34+ AML cells across patients with various genetic backgrounds. Genes upregulated in ROS-low cells were associated with negative regulation of signaling pathways (SPRY1, DUSP10, PIK3IP1, DDIT4) and were enriched for stemness-associated genes (ABCB1, MEIS1, CD109, GFI1B). Downregulated genes in ROS-low cells were related to increased cell differentiation. Functional validation of the drug efflux transporter ABCB1 demonstrated that ROS-low cells have strikingly higher ABCB1 activity compared to ROS-high cells, which was previously associated with poor prognosis in AML. Functional analysis demonstrated that CD34+ AML cells with low ROS levels have an increased sensitivity to the BCL2 inhibitor Venetoclax despite the fact that we observed comparable levels of BCL2 expression in ROS-low and ROS-high cells. These findings suggest that the low mitochondrial activity in ROS-low cells makes them more vulnerable to inhibition of important mitochondrial regulators. In summary, our data from chapter 5 highlights that CD34+ AML cells with low ROS levels have stemness-related features, and thereby most likely coincide with the LSC population, which can be efficiently targeted by BCL2 inhibition.

detail. This included HIF-1α-, AMPK-, mTOR-, FOXO- and SIRT- signaling, as well as of crucial players in DNA damage response pathways such as ATM. Moreover, it was highlighted that the metabolic state of LSCs implicates certain vulnerabilities of these cells. LSCs are highly dependent on their remaining levels of mitochondrial activity, and thereby rely on pathways that regulate mitochondrial integrity. The anti-apoptotic protein BCL2 has an important role in maintaining the mitochondrial membrane potential under stress conditions and was shown to promote LSC maintenance. Similar, proteins involved in mitophagy such as FIS1 have been found to be critical for LSC survival, and thereby serve as promising new targets for AML therapy.

Even though previous studies demonstrated that LSCs have lower ROS levels compared to the total AML cell population, it remained unclear if ROS levels within the stem cell enriched CD34+ AML subtraction are indicative for stemness properties. In chapter 5, we investigated in detail how ROS levels within the CD34+ AML cell population correlate with cellular functions and characteristics such as morphology, metabolic activity, gene expression and drug responsiveness. In our study, we compared features of CD34+ AML cells that were sorted into ROS-low and ROS-high cell populations, which were defined as the cell fractions with the 15% lowest and highest signal intensity for a fluorescence-based ROS dye. We observed that ROS-low cells were remarkably smaller compared to ROS-high cells, with a prominent nucleus and scarce cytoplasm, and enriched for CD34+/CD38- cells. Furthermore, ROS-low cells had a decreased metabolic
Summary, discussion and future perspectives

Discussion and future perspectives

Interference with p53 inactivation as a therapeutic strategy in AML

The data described in chapter 2 demonstrate that reduction of CITED2 levels in AML cells results in an increased amount of p53 protein and increased levels of p53-mediated apoptosis. Reactivation of p53 function is a particular important strategy for AML therapy. Approximately 90% of de novo AML cases do not present with mutations in the TP53 gene, but often with p53 inactivation due to dysfunctions in the p53 regulatory network. TP53, located on the short arm of chromosome 17, regulates a multitude of genes involved in apoptosis, DNA repair, growth arrest and cell differentiation, and thereby represents an important tumor suppressor. Despite the presence of the p53 wild type protein, p53 functions can be impaired due to abnormalities in p53-regulator proteins. P53 is bound and inactivated by its endogenous inhibitors mouse double minute 4 homolog (MDM4 or MDMX) or the mouse double minute 2 homolog (MDM2), which are both often overexpressed in p53 wild type AMLs. Pharmacological disruption of these interactions serves as a promising strategy to restore p53-mediated tumor suppressor functions. Molecules for p53-reactivation via MDM2 or MDMX inhibition are currently investigated in early phase clinical trials. Our study described in chapter 2 suggests that reduction of CITED2 levels results in decreased formation of p53-MDM2 complexes, indicating that CITED2 is part of the p53 regulating network critical for AML cell survival.

