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The role of microRNA in FOXO1 repression in classical Hodgkin Lymphoma

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Abbreviations

°C	Degrees Celsius
3´UTR	3´ untranslated region
4-OHT	4-Hydroxytamoxifen
5´UTR	5´ untranslated region
AAAA	polyadenylate-tail
AAV	Adenoassociated virus
ABVD	Adriamycin, Bleomycin, Vinblastine, Dacarbazine
AGO	Argonaute protein
AKT	(human) Protein kinase B
AML	Acute myeloid leukemia
Amp^r	Ampicillin resistence gene, β-lactamase gene
approx.	approximately
APRIL	A proliferation-inducing ligand
APS	Ammonium persulfate
ARE	adenylate-uridylate-rich elements
ASCT	Autologous stem cell transplantation
ATP	Adenosine triphosphate
AU-rich	Adenine-Uracil-rich
BCL3	B-cell lymphoma 3
BCMA	B-cell maturation antigen
BCR	B-cell receptor
BEACOPP	Bleomycin, Etoposide, Adriamycin, Cyclophosphamide, Oncovin (=Vincristin), Procarbazine, Prednisone
bp	base pairs
BPhB	Bromophenol blue
BSA	Bovine serum albumin
c-Myc	V-Myc myelocytomatosis viral oncogene homolog
c-Raf	rapidly accelerated fibrosarcoma, isoform C
C. elegans	*Caenorhabditis elegans*

CCL	Chemokine (C-C motif) ligand
CCL5	Chemokine (C-C motif) ligand 5, also known as RANTES
CD	cluster of differentiation
CD15	cluster of differentiation 15, mediates phagocytosis and chemotaxis, present on almost all HRS cells
CD19	cluster of differentiation 19, B cell marker
CD20	cluster of differentiation 20, B cell marker
CD3	cluster of differentiation 3, T cell co-receptor
CD30	cluster of differentiation 30, tumor marker for Hodgkin Lymphoma, embryonal carcinoma and anaplastic large cell carcinoma
CD30L	cluster of differentiation 30 ligand
$CD4^+$ Th cell	cluster of differentiation 4 positive T helper cells
$CD4^+$ Treg	cluster of differentiation 4 positive regulatory T cells
CD40	cluster of differentiation 40, costimulatory protein on antigen presenting cells
CD40L	cluster of differentiation 40 ligand
$CD8^+$ T cell	cluster of differentiation 8 positive cytotoxic t cells
CD95L	cluster of differentiation 95 ligand
cDNA	complementary DNA
CDR1	Cerebellar degeneration-related protein 1
cHL	classical Hodgkin lymphoma
CK1	Casein kinase 1
CO_2	Carbon dioxide
con	Negative control
CSF-1	colony stimulating factor 1, macrophage colony-stimulating factor
CT	X-ray computed tomography
CUP	Cancer of unknown primary
CV	cardiovascular
CYLD	CYLD Lysine 83 deubiquitinase
D. melanogaster	*Drosophila melanogaster*

DBD	DNA binding domain
ddH_2O	Double destilled water
DGCR8	DiGeorge critical region 8, also known as Pasha, Partner of Drosha, in *D. melanogaster* and *C. elegans*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA dependent protein kinase
dNTP	Deoxynucleoside triphosphate
Dox	Doxycycline
DSB	double strand breaks
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
dsRNA	Double stranded ribonucleic acid
dT	Deoxythymidine
DT	Doubling time
DYRK1	Dual specificity tyrosine-phosphorylation-regulated kinase 1
E. coli	*Escherichia coli* (bacterium)
e.g.	*exempli gratia* [lat.]
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGR1	Early growth response protein 1
EGFR	epidermal growth factor receptor
Elk1	E26 transformation-specific domain-containing protein 1
ERK	Extracellular signal-regulated kinase, also known as MAP Kinase (mitogen-activated protein kinase)
ESR	Erythrocyte sedimentation rate
et al.	*et alii* [lat.]
EV	empty vector
Exp5	Exportin 5
Eμ-mmu-miR155	Transgenice mice expressing murine (mmu, mus musculus) miR-155 under the control of a V_H promoter-

	Ig heavy chain Eμ enhancer
Ezrin	Cytovillin, Villin-2, cytoplasmic peripheral membrane protein
FBS	Fetal bovine serum
FDG-PET	Fluorodeoxyglucose – positron emission tomography
fkh	Fork head
FLT3	Fms-like tyrosine kinase 3
FOX	Forkhead box
FOXO	Human Forkhead box proteins class O
FOXO1	Human Forkhead box protein O1
Foxo1	Murine Forkhead box protein O1
FOXO1(A2)ER	Vector expressing human FOXO1 coupled to the murine estrogen receptor α ligand binding domain
FOXO3a	Human Forkhead box protein O3a
Foxo3a	Murine Forkhead box protein O3a
FOXP3	Human Forkhead box protein P3
FXR1	Fragile X mental retardation-related protein 1
g	gram
G-CSF	Granulocyte colony stimulating factor
GC	Germinal center
GFP	Green fluorescent protein
GHSG	German Hodgkin Study Group
GM-CSF	granulocyte macrophage colony-stimulating factor
GRB	growth factor receptor-bound protein
Gy	Gray (unit of ionizing radiation dose)
h.i.	heat inactivated
H_2O	water
HCl	Hydrochloric acid
HCV	Hepatits C Virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, buffer
HL	Hodgkin Lymphoma
hluc+	Firefly luciferase

HLA-G	human leucocyte antigen G
hRluc	*Renilla* luciferase
HRS	Hodgkin-Reed-Sternberg
HRP	Horseradish peroxidase
hsa	*homo sapiens*, human species
HSV-TK	Herpes simplex virus thymidine kinase
IARC	International Agency for Research on Cancer
IF-RT	Involved field radiotherapy
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL	Interleukin
IL-13	Interleukin-13
IL-13Rα1	Interleukin-13 receptor α1
IMDM	Iscove's Modified Dulbecco's Medium
IκBα	inhibitor of NF-κB alpha
IκBε	inhibitor of NF-κB epsilon
IKKα	IκB kinase alpha
IKKβ	IκB kinase beta
JAK2	Janus kinase 2
KCH_3COO	Potassium acetate
KCl	Potassium chloride
kDa	Kilo Dalton
KP3721	AKT inhibitor
KRAS	Kirsten rat sarcoma viral oncogene homolog
kV	Kilovolts
L&H	lymphocytic & histiocytic
let-7	Letal 7, miRNA, discovered in *C. elegans*
lin-14	Name derived from "abnormal cell LINeage", target of lin-4 in *C. elegans*
lin-4	First discovered miRNA in *C. elegans*
LMP1	latent membrane protein 1 of EBV
LMP2a	latent membrane protein 2a of EBV
LNA	Locked nucleic acid

