Chapter 6

Summary, Discussion

and Future Perspectives

Summary

Non-coding RNAs, including miRNAs and lncRNAs, have become a main research focus in the past two decades. A large number of aberrantly expressed miRNAs and lncRNAs were reported in types of B-cell lymphomas [1-5]. For part of these non-coding RNAs, oncogenic or tumor suppressor roles have been reported in the pathogenesis of B-cell lymphomas [1,6,7], while the underlying mechanisms for most of the noncoding RNAs in B-cell lymphomas have not been elucidated yet. In this thesis, the functions of noncoding RNAs were explored in two distinct subtypes of B-cell lymphoma, i.e. classical Hodgkin lymphoma (cHL) and Burkitt lymphoma (BL). BL is an aggressive germinal center B-cell (GC-B) derived B-cell lymphoma subtype, most common in children. Characteristic for BL is the MYC-Ig translocation, which leads to overexpression of the oncogenic transcription factor MYC [8-10]. Most cases of endemic BL originate from Africa and are associated with Epstein-Barr virus (EBV) [11,12], while in sporadic BL association with EBV is less common with a frequency of 10-20% [13]. HL is a GC-B cell derived lymphoma subtype that is more common in young adults and in individuals above 60 years of age. In contrast to BL, no characteristic chromosomal aberrations have been detected in HL. About 30% of the HL cases in the western world are associated with presence of EBV in the tumor cells.

In this thesis we aimed to (1) identify deregulated and MYC-regulated ncRNAs, (2) determine the effect of a subset of the ncRNAs on growth of lymphoma cell lines, and (3) explore the mechanisms of action of selected candidates on the pathogenesis of B-cell lymphomas.

1 Expression of noncoding RNAs in BL

a) MicroRNA profiling in BL and GC-B cells

In **Chapter 3** we profiled miRNA expression patterns in 4 BL cell lines and GC-B cells isolated from healthy donors using small RNA sequencing. A total of 366 miRNAs were detected in BL cell lines and/or GC-B cells. The top-10 most abundantly expressed miRNAs accounted for ~70% of all reads both in BL cells and in GC-B cells. The expression pattern of the most abundant miRNAs was similar in BL and GC-B cells, with 7 of the top-10 most abundantly expressed miRNAs being shared. Moderated T-test revealed 26 differentially expressed miRNAs, 18 with decreased

and 8 with increased levels in BL compared to GC-B cells. Of the 26 miRNAs, 6 highly expressed miRNAs were selected for validation using qRT-PCR and the aberrant expression pattern could be confirmed for 5 of them in both BL cell lines and primary BL tissues. For the in BL highly expressed miR-378a-3p, we confirmed that it was regulated by MYC in the P493-6 B-cell line model.

b) Identification and prioritization of MYC-regulated lncRNAs

In **Chapter 5**, we aimed to identify lncRNAs with a deregulated expression pattern by profiling 6 BL cell lines and 3 purified samples of GC-B cells using a custom designed microarray. We identified 706 significantly differentially expressed lncRNA probes, 309 with increased and 397 with decreased expression in BL cell lines compared to GC-B cells. To focus on MYC-regulated lncRNAs, we overlapped these candidates with two of our previously published data sets. For follow-up we focused on consistently upregulated and MYC-regulated lncRNAs by overlapping the 309 probes with increased expression in BL cell lines with 392 lncRNA probes upregulated in BL primary cases compared to a MYC low chronic lymphocytic leukemia (CLL) and the 358 MYC-induced lncRNA probes identified in the P493-6 cell line [14]. This overlap resulted in 44 probes with a MYC-induced and upregulated expression pattern. Re-evaluation of the specificity of the probes and annotation of the corresponding transcripts revealed (1) 3 probes corresponding to a protein coding transcript, (2) 29 probes originating from 15 loci corresponding to a lncRNA and a highly homologous protein coding transcript, and (3) 12 probes from 14 loci specific for lncRNA transcripts. Further validation by qRT-PCR resulted in a candidate list including 20 probes representing 19 lncRNA loci that were MYC-induced and upregulated in BL. The MYC-induction of the lncRNAs was further validated in the P493-6 cell line and 18 of the 19 lncRNAs showed an early response to MYC induction.

2 Noncoding RNA gain- and loss-of-function screens

a) Design of lentiviral libraries for functional screen

Based on published data and our own profiling data in BL (**Chapter 3**) and cHL [15], we selected 58 miRNAs with increased expression for a loss-of-function and 44 miRNAs with decreased expression for a gain-of-function screening study to explore their roles in growth of BL and cHL. In the loss-of-function screen, the included candidates were mainly miRNAs with oncogenic properties, high expression, and/or

increased levels in BL and cHL as compared to their normal counterparts or other B-cell lymphomas [4,15-19]. In the gain-of-function screen, the selection criteria for miRNA candidates mainly included miRNAs with tumor suppressor roles and/or decreased levels in BL and/or cHL.