TP53 mutations are present in less than 10% of de novo AMLs, but are observed with a frequency of ~30% in therapy related AMLs (t-AMLs) and with a frequency of ~70% in complex karyotype AMLs. The majority (70-80%) of TP53 mutations are missense mutations in “hot-spot” codons resulting in amino acid changes and consequently altered protein structure, folding and stability. These mutations are not only associated with loss of p53 tumor suppressive function (LOF), but also acquire gain of function (GOF) properties that promote tumor aggressiveness and correlate with poor prognosis. Other types of TP53 mutations include genomic loss of TP53, as it is observed in patients with deletions of 17p. Notably, patients with germline mutations in the TP53 gene only rarely develop AML, but are rather associated with different types of solid tumors. Also, TP53 mutations are more common in t-AMLs than de novo AMLs, which suggests that inactivated or altered p53 function is not sufficient for leukemic transformation, but instead results in increased cellular stress resistance. In combination with other alterations or stressors such as cytotoxic therapy, this increased resistance likely promotes leukemogenesis. In our study, we observed that knockdown of CITED2 results in increased stabilization of p53. We therefore hypothesize that increased CITED2 expression observed in AML has the opposite effect. High CITED2 levels might interfere with p53 activation and confer, in certain way similar to TP53 mutations or to non-mutational alteration in the p53 regulatory network, a survival advantage to cells under stress conditions.
Chapter 6

The role of p53 and potentially of CITED2-mediated p53 regulation in clonal HSC expansion

P53 was shown to have crucial roles in regulating HSCs in both steady-state and stress conditions. During steady state hematopoiesis, p53 regulates the balance between HSC quiescence and self-renewal. In vivo studies demonstrated that absence of p53 promotes self-renewal, since Tp53-null mice where shown to have a two-to threefold increase of cells capable of long-term blood reconstitution.18,19 In stress conditions, p53 induces expression of genes that protect the genetic integrity of cells, including genes involved in growth arrest, DNA repair and apoptosis.20 Recently, Chen et al showed that mice with a heterozygous expression of the p53 mutant p53R248W don’t have an increased number of long-term HSCs, but that mutant HSCs have a significantly increased repopulation capacity compared to wild-type HSCs, as demonstrated by competitive bone marrow transplantation assays.21 This likely reflects an increased resistance of p53 mutant cells to transplantation induced stress. Notably, both p53R248W/+ and p53/- mice did not show an impaired differentiation of the myeloid or lymphoid lineage,21,22 indicating that absent or altered p53 function in HSCs does not cause leukemic transformation, but rather results in outgrowth of those cells over time due to altered self-renewal and survival linked to an increased stress resistance. Interestingly, a similar phenotype was previously observed in mice transplanted with human CD34+ cord blood cells overexpressing CITED2. In this study, mice that received CITED2-high cord blood cells showed a significantly higher contribution of human cells to hematopoiesis than mice that received control cells, without affecting differentiation.23 Even though CITED2-mediated effects on increased engraftment could also have other reasons, such as altered cell quiescence, high CITED2 levels might protect primitive cells – similar to p53 alterations - from stress-induced apoptosis. This notion is supported by the finding that CITED2-high CD34+CD38- cells have a lower amount of AnnexinV-positive cells than control cells.23