LP	lymphocyte predominant
M7Gppp	7-methyl-guanosine-containing cap
M-MLV	Moloney Murine Leukemia Virus
M-MLV RT	Moloney Murine Leukemia Virus Reverse Transcriptase
mA	Milliampere
MEK	Mitogen/Extracellular signal-regulated kinase, also known as MAP2K (mitogen-activated protein kinase kinase)
MF	Mycosis fungoides
mg	Milligram
Mg^{2+}	Magnesium
$Mg(CH_3COO)_2$	Magnesium acetate
MIF	macrophage migration inhibitory factor
MIM	Missing in metastasis (protein)
min	Minutes
µF	microfarad
µg	microgram
µM	micromolar
miR	microRNA, guide strand of the miRNA duplex
miR*	Passenger strand of the miRNA duplex
miRISC	microRNA induced silencing complex
miRNA	microRNA
mirSVR	microRNA support vector regression, score to predict target downregulation by miRNAs
mL	Milliliter
mM	Millimolar
mmu	*mus musculus*, mouse species
MRE	miRNA recognition element
mRNA	Messenger ribonucleic acid
MRX34	miR-34-mimic
mTOR	Mammalian target of rapamycin
MUT	mutated
N-cadherin	Neural Cadherin

Na_2HPO_4	Disodium phosphate
NaCl	Sodium chloride
NaF	Sodium flouride
NaN_3	Sodium azide
$NaVO_3$	Sodium metavanadate
NEMO	NF-κB essential modulator
NES	Nuclear export signal
NF-κB	Nuclear factor kappa B, nuclear factor kappa-light-chain-enhancer of activated B-cells
NGF	nerve growth factor
NHL	Non-Hodgkin Lymphoma
NK cell	natural killer cell
NLPHL	Nodular lymphocyte-predominant Hodgkin lymphoma
NLS	Nuclear localization signal
NSCLC	Non-small cell lung cancer
nt	Nucleotide
O_2	Oxygen
Oligo(dT)18 Primer	synthetic single-stranded 18-mer deoxythimidine primer
oncomiR	Oncogenic microRNA
ori	origin of replication
p-	phospho-, phosphorylated
p50	NF-κB1, Nuclear factor kappa B 1
p65	RelA, V-Rel avian reticuloendotheliosis viral oncogene homolog A
PACT	Protein activator of PKR
pan	*pan troglodytes*, chimpanzee species
PAX3	Paired box 3
PAX5	Paired box 5
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline with Tween
PCR	Polymerase chain reaction
PD-1	programmed cell death protein 1
PD-1L	programmed cell death protein 1 ligand

PDCD4	Programmed Cell Death 4 (Neoplastic Transformation Inhibitor)
PDK1	Phosphoinositide-dependent kinase 1
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity in a solution
PhastCons	target site conservation score, part of software "Phylogenetic Analysis mit Space/Time models" designed to measure conservation of nucleotide positions among vertebrates
PI3K	Phosphatidylinositol-4,5-bisphosphate 3 kinase
PIP_2	phosphatidylinositol-4,5-bisphosphate
PIP_3	phosphatidylinositol-3,4,5-triphosphate
piRNA	Piwi-interacting ribonucleic acid
PIWI	P-element induced wimpy testis
PKB	Protein kinase B
PKR	Protein kinase R
poly(A)	polyadenylate
PP_i	Pyrophosphate
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
pRTS-IκBα	pRTS-1 vector, containing a conditionally regulated IκBα mutant expressed upon treatment with doxycycline
PTEN	Phosphatase and tensin homolog
PTMs	post-translational modifications
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Raf	rapidly accelerated fibrosarcoma, a MAP3K (mitogen-activated protein kinase kinase kinase)
Ran-GTP	Ras-related nuclear protein bound to GTP (guanosine triphosphate)
RANK	receptor activator of NFκB
Ras	rat sarcoma
REL	V-Rel avian reticuloendotheliosis viral oncogene

	homolog, c-Rel
RelB	V-Rel avian reticuloendotheliosis viral oncogene homolog B
RGS17	Regulator of G-protein signaling 17
RIP	receptor interacting protein
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	Ribonucleic acid interference
RNAse	Ribonuclease
RPL13A	Ribosomal protein L13A, housekeeping gene
RPMI	Cell culture medium developed by Roswell Park Memorial Institute
RSK	Ribosomal S6 kinase
RT	Reverse Transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
s	second
SCT	stem cell transplantation
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEER	Surveillance Epidemiology and End Results
Ser	Serine
SGK	Serum and glucocorticoid inducible kinase
siRNA	Short interfering ribonucleic acid
SIRT1	Sirtuin (silent mating type information regulation 2 homolog) 1, NAD-dependent deacetylase sirtuin-1
SMAD	Name derived from homologous genes in *C.elegans* called SMA (small body size) and in *D. melanogaster* called MAD (mothers against decapentaplegic)
SOCS	suppressor of cytokine signaling
SOS	Son of sevenless
SRE	Serum response element
STAT	Signal transducer and activators of transcription
SV40	Simian virus 40