To study the effect of MYC-induced lncRNAs with increased expression in BL cells, 38 shRNAs for 16 of the 19 MYC-regulated lncRNAs (1-5 shRNAs per locus) were designed and subcloned into vector. The miRNA inhibition pool and the lncRNA shRNA pool were generated in the same vector (miRZip) and for the screening we made one pool containing all constructs, including some shRNA constructs relevant for other ongoing projects not related to the work presented in this thesis. For the gain-of-function screen, 44 miRNA precursors were subcloned into pCDH overexpression vector. B-cell lymphoma cells were infected with the lentiviral libraries aiming at a GFP percentage of 10 to 15%. Cells were harvested at multiple time points for a period of up to 40 days to isolate DNA for NGS-based analysis of construct abundances. Constructs with decreased or increased abundance over time relatively to day 5 or 6 were selected for follow-up experiments.

b) Functional miRNA screen in cHL

In **Chapter 2**, we identified miRNAs relevant for growth of 3 cHL cell lines, i.e. L540, L428, and KM-H2, in duplicate infections using the lentiviral pool of 63 miRNA inhibitor constructs (including 5 negative controls). As a control, we also infected cells with a lentiviral pool of 222 barcoded control constructs. Infected cells were harvested at day 5, 13, and 21 after sorting GFP-positive cells at each time point. The control screen with the barcoded control constructs showed no consistent changes in abundance over time. Using an adapted IQR test significant changes in abundances were detected for 4 miRNA inhibitors (miRZip-21-5p, miRZip-449a-5p, miRZip-625-5p, and miRZip-let-7f-2-3p), indicating a positive effect of the respective miRNAs on cHL cell growth (**Table 1**). For one of the 4 miRNAs, miR-21-5p, an increased expression was observed in cHL compared to GC-B cells. The effect of miR-21-5p inhibition on cHL cell growth was confirmed by GFP growth competition assay and on apoptosis using Annexin-V staining.

Table 1. miRNA inhibitors with a significant change in abundance

Construct	Infections on cHL cell lines						Infections on BL cell lines					
	KM-H2		L540		L428		ST486		DG75		CA46	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
miRZIP-449a-5p	-	-	-	-	-	-	-	-	-	-	-	-
miRZIP-let-7f-2-3P	-	-	-	-			-	-	-	-	-	-
miRZIP-21-5p	-	-		-		-	-		-	-		
miRZIP-let7e-5p		-		-				-	-	-		-
miRZIP-106b-5p	-			-	-			-	-	-		-
miRZIP-190-5p			-	-			-	-	-	-		
miRZIP-625-5p	-	-	-		-		-		-			
miRZIP-378a-3p	-		-						-	-		
miRZIP-15a-5p			-	-							-	-
miRZIP-142-3p	-		-						-			
miRZIP-20a-5p	-			-								
miRZIP-18a-5p									-		-	-
miRZIP-30e-5p							-	-				-
miRZIP-494-3p									-	-	-	-
miRZIP-9-5p							-	-	-			

- indicates a significantly decreased abundance over time. MiRNA inhibitor constructs shown in bold showed a consistent effect in at least two infections of both cHL and BL

c) Functional miRNA screen in BL

In **Chapter 4**, we carried out both a gain-of-function and a loss-of-function screen to identify BL cell growth-related miRNAs in two BL cell lines, ST486 and DG75. Based on our experience in the cHL screen, we further optimized our screening protocol. Firstly, GFP positive cells were sorted only once on day 5 (miRNA inhibitors) or day 6 (miRNA overexpression constructs) to avoid bias that might be introduced by differences in the sorting settings per day. This was important especially for the miRNA overexpression screen for which GFP intensities were relatively low. Secondly, the infected cell populations were cultured for a period up to 40 days, to enhance detection of miRNAs with milder or later effects on cell growth. By analyzing the changes in abundance of the constructs, we identified 10 miRNA inhibitor constructs (miRZip-let-7f-2-3p, miRZip-190-5p, miRZip-449a-5p, miRZip-9-5p, miRZip-106b-5p, miRZip-21-5p, miRZip-let-7e-5p, miRZip-494-3p, miRZip-30e-5p, and miRZip-378a-3p) that were significantly depleted in both independent infections of at least one of the two BL cell lines (**Table 1**). This implicated a negative effect on BL cell growth upon inhibition of these 10 miRNAs. None of miRNA inhibitor constructs showed a significant increase in abundance over time.

In the gain-of-function screen, the abundance of 5 overexpression constructs (pCDH-miR-26a, pCDH-miR-26b, pCDH-miR-34a, pCDH-miR-34c, and pCDH-miR-150) showed a significant depletion, while 3 constructs (pCDH-miR-155, pCDH-miR-222, and pCDH-miR-151a) showed a significant increase in abundance over time (**Table 2**). The effect observed for 4 miRNAs, i.e. miR-26a/b-5p, miR-150-5p, and miR-155-5p, on BL cell growth was consistent with previously published data and our own results [20-24]. GFP growth competition assay with individual constructs confirmed the results of the screens for 15 of the 18 constructs.

For our functional follow-up study, we focused on miRNA candidates that were differentially expressed in BL and had an expression level of >1,000 RPM either in BL or GCB cells. This cut off was based on a previous publication showing that miRNAs with an expression level above 1,000 RPM are more likely to be expressed at functionally relevant levels [25]. This selection resulted in 4 miRNA candidates, i.e. miR-378a-3p from the miRNA inhibitor library and miR-150-5p, miR-26a-5p, and miR-26b-5p from the miRNA overexpression library. As the roles of miR-378a-3p (**Chapter 3**) and miR-150-5p in BL [21] were studied previously by us, we focused on miR-26a/b-5p for further functional studies.