Apparently, both HSCs with impaired p53 functions and HSCs with high CITED2 expression gain a survival advantage over other HSCs, but are still capable of normal differentiation – a condition that can lead to clonal hematopoiesis (CH). CH is defined as a state where a single mutant HSC contributes to a significant clonal proportion of mature blood lineages.24 In the multistep-process of AML development, CH often precedes malignant transformation,25,26 however, it is also observed in healthy individuals. CH is related to age and observed in 10% of people over the age of 65 and 20% of people older than 90 years.26 Somatic mutations detected in CH overlap with mutations recurrently found in AML and often include the genetic modifiers DNMT3A, TET2 and ASXL1. If CH is observed in combination with leukemia-associated mutations, but signs of cytopenia or malignant transformation are absent, the condition is referred to as clonal hematopoiesis of indeterminate potential (CHIP).27 Originally, CHIP was defined as the presence of clonal blood cell populations harboring leukemic driver mutations with an variant allele frequency (VAF) of ≥2%,27 but recent studies indicated that also a lower VAF...
cutoff is associated with an increased risk for developing AML.\textsuperscript{28} Presence of more than one leukemia-associated mutation and a bigger clone size (VAF>10\% for DNMT3A and TET2 mutations) increase the chance to progress to AML, and both of these parameters correlate with age.\textsuperscript{29}

Among the top five genes that are recurrently found mutated in individuals with CHIP is \textit{Tp53},\textsuperscript{25,26,30,31} highlighting the role of dysfunctional p53 in conferring a competitive advantage to HSCs. Thus, p53 mutations represent an initiating event that primes HSCs for malignant transformation, however, acquisition of additional alterations for leukemia onset is required. Likely, also factors that regulate p53 abundance and activity, including CITED2, play a role in clonal expansion of HSCs, thereby increasing the risk for leukemic transformation.

\textbf{Targeting CITED2- prospects and limitations}

Besides the role of CITED2 as a p53 regulator, this thesis further describes the impact of CITED2 on AKT-signaling and BCL2 expression. As shown in chapter 2, downregulation of CITED2 in leukemic cells resulted in decreased levels of phosphorylated (and thus activated) AKT and decreased expression of BCL2. This observation highlights that CITED2 has a central role in a network of three signaling pathways frequently deregulated in AML and associated with poor prognosis and therapy resistance. Constitutive activation of AKT signaling is found in 50-70\% of AML patients\textsuperscript{32} and has an important role for the initiation, proliferation and maintenance of leukemic cells.\textsuperscript{33–38} Furthermore, activation of AKT is associated with chemoresistance,\textsuperscript{39–42} which is at least partly explained by the role of AKT in the repair of (therapy-induced) DNA damage.\textsuperscript{43} Increased AKT activation likely promotes leukemic cell survival during chemotherapy by enhancing the DNA repair capacity. LSCs were shown to have lower levels of activated AKT compared to more differentiated blasts,\textsuperscript{44} however, the remaining levels of AKT still seem to contribute to their oncogenic properties, as Quotti Tubi et al. demonstrated that targeting AKT signaling can sensitize LSCs for chemotherapeutic treatment.\textsuperscript{45} Inhibition of AKT signaling alone appears to be not sufficient for effective AML treatment, since clinical trials with inhibitors for AKT, PI3K and MTOR mostly failed.\textsuperscript{33} However, inhibition of AKT likely is beneficial as part of combination treatments. Alternatively, targeting of molecules that affect multiple pathways relevant in AML – such as CITED2 – could represent interesting therapy approaches. This thesis further describes that knockdown of CITED2 in leukemic cells results in reduced expression of the anti-apoptotic protein and mitochondrial regulator BCL2. The apoptotic phenotype of CITED2-knockdown cells could be completely rescued by simultaneous upregulation of BCL2, indicating that a CITED2/BCL2-axis may play an important role for AML cell survival. As described in detail in chapter 4, BCL2 function is crucial for the maintenance of LSCs, mainly due to its role on regulating OXPHOS.\textsuperscript{46} Moreover, overexpression of BCL2 is associated with chemotherapeutic resistance and corresponds with poor overall survival.\textsuperscript{47,48}