SYBR Green	Cyanine dye for DNA labelling
TAD	Transactivation domain
TAE	Buffer made of Tris base, acetic acid and EDTA
TARC	thymus and activation regulated chemokine, CCL17
TBS	Tris-buffered saline
TBST	Tris-buffered saline with tween
TEMED	N,N,N',N'-Tetramethylethylendiamin
TG101348	JAK2 inhibitor
TGF-β	transforming growth factor beta
Thr	Threonine
TNFα	Tumor necrosis factor α
TNFAIP3	tumor necrosis factor alpha-induced protein 3 gene
TRAF	tumor necrosis factor alpha receptor associated factor gene
TRBP	Tar RNA binding protein
TRIS	Tris(hydroxymethyl)-aminomethan
TRKA	tropomyosin receptor kinase A
Tween	Polysorbat
Tyr	Tyrosine
U0126	MEK1/2 inhibitor
U6	Spilceosomal RNA U6, highly conserved
V	Volts
vol/vol	Volume fraction
WHO	World Health Organization
WT	Wild type
ZIP	Zrt- And Irt-Like Protein 1, Zinc/Iron Regulated Transporter-Like
ZnT	Solute Carrier Family 30 Zinc Transporter

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1 Introduction

1.1 Hodgkin lymphoma

Over 180 years ago Dr. Thomas Hodgkin was the first to describe several cases of a disease that made “the absorbent glands” (lymph nodes) and the spleen appear morbid and, subsequently, this “appearance” has been named Hodgkin’s disease (Hodgkin 1832; Küppers 2009). After it was realized that this disease was a lymphoid malignancy, the term Hodgkin lymphoma (HL) was preferred over Hodgkin’s disease (Stein et al. 2001). In 2013 it represented 11.8% of all types of lymphoma diagnosed. Other types of lymphoma are referred to as Non-Hodgkin lymphomas (NHL) in general. The word “lymphoma” designates a cancer of the lymphatic system that develops from B or T lymphocytes when they undergo a malignant change and multiply out of control (Facts 2013 [Internet] 2013). In the case of HL the tumor most often develops from B lymphocytes (Stein et al. 2001). This nsight, at least for classical Hodgkin Lymphoma, was difficult to obtain, as the tumor cells have lost the majority of their B cell phenotype. Only very rarely they derive from T cells (Küppers et al. 2012).

1.1.1 Histology

Hodgkin Lymphoma is divided into two main categories: classical Hodgkin lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL), although it should be noticed that according to (Küppers [Internet] 2013) NLPHL should be considered as a separate disease. Classical Hodgkin lymphoma can be further subdivided into nodular sclerosis, mixed cellularity, lymphocyte-rich and lymphocyte-depleted (Stein et al. 2001). Figure 1 shows an overview of the several subtypes including their prevalences.

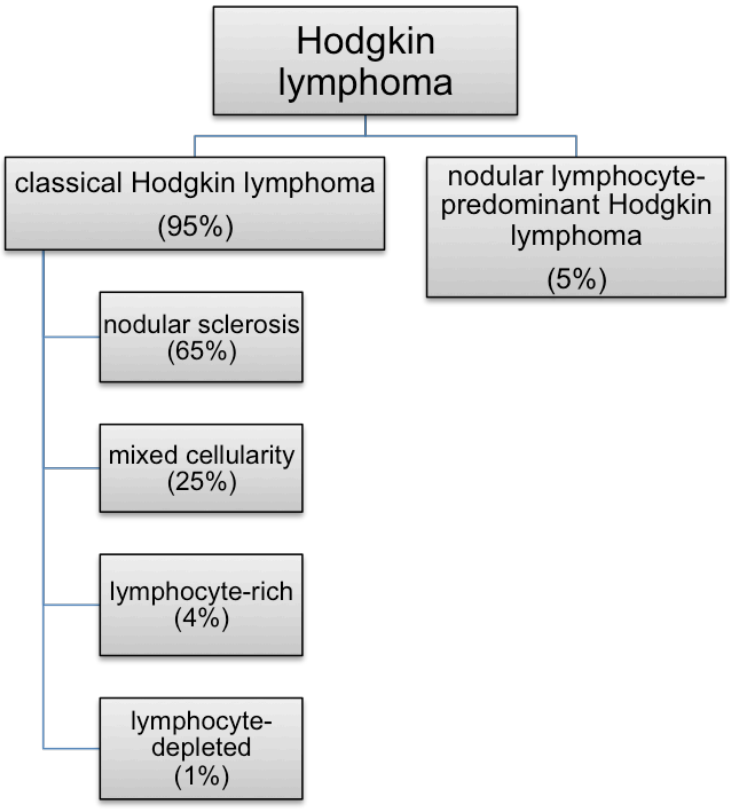

Figure 1 | Illustration of subdivisions of HL by morphology and phenotype of lymphoma cells as well as composition of the cellular infiltrate. Percentage of cases adapted from http://www.ghsg.org/histologie, last accessed on September 8, 2013.

Interestingly, tumor cells in HL are very rare and make up only about 0.1 - 2%. In cHL the malignant cells are referred to as Hodgkin cells (mononucleated) or Reed-Sternberg cells (multinucleated). Usually they are summed up and then called Hodgkin- and Reed-Sternberg (HRS) cells (Küppers, Engert, and Hansmann 2012). The equivalents in NLPHL are called lymphocytic and histiocytic (L&H) or more recently lymphocyte-predominant (LP) cells (Küppers et al. 1998; Küppers, Engert, and Hansmann 2012). Here we focus on classical Hodgkin Lymphoma. HRS cells are surrounded by an infiltrate of numerous non-neoplastic cells including eosinophils, neutrophils, histiocytes, lymphocytes, plasma cells and fibroblasts, as well as collagen fibers (Stein et al. 2001). This so-called microenvironment is visualized in Figure 2. Due to the very low amount of actual tumor cells, one can imagine the difficulties that come across when working with this tumor entity. For detailed molecular analyses HRS cells need to be microdissected in order to be further processed. Much of the research work is therefore done by using established cell lines that originate from patients suffering from HL.