Table 2. miRNA overexpression constructs with a significant change in abundance

Construct	Infections on cHL cell lines						Infections on BL cell lines					
	KM-H2		L540		L428		ST486		DG75		CA46	
	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd
pCDH-miR-146a	-	-	+	+								
pCDH-miR-200a	-	-	-		-							
pCDH-miR-200c	-	-	-	-	-	-						
pCDH-miR-30a			-	-								
pCDH-miR-449a	-	-	-	-								
pCDH-miR-26a			-				-	-	-		-	-
pCDH-miR-26b							-	-				
pCDH-miR-34a						-			-	-		
pCDH-miR-34c									-	-		
pCDH-miR-150							-		-	-	-	-
pCDH-miR-9							-		-		-	-
pCDH-miR-19b-1			+	+	+							
pCDH-miR-151a									+	+		
pCDH-miR-222							+	+				
pCDH-miR-155							+	+				

+ indicates a significantly increased abundance and - indicates a significantly decreased abundance over time

d) Comparison of miRNA screening results in HL and BL

As HL and BL are both GC-B cell derived malignancies, it might be expected that there is a substantial overlap between the miRNAs that affect growth in both lymphomas. In the screen with miRNA inhibitor constructs, a consistent effect in at least two infections of both cHL and BL has been observed for 9 constructs (shown in bold in **Table 1**). Inhibition of miR-21-5p decreased growth of both cHL and BL cells in the screen. We selected miR-21-5p for follow-up in cHL but not for BL cells. MiR-21-5p was the most abundantly expressed and one of the significantly upregulated (3.4-fold) miRNAs in cHL [26]. In BL, miR-21-5p levels were also high, although lower as compared to the levels in GC-B cells. In a recent report, knockdown of miR-21-5p induced cell cycle arrest by affecting the PI3K/AKT signaling pathway in BL [22]. These findings are in line with the results of our screen in BL cells, indicating that miR-21-5p is also important in BL. Vice versa, we studied miR-378a-3p in BL, but not in cHL. MiR-378a-3p was highly expressed in both BL and cHL compared to GC-B cells. A negative effect upon inhibition was seen in 2 infections in BL and cHL cells. This suggests that miR-378a-3p may also be an essential regulator in cHL. However, the role of miR-378a-3p has not yet been studied in cHL. In addition, members of miR-17~92 cluster and its analogous miR-106b~25 cluster have been reported to be upregulated or highly expressed in HL and/or BL. They have been implicated in HL and BL pathogenesis and are associated with poor prognosis [27,28]. In line with the findings, we observed a decreased abundance for inhibitors of three members of the miR-17~92 / miR-106b~25 clusters (miR-106b-5p, miR-18a-5p and miR-20a-5p), further indicating their oncogenic roles in cHL and/or BL cells. The functional consequences of the other miRNAs affecting growth of both cHL and BL have not been elucidated. In future studies, it is worthwhile to explore the functions of these miRNAs in more detail.

Comparison of the results of our gain-of-function screen in BL, with previous data of a similar screen in cHL [29] revealed no overlap (**Table 2**). Although the impact of specific miRNAs might be different in the two diseases, some differences in experimental setup could also have influenced the observed results. The infected BL cells were sorted only once on day 5 or day 6, while the infected cHL cells were sorted on each harvest day. In addition, the screen was conducted for 27 days in cHL and for 40 days in BL, thus some later effects might have been missed in HL. Besides the functions of miR-26a/b-5p in BL that we presented in the thesis, the roles of miR-150 and miR-155 in BL cell growth were validated previously by us [20,21]. The other

miRNAs identified in cHL and/or BL have not been studied in detail, so further work is needed to explore their mechanisms.

e) LncRNA functional screen in BL

In **Chapter 5**, 16 MYC-induced lncRNAs that were upregulated in BL cells were investigated in a loss-of-function screen. Briefly, 3 BL cell lines, i.e. ST486, DG75, and CA46, were infected in duplicate with the lentiviral pool containing among others the 38 shRNA constructs targeting these lncRNAs, and infected cells were sorted at day 5 and cultured over a period of 40 days. Analysis of construct abundance revealed a significant depletion of 3 shRNAs (MAFG-AS1-sh2, TCONS_l2_00028770-sh1, and TCONS_l2_00007970-sh4) in at least 2 infections of one BL cell line. Abundance of MAFG-AS1-sh2 decreased in both ST486 and CA46, while the abundance of 2 other shRNA constructs decreased in one of the 3 cell lines. The negative effect of knockdown of the lncRNAs was validated by GFP growth competition assay with individual shRNA constructs in 3 BL cell lines. Of the 3 constructs, MAFG-AS1-sh2 showed the most profound effect on BL cell growth and 2 more shRNAs were designed to further confirm the effect of MAFG-AS1 knockdown on BL cell growth.

f) Other genes in the loss-of-function screen

The loss-of-function screens performed in cHL (**Chapter 2**) and BL (**Chapter 4**) cells included besides the above described constructs also 176 shRNA constructs targeting genes relevant for other projects. In cHL, the abundance of 9 of these shRNAs targeting 7 genes, i.e. BAP1 (2 constructs), EZH2 (2 constructs), HNRNPL, SETD2, MEF2C, FLJ42351, and REL (all with one construct per gene), were decreased in at least 4 out of 6 infections (**Table 3**). In BL cells, 14 shRNAs targeting 8 genes showed a decrease in abundance in both infections of at least two BL cell lines, i.e. EZH2, TCF3 (both with 3 constructs), BAP1, MYC (both with 2 constructs), HNRNPL, SETD2, MEF2C and WDR5 (each with one construct). For 7 out of these 8 genes a negative effect was observed also upon sgRNA-induced knockout in the Brunello screen in ST486 cells, further confirming their essential roles in BL. The overlap between cHL and BL was high with a shared negative effect on growth for shRNAs targeting 5 genes, i.e. BAP1, EZH2, HNRNPL, SETD, and MEF2C. For 3 of these genes, i.e. MYC, EZH2 and REL, their relevance for BL and/or cHL has been reported previously [21,30-33]. The negative effect of MYC knockdown on BL was in line with previously reported oncogenic properties of MYC in B-cell lymphomas [21,30,32], but it has not