In summary these data highlight that targeting CITED2 functions simultaneously hits multiple pathways that have a crucial role for AML cell
survival and maintenance. A recent study demonstrated that the p53 apoptotic network contributes to resistance of leukemic cells to the BCL2 inhibitor Venetoclax,49 and thus targeting both BCL2 and p53 simultaneously might eliminate more effective LSCs. However, the downside of targeting CITED2 is that this protein is also essential for the survival of normal HSCs. Our group previously showed that knockdown of CITED2 in human CD34+ cord blood cells significantly impairs their colony forming ability.23 Additionally, murine gene knockout studies showed that deletion of CITED2 in HSCs results in multilineage bone marrow failure and that CITED2 is only dispensable for committed hematopoietic cells.50 In ideal circumstances, agents used for AML therapy should target LSCs and leukemic blasts, while sparing out the healthy compartment. However, also current chemotherapeutic treatment strategies are harmful to normal HSPCs, but apparently fail frequently to efficiently target the AML cells that drive disease relapse. Additionally, it would be interesting to study pharmacological CITED2 inhibition in leukemic cells, which likely has different effects compared to a complete gene knockdown as it was done in our study. CITED2 consists of several domains which enable the interaction of CITED2 with multiple other proteins. The detailed molecular mechanisms of CITED2 actions are still not elucidated and it remains to be investigated which interactions of CITED2 with other proteins are driving its pro-survival role for leukemic cells. For example, it would be interesting to investigate if interference with the CITED2-SMAD2/3 interaction affects leukemic cell survival and maintenance. High expression of SMAD3 was associated with an adverse prognosis in AML patients undergoing chemotherapy, and the SMAD3 interaction partner SOX4 was shown to be involved in regulating LSC self-renewal.51,52 Furthermore, HSCs and LSCs might have an altered dependency on CITED2-mediated regulation of CBP/p300 targets. Shedding light on these questions might help to identify a therapeutic window of CITED2-based targeting.

Potential roles of the PU.1/CITED2 axis in preleukemic stages

Development of AML is a multistep-process that requires acquisition of multiple genetic alterations.53,54 The phase preceding malignant transformation, in which leukemia-associated mutations are already present but don’t cause a block in differentiation, is referred to as preleukemic phase or preleukemia. In general, CHIP and preleukemia have similar characteristics such as presence of mutations recurrently found in leukemia and alterations in self-renewal, proliferation and stress-resistance of HSCs.55 However, in contrast to CHIP, the term “preleukemia” is usually used if a certain condition definitively results in progression to malignant transformation. For instance, in human studies, the name “preleukemic phase” is usually used if a certain condition definitively results in progression to malignant transformation. For instance, in human studies, the name “preleukemic phase” is usually used if a certain condition definitively results in progression to malignant transformation. For instance, in human studies, the name “preleukemic phase” is usually used if a certain condition definitively results in progression to malignant transformation. For instance, in human studies, the name “preleukemic phase” is usually used if a certain condition definitively results in progression to malignant transformation. For instance, in human studies, the name “preleukemic phase” is usually used if a certain condition definitively results in progression to malignant transformation. For instance, in human studies, the name “preleukemic phase” is usually used if a certain condition definitively results in progression to malignant transformation.
includes PU.1-knockdown (PU.1<sup>KD/KD</sup>) mice, which show reduced expression of PU.1 and develop AML with a latency of approximately six months, with additional mutations present in the malignant cells.<sup>57</sup>