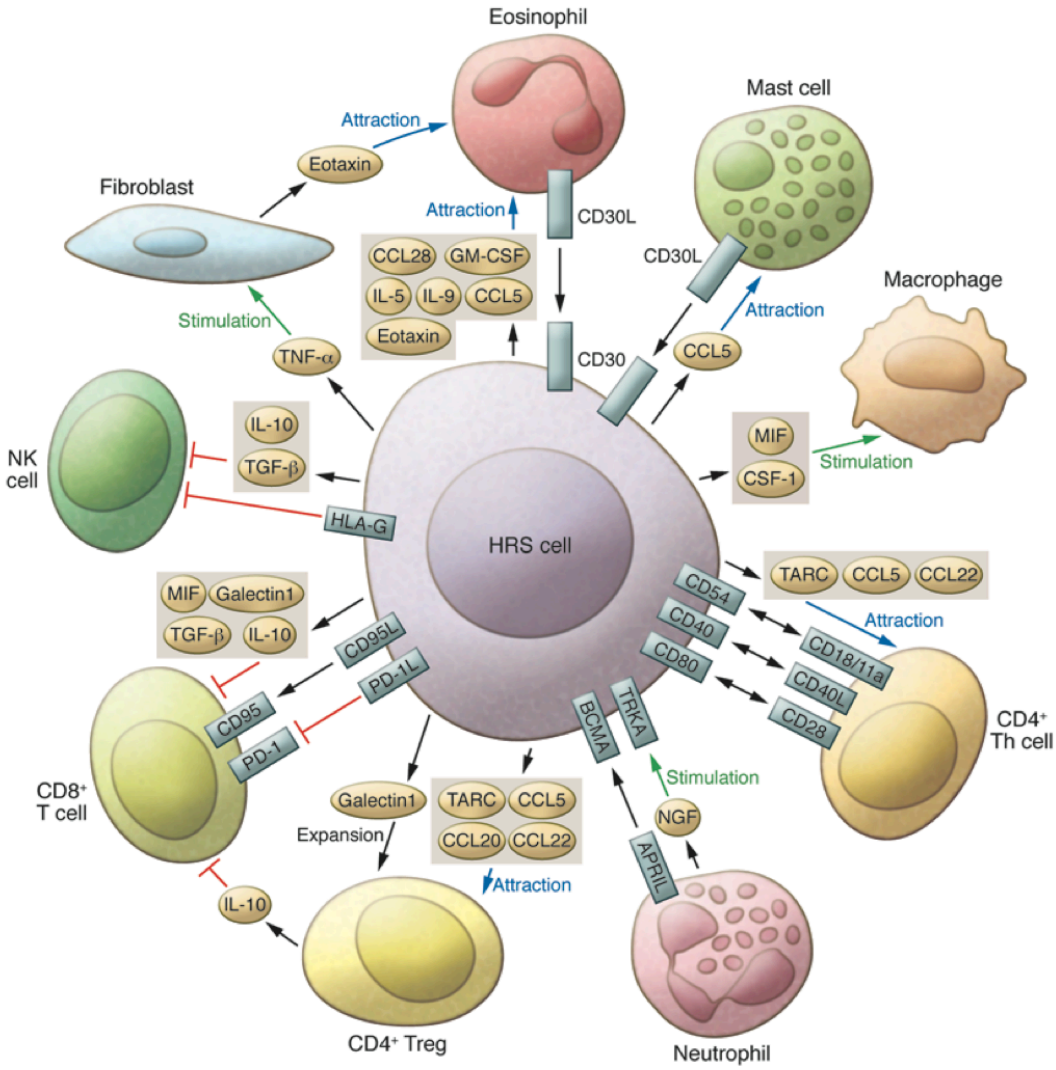

Figure 2 | Illustration of Hodgkin Lymphoma microenvironment. Hodgkin and Reed-Sternberg (HRS) cells attract a variety of non-oncogenic cells by secretion of cytokines and chemokines. Multiple receptor-ligand interactions between HRS and their microenvironmental cells sustain tumorigenic assembly. APRIL (A proliferation-inducing ligand), BCMA (b-cell maturation antigen), CCL (CC-motif Chemokine), CD (cluster of differentiation), CD30L (CD30 Ligand), CD40L (CD40 Ligand), $CD4^+$ Th cell (CD4 positive T helper cells), $CD4^+$ Treg (CD4 positive regulatory T cells), $CD8^+$ T cell (CD8 positive cytotoxic t cells), CD95L (CD 95 ligand), CSF-1 (colony stimulating factor 1, macrophage colony-stimulating factor), GM-CSF (granulocyte macrophage colony-stimulating factor), HLA-G (human leucocyte antigen G), IL (interleukin), MIF (macrophage migration inhibitory factor), NGF (nerve growth factor), NK cell (natural killer cell), PD-1 (programmed cell death protein 1), PD-1L (programmed cell death 1 ligand), TARC (thymus and activation regulated chemokine, CCL17), TGF-β (transforming growth factor beta), TNF α (tumor necrosis factor alpha), TRKA (tropomyosin receptor kinase A). Republished with permission of American Society For Clinical Investigation, from Hodgkin Lymphoma, Küppers et al., volume 122, edition number 10, 2012; permission conveyed through Copyright Clearance Center, Inc. License ID: 3950180147539.

1.1.2 Molecular Biology

HRS cells of cHL “are probably derived from germinal center (GC) B cells that have acquired disadvantageous immunoglobulin variable chain gene mutations and normally would have undergone apoptosis, whereas L&H cells of NLPHL appear to derive from antigen-selected germinal center B cells” (Küppers 2009). Only very few cases seem to be derived from peripheral post-thymic T lymphocytes (Stein et al. 2001). However, a caveat can be issued when taking into account that some T cell lymphomas have been found to co-express CD30 and CD15, which both are considered a strong argument towards the diagnosis of HL (Barry et al. 2003). The GC B cell origin of cHL has been controversial for a long time, not least because HRS cells have lost their B cell phenotype (Schwering et al. 2003) and Ig gene rearrangements were also found in T cell and myeloid malignancies (Mark et al. 1995; Szczepański et al. 1999). The evidence to prove the GC B cell origin was given when it was shown that HRS cells bear clonally rearranged and somatically mutated Ig heavy and light chain genes that in about one quarter of cases render these genes non-functional by e.g. nonsense mutations (Kanzler et al. 1996; Küppers [Internet] 2013; Küppers et al. 1994). Acquiring such destructive mutations would normally cause immediate cell death by apoptosis, which is the reason why it is believed that those cells are rescued from their apoptotic fate by some transforming event, one of which could be caused by Epstein Barr Virus (EBV) infection (Küppers [Internet] 2013). Infected HRS cells are generally found in about 40% of cases (depending on the geographical background) and display several oncogenic features caused by the infection (Kapatai and Murray 2007; Küppers 2013; Weinreb et al. 1996). The EBV latent membrane protein 1 (LMP1) mimics an activated CD40 receptor causing constitutive activation of NF-κB (Kilger et al. 1998) and latent membrane protein 2a (LMP2a) mimics a tonic B cell receptor (BCR) signal (Bechtel et al. 2005; Doench et al. 2003; He and Hannon 2004; Mancao and Hammerschmidt 2007) both leading to the survival of the cells. Remarkably, all HRS cell clones harboring destructive Ig V gene mutations that prevent BCR expression were found to be EBV-positive (Bräuninger et al. 2006).