been reported as an important regulator of growth of cHL cells. The negative effect of inhibition of EZH2, a known oncogene in B-cell lymphoma, in both BL and cHL is consistent with previous studies [31,33]. Moreover, inhibition of EZH2 was indicated as a possible therapeutic strategy for the treatment of lymphomas [34,35]. REL, a subunit of NF-κB, is frequently overexpressed in HL and the 2p16.1 REL locus was identified as a susceptibility locus for cHL [36,37]. The functional relevance of REL on BL growth is unclear. A recent report showed that deletion of REL in BL cells resulted in loss of Bach2 expression, which is a tumor suppressor [38]. The transcription factor TCF3 promotes cell-cycle progression by trans-activating CCND3, and promotes survival of BL cells by intensifying B-cell receptor (BCR) signaling through the PI3K pathway [13,39]. In a GWAS analysis, TCF3 was identified as a susceptibility locus of HL [40]. In contrast to its growth promoting effects in BL, overexpression of TCF3 in cHL induced apoptosis and cell cycle arrest [41]. These opposite effects might be related to a different set of genes targeted by TCF3 in BL and HL. Overall, these data provide additional leads for further studies on essential genes in BL and cHL.

Table 3. ShRNA constructs with a decreased abundance in BL and/or cHL cells.

Gene	Construct	Infections on cHL cell lines						Infections on BL cell lines						Brunello screen in ST486 (FC)
		KM-H2		L540		L428		DG75		CA46		ST486		
		1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	1st	2nd	
EZH2	sh2			-	-	-	-	-	-	-	-		-	-9.4
	sh4			-	-	-	-	-	-	-	-	-	-	
	sh5							-	-	-	-	-	-	
TCF3	sh1							-	-	-	-	-	-	-4.3
	sh2							-	-	-	-	-		
	sh4							-	-	-	-	-	-	
BAP1	sh3	-	-	-	-	-	-	-	-	-	-	-	-	-8.2
	sh4	-	-	-	-	-	-	-	-	-	-	-	-	
MYC	sh1			-	-			-	-	-	-	-	-	-11.1
	sh3							-	-	-	-	-	-	
HNRNPL	sh2	-	-	-	-	-	-	-	-	-	-	-	-	-3.4
MYB	sh2	-			-			-	-	-			-	-8.4
SETD2	sh3	-	-	-	-	-	-	-	-	-		-	-	-5.5
WDR5	sh1									-	-	-	-	ns
FLJ42351	sh2	-		-	-	-	-	-	-		-	-		Not included
MEF2C	sh1	-	-	-	-			-	-	-	-	-	-	ns
REL	sh1	-	-		-	-	-			-	-	-		ns

- indicates a significant decreased abundance; FC – fold change; ns = not significant

2 Studying noncoding RNA functions

a) MiRNA target identification strategies

The function of a miRNA depends on its cell type-specific target genes. Thus, to unravel the mechanism underlying the effect on growth of BL and cHL, it is essential to identify the relevant target genes. A commonly used approach is to pull down the RNA-induced silencing complex (RISC) and identify the transcripts that are bound to it. The most common protein used for the immunoprecipitation (IP) of the RISC complex is one of the Argonaute proteins. The Ago protein family consists of 4 members, of which Ago1 and Ago2 are highly expressed, while Ago3 and Ago4 are expressed at low levels in human [42]. In this thesis, we performed Ago2-RIP-Chip analysis to identify cell type-specific target genes of selected miRNAs in an unbiased way. Luciferase reporter assays and western blotting were used to confirm interactions of specific miRNAs and target genes.

b) MiR-21-5p target identification in cHL

In **Chapter 2**, we identified miR-21-5p as a miRNA that supports growth of cHL cells. To explore the functional mechanism, we identified potential miR-21-5p target genes based on our previously published Ago2-RIP-Chip data [15] from 3 cHL cell lines. In total, we identified 1,294 genes that were IP-enriched in at least 2 out of 3 cHL cell lines. Among these were 36 previously proven and/or TargetScan predicted targets of miR-21-5p. Gene Ontology analysis revealed a function related to cell growth or apoptosis for 13 of the Ago2-IP enriched targets. 4 of the 13 genes, i.e. BTG, PELI1, TIAM1, and SMAD7, had a decreased expression in cHL compared to GC-B cells. Thus, these 4 miR-21-5p targets were the most promising targets to explain the phenotype observed upon miR-21-5p inhibition. Based on the high expression of BTG2 and PELI1 in GC-B cells, these 2 genes were selected for further validation by luciferase reporter assays in HEK293-T cells. Targeting by miR-21-5p was confirmed in the reporter assays for both genes, but at the protein level this could only be confirmed for PELI1 and not for BTG2.