In order to investigate factors that might promote maintenance of preleukemic cells or potentially accelerate malignant transformation, we investigated the effects of CITED2 overexpression in preleukemic murine PU.1<sup>KD/KD</sup> cells (chapter 3 of this thesis). We observed an outgrowth of CITED2-PU.1<sup>KD/KD</sup> cells in in vitro assays and increased engraftment of CITED2-PU.1<sup>KD/KD</sup> HSPCs in transplantation assays compared to control-PU.1<sup>KD/KD</sup> HSPCs. This likely indicates that CITED2-PU.1<sup>KD/KD</sup> HSPCs have altered self-renewal and survival properties, which is in line with known functions of CITED2 in HSC maintenance and increased p53-dependent stress resistance (see chapter 2). However, whereas full deletion of Tp53 in PU.1<sup>KD/KD</sup> mice was previously shown to result in more aggressive AML,<sup>22</sup> CITED2 overexpression in PU.1<sup>KD/KD</sup> HSPCs was not sufficient for accelerated leukemic transformation. Lack of accelerated AML development in PU.1<sup>KD/KD</sup> cells and CITED2-PU.1<sup>KD/KD</sup> cells might coincide with the observation that mutations associated with impaired PU.1 expression or function are frequently observed in early phases of AML evolution, long before the event of malignant transformation. Mutations in the gene encoding PU.1 itself are rare,<sup>58,59</sup> but several mutations associated with premalignant stages (e.g. DNMT3A, TET2, AML1-ETO) are associated with (modest) reduction of PU.1 levels, which is known to be sufficient for promoting leukemogenesis in combination with additional hits.<sup>60</sup>

Overexpression of the DNMT3A mutant R882H in murine HSPCs was shown to result in decreased expression of several differentiation-associated genes, including the gene encoding PU.1.<sup>61</sup> Furthermore, PU.1 was shown to directly interact with DNMT3A for target gene repression,<sup>23,62</sup> a process that seems to be altered in the presence of mutant DNMT3A. PU.1 was also shown to directly interact with TET2 for modulating target gene expression during monocyte-to-osteoclast differentiation.<sup>63</sup> Therefore, TET2 mutants potentially also affect PU.1 function during myeloid differentiation, however, experimental evidence for this hypothesis is still lacking. Another mutation that was clearly associated with reduction of PU.1 levels is AML1-ETO,<sup>64,65</sup> which is one of the most frequent translocation products found in de novo AML.<sup>66</sup> The AML1-ETO fusion protein, generated by the t(8;21)(q22;q22) rearrangement, can be detected in AML patients long before the moment of diagnosis.<sup>67–69</sup> Expression of AML1-ETO in human CD34<sup>+</sup> HSPCs resulted in enhanced self-renewal and dysregulated differentiation without leukemic transformation, and thus generation of a preleukemic condition.<sup>70</sup> This is in line with studies showing that expression of AML1-ETO alone in both murine and human HSPCs is not sufficient for leukemic transformation.<sup>66,71–74</sup> At the time of AML diagnosis, AML1-ETO is frequently found together with other mutations that promote cell proliferation and confer oncogenic cooperativity such as CBL mutations,<sup>75</sup> RAS mutations,<sup>76</sup> or activating c-KIT mutations.<sup>77</sup> This strongly suggests that overexpression of CITED2 in PU.1<sup>KD/KD</sup> cells promotes...
maintenance of preleukemic cells, but that additional hits are required for leukemic transformation. Increased maintenance of CITED2-PU.1\textsuperscript{KD}/KD cells potentially increases the risk for their malignant transformation, and it would be interesting to address this hypothesis in future studies.

Studying the biology of preleukemic HSCs is highly relevant, since LSCs emerge from these cells. According to the clonal evolution model of AML, interference with expanded preleukemic HSCs might prevent the formation of transformed LSCs. Furthermore, preleukemic HSCs might play a role in disease relapse. AML relapse can originate from a subclone that was already present at diagnosis,\textsuperscript{78,79} or from preleukemic cells that often survive chemotherapy,\textsuperscript{55} and therefore represent an increased risk of disease recurrence - even if LSCs are successfully eliminated.