Furthermore it was shown that HL cell lines have undergone Ig class switch recombination (Irsch et al. 2001) and that composite lymphomas of a HL and a mature B cell lymphoma are frequently clonally related, indicating that they derive from a common GC B cell precursor (Bräuninger et al. 1999; Marafioti et al. 1999).
Pathological diagnostics are based on the morphological appearance of the HRS cells histologically and are secured by immunohistological stainings for the marker molecules CD30, CD15, LMP1, PAX5, CD20 and CD3 (GHSG Referenzpathologie [Internet] 2013), of which CD30 is the most frequent surface molecule. HRS cells are positive for CD30 in nearly all cases (Stein et al. 2001). A comparison of immunophenotypes of cHL and NLPHL can be found in Table 1.
Deregulated signaling pathways that are considered hallmarks of HRS cells include consitutive activation of NF-κB, JAK/STAT, PI3K/AKT as well as MAPK/ERK pathways (Bargou et al. 1997; Dutton et al. 2005; Hinz et al. 2002; Kube et al. 2001; Skinnider et al. 2002; Zheng et al. 2003). Additionally, inactivation of tumor suppressor genes like FOXO1 are also frequent in cHL (Vogel et al. 2014; Xie et al. 2012).

Table 1 | Comparison of immunophenotypes of classical Hodgkin Lymphoma and nodular lymphocyte-predominant Hodgkin Lymphoma. BCR (b cell receptor), cHL (classical Hodgkin Lymphoma), EBV (Epstein Barr virus), Ig (Immunoglobulin), NLPHL (nodular lymphocyte-predominant Hodgkin Lymphoma). (Ha and Kim 2014; Küppers et al. 2002; Stein et al. 2001; Yung and Linch 2003).

Immunophenotype	**cHL**	**NLPHL**
CD30	positive	negative
CD15	usually positive	negative
CD20 (B cell marker)	occasionally positive	usually positive
BCR	absent	present
other B cell antigens	usually negative	usually positive
EBV infected	frequently (about 40%)	negative
mutated Ig genes	yes	yes
intraclonal diversity	very rarely	frequently
“crippling” mutations	approx. 25% of cases	rarely

1.1.3 Clinical Aspects

At early stages HL usually presents with painless enlargements of one or more lymph nodes (Facts 2013 [Internet] 2013). About 70% of these enlargements in HL occur peripheral but close to the truncus, mainly cervical. Other unspecific symptoms are unexplained weight loss, fever and night sweats (so called B symptoms). Intermittent fever (Pel-Ebstein fever), although infrequent, is typical for HL. Also typical, but even less frequent, is pain at the sites of the nodal disease after drinking of alcohol (Herold 2011). Patients may present with burning pruritus in 10-30% of cases, usually on the lower legs (Pusl and Beuers 2006). The reason for this sensation is not entirely understood. Strikingly, several dermatologic manifestations have been identified including eczema, erythema nodosum and mycosis fungoides (MF). A study from Rubenstein and Duvic from 2006 shows that mycosis fungoides, which was found to be present in 1% of the 1049 monitored HL patients, was more than 290 times more common in HL patients than in the general population (Rubenstein and Duvic 2006). Mycosis fungoides itself is a type of cutaneous lymphoma and a link between HL and MF could possibly indicate a common way of lymphocytic dysregulation. Further symptoms may be hepato- and / or splenomegaly, neurological, endocrine, skeletal or pulmonary impairments (Herold 2011).

1.1.4 Diagnosis, Staging and Treatment

If clinical aspects (anamnesis and physical examination) suggest the suspicion of lymphoma, an excisional biopsy of the affected lymph node should be performed. This biopsy should then be diagnosed by a review pathologist who is highly experienced in the field of lymphoma diagnostics (GHSG Lymph Node Biopsy [Internet] 2013). Staging of the patient is usually based on the Ann Arbor classification from 1971 which focuses on disease spread (Carbone et al. 1971). Notably, this classification was the origin of the well known “B-symptoms”, which comprise unexplained fever, night sweats and unexplained weight loss of more than 10% of body weight in the last 6 months and is now generally used to abbreviate these symptoms (Eichenauer et al. 2013). Roughly 40% of HL patients suffer from these symptoms (Küppers et al. 2012). In contrast to former times,

non-invasive methods are presently used for staging purposes. Computed tomography (CT) of the neck, chest, abdomen and pelvis is routinely used to detect masses in HL patients and is enhanced by using FDG-PET that allows a statement about the metabolic activity and therefore viability of tissues. Assessment of the bone marrow is important for evaluation prior to therapy as well as staging although involvement of the bone marrow occurs only in 5% of patients (Eichenauer et al. 2013). Some clinics may also be using the Cotswolds modifications that were proposed 1989 at the Cotswold meeting and also consider prognostic factors such as bulky disease (palpable lymph node >10 cm or mediastinal mass > 1/3 of of the transverse diameter of the thorax at the level of vertebrae T5/6), denoted by an X designation as well as extranodal lymph node involvement, denoted by an E designation (Lister et al. 1989).