c) MiR-378a-3p target identification in BL

In **Chapter 3**, we identified miR-378a-3p target genes relevant for BL. Inhibition of miR-378a-3p significantly decreased cell growth for 3 out of 4 BL cell lines in GFP cell

growth competition assays. We followed multiple approaches to identify miR-378a target genes. Firstly, we tried to identify miR-378a-3p targets using available Ago2-RIP-Chip data from wild type BL cell lines. A total of 1,053 probes were IP-enriched in 3 BL cell lines, and 19 genes, including 2 known targets of miR-378a-3p, i.e. TOB2 and ZFP36L2, were relevant to cell growth, apoptosis, and cell cycle. However, we could not confirm targeting of these two genes by miR-378a-3p in BL cells. To further establish enrichment in the Ago2-IP fraction, we performed qRT-PCR on total and Ago2-IP fractions of BL cells infected with miR-378a-3p inhibition or overexpression constructs. This revealed no significant changes in Ago2-IP enrichment for the two known targets or any of the other 17 growth related genes. Next, we used the same Ago2-IP samples for a genome wide microarray experiment, to identify BL relevant miR-378a-3p target genes in comparison to negative control infected cells. This revealed 63 genes with >2-fold increase in IP/T ratio in miR-378a-3p overexpressing BL cells and 21 genes with >2-fold decrease in IP/T ratio in cells infected with miR-378a-3p inhibitor as potential targets of miR-378a-3p. The overlap between the two approaches was limited to just one gene, i.e. MYCBP. This gene was not enriched in the Ago2-IP fractions of the wild type cells. Despite the limited overlap between the genes identified in the cells overexpressing miR-378a-3p or with miR-378a-3p inhibition, we selected 7 genes, including MYCBP, CISH, BCR, TUBA1C, FOXP1, MNT, and IRAK4, for further follow-up studies. These 7 genes were selected based on the enrichment in the IP fraction combined with presence of at least one miR-378a-3p binding site and a Gene Ontology term related to cell growth, apoptosis, or/and cell cycle. In addition, we also included the lncRNA JPX, as it showed a strong enrichment upon miR-378a-3p overexpression and contained an 8-mer seed binding site for miR-378a-3p. Luciferase reporter assays confirmed targeting of 4 genes, i.e. IRAK4, MNT, FOXP1, and JPX, by miR-378a-3p. Thus, these genes might in part explain the phenotype observed upon inhibition of miR-378a-3p in BL. Validation experiments to confirm the relevance of these genes are ongoing.

d) MiR-26b-5p target identification in BL

In **Chapter 4**, we identified 18 miRNAs that affected growth of BL cells and selected miR-26b-5p for a more in depth study. MiR-26b-5p was selected because it is a MYC-repressed miRNA with a significantly decreased expression in BL cell lines and primary BL tissues compared to GC-B cell controls. Ago2-RIP-Chip upon overexpression of miR-26b-5p in ST486 and DG75 revealed 94 and 59 genes with

a >2-fold increased IP/T ratio compared to empty vector infected cells, respectively. The overlap between the Ago2-IP enriched genes between the 2 cell lines was considerable with 47 genes in overlap. To efficiently prioritize miR-26b-5p target genes relevant for BL cell growth, we performed a genome-wide CRISPR/Cas9 knockout screen for all protein-coding genes in ST486 cells. This Brunello library consisted of sgNRAs for ~20,000 genes with each gene being targeted by four single guide (sg)RNAs. Similarly with the principle of the high throughput screens for identification of cell growth-related miRNAs and lncRNAs, the changes in abundance of the sgRNA constructs were monitored over time. Of the 47 Ago2-IP enriched genes identified in both BL cell lines, 42 were protein coding genes and included in the Brunello library. Comparing the results of the Ago2-IP enriched genes with the genes affecting growth of BL based on the Brunello screen, revealed an overlap of 8 genes, including 2 known miR-26b-5p targets, i.e. EZH2 and KPNA2. So, these 8 genes might be targeted by miR-26b-5p and relevant for the observed phenotype. We selected 5 genes with the strongest depletion in the Brunello screen (FC < -5.0) for further validation. EZH2, ranked at the 2nd position, was not included as it has already been proven to be an essential gene for BL cells and a validated target of miR-26b-5p in BL [31]. For the 4 remaining genes, i.e. COPS2, NOL12, MRPL15, and KPNA2, a negative effect on cell growth was confirmed upon knockout of these 4 genes using CRISPR/Cas9 based GFP growth competition assays in ST486 and DG75. Luciferase reporter assays for 6 predicted target sites in these 4 genes confirmed targeting of miR-26b-5p only for KPNA2. This indicated that KPNA2 might be relevant for the phenotype observed for miR-26b-5p. Together with proven miR-26b-5p target EZH2 and the by KPNA2 positively regulated genes MYC, OCT4 and C-jun a complex network is formed (Figure 1A). To confirm the network, the KPNA2, EZH2, and MYC protein levels were analyzed upon overexpression of miR-26b-5p in ST486 and DG75. Decreased protein levels were observed for all three genes, further supporting our findings in Chapter 4 (Figure 1B).

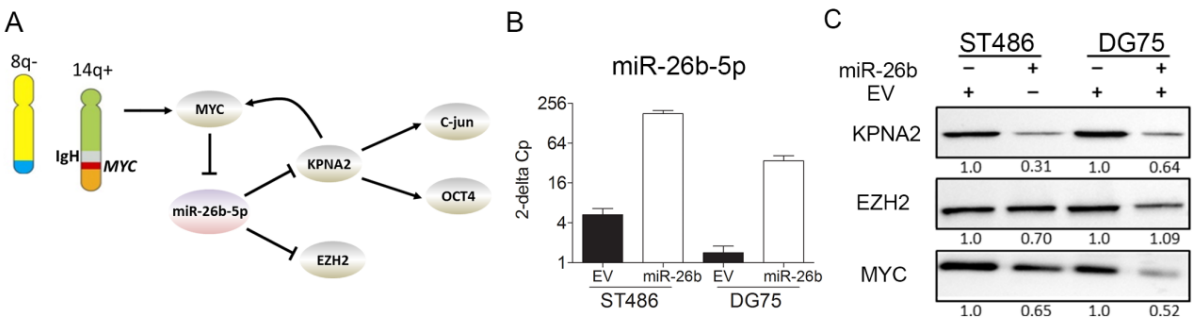

Figure 1. The MYC-miR-26b-5p-KPNA2/EZH2 network in BL. (A) Model of the effect of MYC-mediated suppression of miR-26b-5p expression in BL. (B) MiR-26b-5p levels upon overexpression in BL cell lines ST486 and DG75. (C) KPNA2, EZH2, and MYC protein levels upon miR-26b-5p overexpression. Protein levels were normalized to total protein loading.