**A different mitochondrial content likely plays a major role for LSC-specific features**

In addition to mediating resistance to p53-mediated apoptosis, the stemness protein CITED2 was also shown to be involved in mitochondrial regulation. \textit{Cited2} deletion in murine HSCs resulted in loss of their stemness potential, accompanied by signs of increased mitochondrial activity such as elevated mitochondrial membrane potential, elongation of mitochondrial shape and elevated ROS production.\textsuperscript{1} Chapter 4 and 5 of thesis, as well as numerous previous studies, describe that LSCs have different metabolic properties compared to the non-LSC population of leukemic blasts, including a characteristically low mitochondrial activity and low levels of ROS. We demonstrated that ROS-low LSCs have a reduced mitochondrial content, low levels of ATP and a different gene expression signature that suggests an increased stemness potential of ROS-low LSCs compared to their ROS-high counterparts, which is in line with \textit{in vivo} studies.\textsuperscript{46} It is unlikely that this difference can be explained by differences in genetic background in view of the common molecular mutations in both fractions. Recently it was shown that phenotypic variability in cells with an identical genetic background is strongly dictated by the mitochondria content.\textsuperscript{80} Differences in mitochondrial content and function were found to account for approximately half of the differences observed on protein level between cells. This observation is closely linked to the fact that eukaryotic gene expression is an energy demanding process and that the majority of cellular ATP is used for mRNA- and protein synthetisis.\textsuperscript{81} A different number of mitochondria, which are the most potent ATP source in eukaryotic cells, was found to influence mRNA abundance, translation and alternative splicing.\textsuperscript{80} Global gene expression rates were suggested to be proportional to cell growth,\textsuperscript{82} and our observation that ROS-low LSCs are significantly smaller than ROS-high cells supports the notion that LSCs might have in general lower transcription- and translation rates compared to other leukemic cells. Sources of mitochondrial heterogeneity include asymmetric partitioning at cell division, cell cycle stage and mitochondrial dynamics,\textsuperscript{83} which has been shown to be altered in LSCs.\textsuperscript{84} A study by de Almeida et al. suggested that HSCs have a relatively high mitochondrial mass, however, the activity of these mitochondria was shown to be low, which likely has
A low mitochondrial content and/or activity can influence gene expression not only by providing less energy for transcription and translation, but also by producing less ROS which serve as signaling molecules. Recently, Pollyea et al demonstrated that standard induction chemotherapy results in elevation of ROS, but does still not efficiently target LSCs. This indicates that having low levels of the signaling molecule ROS is not the main mechanism that drives chemoresistance in LSCs. Thus, the detailed molecular consequences of low mitochondrial content and chemotherapy resistance are still not known.

**Concluding remarks**

High rates of disease relapse represent the current challenge of AML therapy. However, recent research is starting to shed light on the nature of the cells that drive AML relapse after initial reduction of leukemic blasts by various therapy strategies. LSCs likely survive conventional chemotherapy due to their unique metabolic characteristics and maintenance of their high expression of stemness-associated genes. Detailed analysis of the LSC biology is highly relevant to identify potential vulnerabilities of this cell population and to improve current treatment strategies. Based on data of this thesis and previous studies we learned that therapy-naïve leukemic cells with the lowest mitochondrial activity have the highest expression of stemness genes and the highest repopulation capacity. However, at the moment it is still not entirely clear how these features change during chemotherapy or disease relapse, and it would be interesting to investigate further.
to address these questions in future studies. Furthermore, it is important to fully understand the process of AML evolution. Inference with alterations in preleukemic cells that promote increased self-renewal and stress resistance might prevent malignant transformation or disease relapse. In this thesis we learned that high expression of CITED2 is not sufficient for leukemic transformation, but that this protein might have important roles in interfering with p53-mediated apoptosis and maintenance of preleukemic cells. Research that focuses on the fundamental molecular mechanisms of AML development and maintenance is essential to eventually improve AML patient survival.

References


45. Quotti Tubi, L. et al. Protein kinase CK2 regulates AKT, NF-kappaB and
**Chapter 6**


64. Huang, G. et al. The ability of MLL to bind RUNX1 and methylate H3K4 at PU.1 regulatory regions is impaired by MDS/AML-associated RUNX1/AML1 mutations. Blood 118, 6544–6552 (2011).


Summary, discussion and future perspectives