Treatment has improved drastically over the years. Treatment modalities now are dependent on the treatment groups according to their stage of disease together with the presence of risk factors (bulky disease, extranodal disease, elevated ESR, ≥ 3 nodal areas) and include involved field radiotherapy (usually 20-30 Gy) as well as chemotherapy. There are mainly 2 chemotherapeutic schemata: ABVD (Adriamycin, Bleomycin, Vinblastine, Dacarbazine) and BEACOPP (Bleomycin, Etoposide, Adriamycin, Cyclophosphamide, Oncovin = Vincristine, Procarbazine, Prednisone) (Eichenauer et al. 2013). The latter exists as baseline and escalated therapy, in which the baseline therapy is escalated with G-CSF to prevent prolonged neutropenia and severe infections and that also includes higher doses of Etoposide, Adriamycin and Cyclophosphamide (Diehl 1993). Surgery is not performed in order to cure the patient (GHSG Therapie [Internet] 2016). Autologous stem cell transplantation (ASCT) is applied for primary refractory or relapsed HL with progression free survival rates at 5 and 10 years of both 48% and overall survival of 53% and 47%, respectively (Majhail et al. 2006).

Currently, the main goal in finding new treatment options for Hodgkin Lymphoma is to reduce toxicity and at the same time maintain or even improve efficacy (Küppers et al. 2012). Latest developments are pursuing a targeted therapy approach. For example Brentuximab Vedotin (anti-CD30 antibody coupled with the antimitotic agent monomethyl auristatin E) is a new drug approved for relapsed or refractory HL (Dinner and Advani 2013; Lunning and Younes 2013; Moskowitz

2012). Also other drugs such as Ofatumumab (Anti-CD-20 antibody) or Everolimus (mTOR inhibitor) are presently undergoing clinical studies (Küppers et al. 2012).

1.1.5 Epidemiology and Prognosis

According to the GLOBOCAN 2012 project, the estimated 1-year-prevalence (per 100,000) for the world, Europe and Germany are 0.8, 2.1 and 1.9 respectively for both sexes. Therefore it is more common in Europe than in world average, but within Europe, Germany seems to be slightly less affected. Also, men are more frequently affected in general (GLOBOCAN 2012 Tables [Internet] 2016). Interestingly, there are two age-peaks: one in the 3rd and another one in the 7th decade of life, as it is shown in Figure 3. This makes HL a disease of also the young adult in contrast to NHL, which is mainly a disease of the elderly (*SEER Cancer Statistics Review 1975-2010 Hodgkin Lymphoma* [Internet] 2013; *SEER Cancer Statistics Review 1975-2010 Non-Hodgkin Lymphoma* [Internet] 2013). Even though the 5-year survival rates are very high, in fact about 85% of patients suffering from HL can be cured, there are still about 15% of patients that will die from this condition (*SEER Cancer Statistics Review 1975-2010 Hodgkin Lymphoma* [Internet] 2013). This is a tragic finding especially when considering that a high percentage of those die at a very young age. Another main problem is the high risk of secondary neoplasia. About 15% of successfully treated patients gain secondary tumors, most importantly mammary carcinoma, thyroid carcinoma, AML or secondary NHL 20 years after treatment due to the general toxicity of radio- and chemotherapy. Other adverse effects of the treatment include cardiac, pulmonary and gonadal toxicity (the latter accounts for infertility and amenorrhea), and thyroid malfunctions (Herold 2011). For that reason research on HL is still a highly indispensable duty.

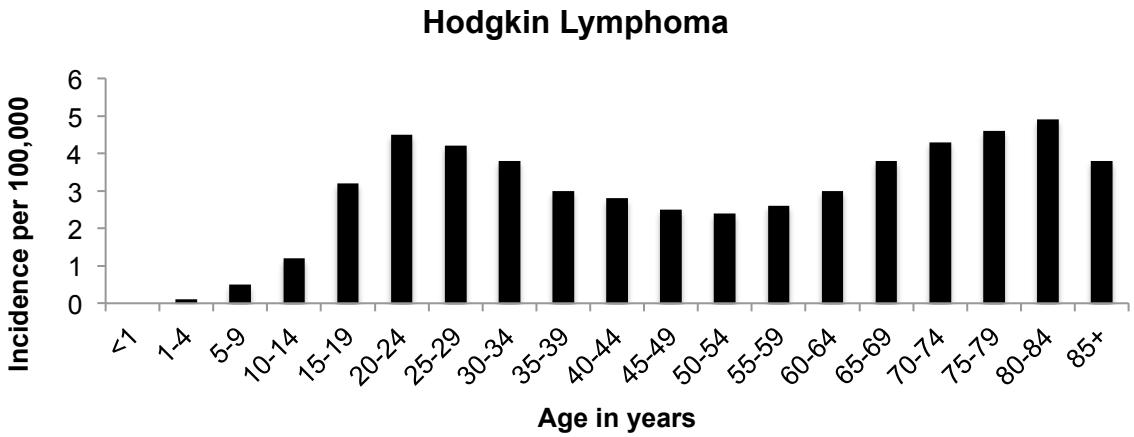

Figure 3 | Distribution of Hodgkin Lymphoma incidence per 100,000 related to age groups. SEER 18 Areas (USA). Age-specific rates 2006-2010. All races, male & female. Data from http://seer.cancer.gov/csr/1975_2010/results_merged/sect_09_hodgkins.pdf, last accessed on September 8, 2013.

1.2 FOXO1

FOXO1 is a member of the forkhead box family, a family of transcription factors that are characterized by their conserved DNA-binding domain called “forkhead box” (Kaestner et al. 2000). The name derived from the first FOX transcription factor identified in 1989 in *Drosophila melanogaster* – the gene was then called “fork head” (fkh) due to the two spiked-head structures found in embryos of the Drosophila fkh mutant (Carlsson and Mahlapuu 2002; Weigel et al. 1989). After that, many other members of this family emerged in species from yeast to human, making it difficult to name the newly characterized transcription factors. This problem was addressed at the first International Meeting on Forkhead / Winged Helix Proteins in La Jolla. The unified nomenclature was agreed to be Forkhead box (FOX) (Kaestner et al. 2000). They comprise 19 subgroups named A to S (Lehmann et al. 2003). FOXO1 is a member of the O subgroup of forkhead box transcription factors, which consists of 4 members in mammals (FOXO1, FOXO3, FOXO4, FOXO6) (Calnan and Brunet 2008). FOXO proteins are composed of specific domains, as shown in Figure 4: a highly conserved DNA binding domain (DBD), a nuclear localization signal (NLS), a nuclear export signal (NES) and a

transactivation domain (TAD). Additionally, three conserved phosphorylation sites for AKT/PKB are a common feature in FOXOs, of which the first two create binding sites for 14-3-3 proteins that facilitate nuclear export (Obsil and Obsilova 2008).