e) Functional study of MAFG-AS1 in BL

In **Chapter 5**, the relevance of 3 selected MYC-regulated lncRNAs was confirmed by GFP growth competition assays in BL cell lines. All 3 lncRNA transcripts showed a predominant nuclear localization in BL cell lines, indicating a potential gene regulatory role at the transcriptional level. This is in line with our Ago2-RIP-Chip data, showing that none of these lncRNA transcripts showed enrichment in the Ago2-IP of BL cell lines. This suggests that they are less likely to interact with miRNAs. In contrast, two previous studies [43,44] indicated presence of MAFG-AS1 transcript in the cytoplasm of lung adenocarcinoma and colorectal cancer cells and showed interaction of this transcript with miRNAs, postulating a role as a competing endogenous (ce)RNA. Combined with our findings, this suggests different cell type-specific functions for this lncRNA. A cell type-specific role of lncRNAs has been shown previously for multiple MYC related lncRNA genes [45]. Among the 3 lncRNAs identified in **Chapter 5**, MAFG-AS1 showed the most profound effect on BL cell growth. So, we focused follow-up experiments on MAFG-AS1. We first assessed a possible cis-regulatory role of MAFG-AS1. Two of its neighboring genes, i.e. MAFG and PYCR1, were upregulated in BL cells compared to GC-B cells. However, knockdown of MAFG-AS1 did not affect the expression of the 2 neighboring genes. Interestingly, by analyzing 2 chromatin immunoprecipitation data sets from P493-6 and BL cell lines [14,46,47], 2 MYC binding sites were observed within 10kb of the transcription start sites of MAFG-AS1, PYCR1 and MAFG, suggesting that the enhanced expression of the MAFG-AS1 neighboring genes, as well as MAFG-AS1, might be the result of regulation by MYC.

Future perspectives and discussion

Up to date, a large number of ncRNAs with an aberrant expression pattern have been identified in cHL and BL. However, annotation of their function and their relevance for the pathogenesis of B-cell lymphomas remains largely unknown. Although we explored the mechanisms of some ncRNAs in BL and cHL, there is a need for more effective strategies to enable more effective follow-up studies.

1. Genome-wide screens for noncoding RNAs

a) microRNAs

To explore the functions of miRNAs in cHL and BL, we performed gain- and loss-of-function screens to identify cell growth-related miRNAs. Even though we performed pilot experiments to optimize the setup of the approach, some drawbacks were still present and further improvements are needed. The first potential problem is the relatively small size of our libraries and the limited number of negative controls. For small libraries, the normalization or calculation of outliers is more strongly influenced by the number of constructs that have changes in abundance. The miRZip lentiviral pool for loss-of-function screen consisted of 249 constructs, including 5 negative controls, and the pCDH lentiviral pool for gain-of-function screen consisted of 44 miRNA overexpression constructs and only one negative control. Thus, it would be better to extend both libraries to all known miRNAs (currently miRBase v22 contains 1,917 annotated miRNAs) and increase the number of controls in the pool.

For the pCDH overexpression vector, the main problem we encountered was the suboptimal expression cassette for GFP. Although the EF1-alpha promoter is a strong promoter in B cells, the miRNA-IRES-GFP cassette driven by EF1-alpha induces relatively low GFP expression levels, resulting in weak GFP signals compared to non-infected cells. This might have resulted in variable sorting settings, resulting in variable inclusion, especially of the weak GFP positive cells, from sort to sort. Thus, for cells with low GFP signals, read counts may vary due to differences in sorting efficiency which might lead to false positive results and/or inconsistent changes over time.

The gain- and loss-of-function screens could be improved by adding constructs with mutated seed sequences. Although most of the miRZip constructs showed high

expression of the functional strand as shown by the small RNA seq analysis, it remains unclear how efficient these antisense miRNAs are in the cell type of interest, especially for the highly abundant miRNAs. More powerful tools should be introduced for complete inhibition and effective overexpression of miRNAs.

At present, CRISPR-Cas9-dependent systems for gain- and loss-of-function analysis are becoming available and might be more effective for screening studies in B-cell lymphoma [48,49]. CRISPR-Cas9 approaches result in genomic knock-out of miRNAs rather than blocking the mature miRNAs. This will be a more effective approach for loss-of-function screening than the miRZip vector, especially for highly abundant miRNAs. In addition, this also allows inclusion of multiple guides targeting the same miRNA locus and thus more reliable results. Recently, a sgRNA based loss-of-function screen that covered 1,594 annotated human miRNAs was performed to identify fitness-associated miRNAs in Hela cells and gastric carcinoma cells [50] Another recent report showed that CRISPR-Cas9 based miRNA knockdown robustly reduced the expression of miRNAs by up to 96% [51]. In addition, high specificity of the CRISPR-Cas9 system makes it possible to edit specific miRNAs without affecting their homologous family members. The feasibility of such global miRNA loss-of-function screens was shown in myeloid leukemia [52]. For gain-of-function screening, an endonuclease inactive variant of Cas9 (dCas9) coupled to transcriptional activators can be used as an alternative strategy to the pCDH-EF1 overexpression vector [53]. Similarly, dCas9 coupled to transcriptional repressors could also serve as an alternative to inhibit miRNA expression [54].

b) Long noncoding RNAs

In the lncRNA loss-of-function analysis, the first encountered problem was the rather limited number of MYC-induced lncRNA candidates we identified. The procedure of candidate selection could be further improved. As expression of lncRNAs can be highly specific in distinct cell types or disease states, it may be more optimal to base selection of candidates on BL cells only and not include non-germinal center B-cell derived models such as P493-6 B-cells and CLL cases. This will result in a more complete list of MYC-induced lncRNAs relevant for BL pathogenesis.

In this thesis, the efficiency of shRNAs was not validated for individual constructs, so we cannot conclude that lncRNAs that did not give a phenotype in the screen were indeed not essential for BL growth. Thus, a challenge in studying lncRNAs is the lack

of efficient loss-of-function approaches, especially for transcripts that are predominantly localized in the nucleus [55,56]. As an alternative approach, a CRISPR-Cas9 system could also be used for functional study of lncRNAs. This approach may overcome the less effective knockout of nuclear-specific lncRNAs. Another factor that complicates high throughput screening of lncRNA loci is the complexity of their loci, the presence of overlapping or neighboring protein coding gene loci and shared promotor regions. As such, it may often not be clear which part of the lncRNA should be targeted by sgRNAs to obtain a functional lncRNA knockout. To overcome at least part of these issues, it may be worthwhile to apply CRISPR-dCas9 combined with transcriptional activators and repressors [57] [58]. Thus, CRISPR-based loss- and gain-of-function screens both are applicable for lncRNA study in a genome-wide scale. An alternative approach to study MYC-regulated lncRNAs could be focused on a CRISPR/Cas9-mediated screen designed to disrupt MYC binding sites (the E-box sequences) nearby the MYC-regulated lncRNAs. This could represent an alternative strategy to knockdown MYC-regulated lncRNAs and identify those relevant for BL cell growth. Subsequent gene expression analysis upon infection with sgRNAs disrupting the E-box motif also allows confirmation that the selected lncRNA indeed is a direct target of MYC. This approach can – when applied on a genome-wide scale – reveal MYC binding sites and target genes essential for BL cell growth. However, one has to bear in mind that proteins other than MYC can also bind to E-boxes and regulate gene expression.

In this thesis, we mainly focused on the functions of ncRNAs on growth of B-cell lymphomas. However, ncRNAs may be involved in other processes essential for B-cell lymphoma which do not affect proliferation, but for example modulate the cross-talk with the microenvironment. Thus, screens with different readouts, e.g. based on other phenotypes or on membrane markers relevant for targeted therapy, could provide novel insight in the role of ncRNAs in other aspects of B-cell lymphomagenesis.

2. Functional follow up for miRNA studies

In this thesis, the roles of miR-21-5p, miR-378a-3p, and miR-26b-5p were studied in more detail in B-cell lymphomas. As a first strategy we identified miR-21-5p target genes using Ago2-RIP-Chip data from wild type cHL cell lines. This resulted in identification of two miR-21-5p target genes, BTG2 and PELI1, that are relevant to pathogenesis of cHL. However, this approach was not successful for the identification

of miR-378a-3p target genes. Therefore, we changed the strategy to screen for miR-378a-3p targets in BL cells upon miR-378a-3p overexpression and inhibition. It significantly improved the specificity of miRNA target screening and resulted in the identification of 4 novel targets of miR-378a-3p. As an alternative approach, we combined the Ago2-IP for the identification of miR-26b-5p targets with a CRISPR-Cas9 based dropout screen to directly identify target genes that are also relevant for the growth of BL cells.

We validated targeting of genes by specific miRNAs using luciferase reporter assays and western blotting. Targeting of PTG2 and PELI1 by miR-21-5p was validated in cHL. In BL cells, IRAK4, MNT, and FOXP1, and lncRNA JPX were targeted by miR-378a-3p and KPNA2 was validated as a target gene of miR-26b-5p. However, for some other strong candidates selected based on Ago2-IP experiments, such as the putative miR-26b-5p targets COPS2, NOL12, and MRPL15, we could not confirm they were targeted by miR-26b-5p. It is possible that, apart from the canonical miRNA binding sites that we focused on, non-canonical miRNA binding sites may also be relevant, especially for the highly expressed miRNAs, such as miR-26b-5p and miR-378a-3p in BL. It has been shown that miRNAs in human and mice can extensively bind targets in a non-canonical fashion, i.e. their seed region comprises G:U and bulge combinations [59,60]. At present, this type of binding lacks sufficient attention in predicting algorithms and is therefore not easy to identify within a transcript of interest. In contrast to the Ago2-IP approach used in this thesis that does not provide direct information on miRNA – target gene interaction, other methods have been described that can provide such information, such as PAR-CLIP and HITS-CLIP [61,62]. Although more labor-intensive, these methods provide information on the target and the location of the binding site allowing for a more straightforward identification of miRNA – target gene interactions and also provides information of non-canonical miRNA binding sites.