Figure 4 | Schematic illustration of FOXO1 protein. All FOXO proteins show the same functional structure: forkhead DNA binding domain (DBD), nuclear localization signal (NLS), nuclear export signal (NES), transactivation domain (TAD). Phosphorylation sites for AKT/PKB (Protein Kinase B) and SGK (serum and glucocorticoid inducible kinase) marked with (P). 14-3-3 protein binding motifs marked in red. See (Obsil and Obsilova 2008).

1.2.1 Functions and regulation of FOXO transcription factors

FOXO factors play a key role in energy metabolism, development, tumor suppression and longevity. As transcription factors they upregulate specific target genes that are involved in critical cell functions such as cell cycle arrest, apoptosis, DNA repair, autophagy, oxidative stress resistance, stem cell maintenance, differentiation and gluconeogenesis (among others) (Greer and Brunet 2005; Obsil and Obsilova 2008; van der Horst and Burgering 2007). Those functions are diverse and can be even antagonistic which suggests them to be differentially controlled in specific tissues (Greer and Brunet 2005). FOXO protein expression varies in different tissues which supports the previous assumption (Maiese et al. 2009). FOXO1 is expressed relatively ubiquitously, displaying particularly high levels in B and T cells (BioGPS FOXO1 [Internet] 2016). FOXOs show functional redundancy, seen when conditional deletion of Foxo1/3/4 in mice resulted in tumor formation, whereas deletion of a single isoform did not (Paik et al. 2007). On the other hand, they also show non-redundant functions as shown in knockout experiments with mice. Foxo1 knockout mice were found to die during embryogenesis due to defects in angiogenesis, whereas Foxo3a knockout mice only show infertility but are viable (Greer and Brunet 2005; Nakae et al. 2008). The clinical significance of FOXO1 is displayed in Figure 5.

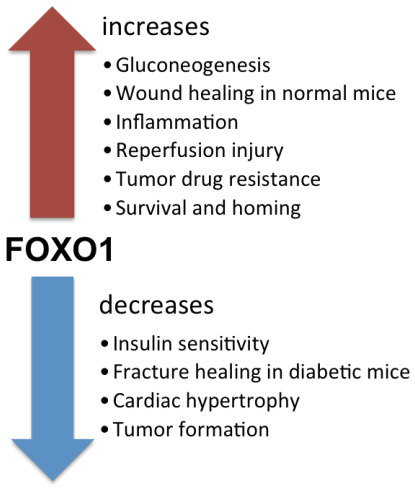

Figure 5 | Illustration of FOXO1 clinical significance. The function of FOXO1 has been investigated in genetically modified mice and in vitro. High levels of FOXO1 either increase or decrease certain clinical events. For example, FOXO1 acts diabetogenically by inhibiting insulin sensitivity and increasing gluconeogenesis. See (Y. Wang et al. 2014).

The regulation of these transcription factors follows a wide range of external stimuli such as several growth factors, insulin, cytokines and many more. This is achieved by post-translational modifications (PTMs) of FOXO protein. These modifications include acetylation, phosphorylation, mono- and polyubiquitination, as far as it is known. PTMs of FOXO lead to changes in its conformation, create or destroy binding motifs for specific binding partners that in return alter FOXO functions, change FOXO protein levels or its subcellular localization (Calnan and Brunet 2008; Greer and Brunet 2005; Maiese et al. 2009; Obsil and Obsilova 2008). The most important PTM seems to be phosphorylation by AKT or SGK (see Figure 4 and 6). Upon Insulin or other growth factor signaling, the PI3K-AKT/SGK-pathway is activated and leads to the phosphorylation of 3 conserved sites (threonine 24, serine 256 and serine 319 in case of FOXO1) causing the sequestration of FOXO in the cytoplasm and thereby preventing it from acting as transcription factor (Biggs et al. 1999; Brunet et al. 1999; Kops et al. 1999; Nakae et al. 1999). Phosphorylation at the N-terminal and Forkhead-domain phosphorylation sites creates binding motifs for the chaperone protein 14-3-3 which then leads to the active nuclear export of FOXO (Brunet et al. 1999; Li et al. 2007; Obsilova et al. 2005; Rinner et al. 2007). 14-3-3 proteins probably expose FOXOs nuclear export sequence due to a conformational change of the molecule and interfere with the function of its nuclear localization signal (Brunet et al. 2002;

Obsilova et al. 2005). Furthermore, phosphorylation of the second site (Serine 256 in FOXO1) introduces a negative charge to the basic residues of the nuclear localization signal, preventing FOXO from reentering the nucleus (Rena et al. 2001). Of note, phosphorylation by AKT does not only lead to the nuclear export of FOXO, but also triggers ubiquitin-mediated degradation (Greer and Brunet 2005; H. Huang et al. 2005; Plas and Thompson 2003). Phosphorylation by other kinases, such as CK1 or DYRK1, at additional sites also contributes to cytoplasmic sequestration (Calnan and Brunet 2008; Greer and Brunet 2005; Rena et al. 2002; Woods et al. 2001).