Another potential powerful tool might be to involve the general public to aid in the identification of canonical and non-canonical miRNA binding sites. The Unmask citizen science game (https://unmask.nl/), developed in collaboration with Games for Health, asks players to match nucleotides between a miRNA and a potential target, and by doing this they can score points depending on the quality of the binding site (**Figure 2**). Once massively played. this may lead to the identification of novel binding sites which can be tested for functionality.

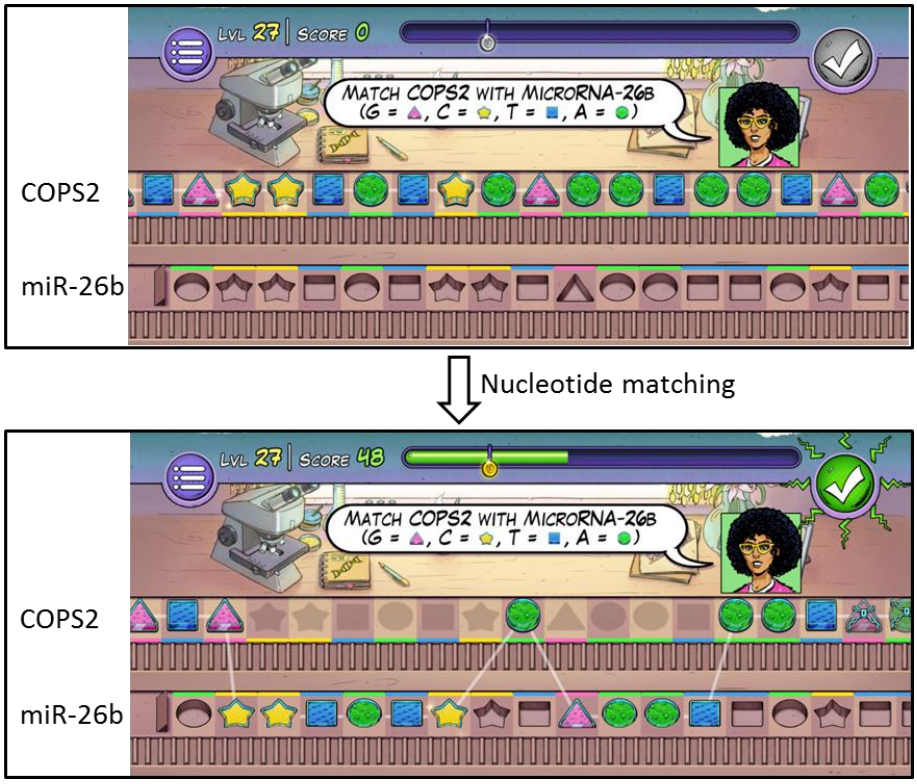

Figure 2. Schematic of the Unmask game. Four different shapes represent (A) adenine, (G) guanine, (C) cytosine, and (T) thymine. Higher scores are obtained when matching more nucleotides between RNA and miRNA sequences.

3. Functional follow up for candidate long noncoding RNAs

Specific follow-up strategies can be applied for lncRNAs depending on the subcellular localization. LncRNA transcripts that are specifically located in the cytoplasm are more likely to have regulatory roles at the post-transcriptional stage, for example by regulating transcript stability or by interacting with miRNAs. Nuclear lncRNAs are more likely to play a role in gene expression regulation at the transcriptional level in cis or trans, for example by interacting with histone modifying complexes, transcriptional regulators, or by binding to DNA. Transcriptome analysis upon overexpression or knockout of specific lncRNAs could help to identify trans-regulated genes on a genome wide scale. Cis-regulatory effects can be identified by targeted qRT-PCR experiments. It has been recognized that lncRNAs can affect gene expression in cis by the very act of transcription rather than the transcript itself. Therefore, to explore cis-regulatory roles, it is important to inhibit the process of transcription at the lncRNA locus rather than targeting the transcript itself. Thus, the shRNA-based techniques that we applied to study cis-regulation for MAFG-AS1 may

not be optimal and future studies should introduce more appropriate strategies such as CRISPR-CAS9 based based techniques. To identify the binding partners of the lncRNA (RNA, DNA and protein), lncRNA pulldown combined with (small) RNA-seg profiling, DNA-seq or LC-MS/MS can be applied [63]. Due to the more diverse functions of lncRNAs as compared to miRNAs, elucidating their functional mechanisms is more challenging.

Concluding remarks

In conclusion, we identified deregulated and MYC-regulated ncRNAs, and determined the effect of these ncRNAs on growth of cHL and BL cells using loss- and gain-of-function screens. For some of the most interesting candidates, functional follow-up studies were done to explore the mechanisms that were relevant to pathogenesis of B-cell lymphomas. Our findings add to the current knowledge about the role of ncRNAs in B-cell lymphomas. A main challenge in studying ncRNAs is to make the step from *in vitro* to *in vivo* studies, such as in patient-derived xenograft (PDX) mouse models [55,56], which have been established for B-cell lymphoma, including BL [57,58]. Xenograft mouse models, combined with transcript loss- and gain-of-function screens, might be interesting options to study more features of B-cell lymphomas such as aggressive behavior or transformation to high grade lymphomas. Such studies are important to establish the potential of targeting these ncRNA as therapeutic approaches. Another potential clinically relevant direction for ncRNAs might be to explore the value of their expression patterns as diagnostic and prognostic biomarkers in lymphoma [59-67]. In recent years, specific miRNAs and lncRNAs moved from bench to clinical trials and these might turn out to have clinical potential [64,65]. However, more work is needed to uncover the exciting world of ncRNA biology and their relevance in B-cell lymphomas.

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