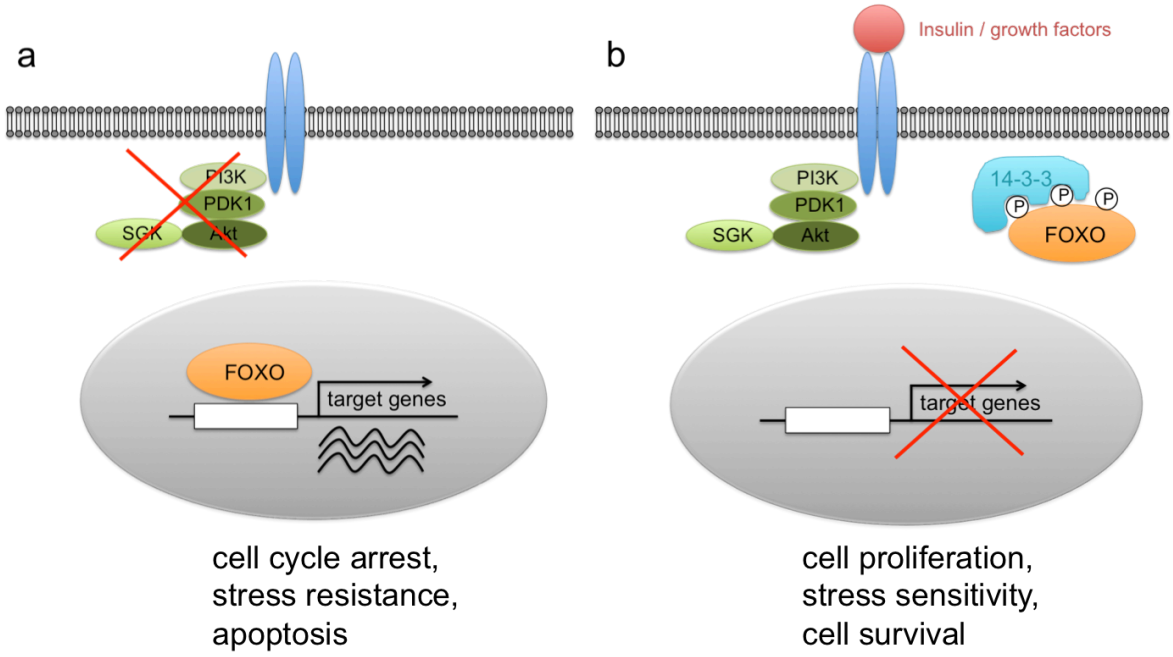

Figure 6 | Illustration of FOXO regulation by insulin or growth factors. a) In the absence of insulin or growth factor signaling, AKT (protein kinase B) and SGK (serum and glucocorticoid inducible kinase) are inactive. FOXO is located in the nucleus, upregulating its specific target genes, ultimately leading to cell cycle arrest and/or apoptosis. b) In the presence of Insulin or growth factor signaling, FOXO is phosphorylated by the PI3K-AKT/SGK pathway and shuttled to the cytoplasm by 14-3-3 proteins. Therefore it is no longer acting as transcription factor, leading to cell proliferation and cell survival. PDK1 (phosphoinositide-dependent kinase 1) PI3K (Phosphatidylinositol-4,5-bisphosphate 3 kinase). See (Greer and Brunet 2005).

1.2.2 The role of FOXO1 in cancer

FOXO1, formerly known as FKHR (forkhead in rhabdomyosarcoma), was first identified in its originally name-giving type of cancer – alveolar rhabdomyosarcoma. While investigating the translocation t(2;13) in alveolar rhabdomyosacoma, an oncogenic fusion protein of PAX3 and FOXO1 was found. The abberant function of this fusion protein, as well as the loss of one FOXO1 allele, are believed to cause the tumorigenic effects in this type of cancer (Galili et al. 1993). By now, FOXO1, among other FOXO transcription factors, is a well-known tumor suppressor in a variety of human cancers, such as cervical cancer, endometrial cancer, prostate cancer and many more (Dong et al. 2006; Monsalve and Olmos 2011; Myatt et al. 2010; Zhang et al. 2015). Recently, our group around Dr. Alexey Ushmorov identified FOXO1 to also be a tumor suppressor in classical Hodgkin Lymphoma (Xie et al. 2012). Furthermore it was found that deletion of three FoxO genes (FoxO1, FoxO3, FoxO4) in mice leads to thymic lymphomas and widespread hemangiomas (Paik et al. 2007). Another group was able to show that expressing a consitutively active form of FoxO1 decreases the size of tumors formed by PTEN-deficient cells in nude mice. However, the anti-tumorigenic effect of FOXO1 in these cells was found to be mediated by its cell cycle arrest targest, rather than its proapoptotic targets (Greer and Brunet 2005; Ramaswamy et al. 2002). In human cancers frequent chromosomal abberations occur at the q14 band of chromosome 13 (13q14), where the FOXO1 gene is situated (Dong et al. 2006; Galili et al. 1993). As for cHL, FOXO1 deletions were detected in 11.3% of the 53 primary HRS specimens and most of the cHL cell lines showed a deletion of at least one chromosome 13q (Xie et al. 2012).

1.2.3 FOXO1 in cHL

FOXO1 expression is low in cHL. This was shown directly by Immunohistochemistry, Immunoblot (FOXO1 protein), quantitative RT-PCR (FOXO1 mRNA), as well as indirectly by measuring mRNA of FOXO1 target genes. Interestingly, other types of B cell malignancies and lymphoid follicles showed high levels of FOXO1, meaning that the loss of FOXO1 is a specific feature of cHL (Xie et al. 2012). We were able to demonstrate that the

chromosomal loss of FOXO1 locus is a recurrent finding in cHL (see chapter 1.2.2) (Xie et al. 2012). Upon experiments using an inducible FOXO1(A3)ER construct encoding a constitutively active FOXO1 protein that could be translocated to the nucleus upon application of 4-OHT, we were able to show that re-inducing FOXO1 to the nucleus induces growth arrest and apoptosis in cHL cell lines, therefore supporting the role of FOXO1 as a tumor suppressor in cHL (Xie et al. 2012).

1.2.4 Targeting FOXOs for cancer therapy

Due to their role as tumor suppressors, FOXOs have been discussed as potential therapeutic targets in cancer (Monsalve and Olmos 2011). Considering the proapoptotic and antiproliferative effects of FOXOs this seems like a logical assumption. Nevertheless, modulating FOXO proteins can become a double-edged sword that bears the possibility of both beneficial and adverse outcomes (Maiese et al. 2008). For example, overexpression of FOXO1 and FOXO3a in prostate cancer cell lines leads to apoptosis, emphasizing their role in the control of prostate tumor cell growth (Maiese et al. 2008; Modur et al. 2002). Supporting studies have shown that the suppression of FOXO3a activity leads to increased prostate tumor cell growth (Lynch et al. 2005; Maiese et al. 2008). However, it has also been found that in this tumor entity FOXO3a positively regulates androgen receptor expression, therefore potentially diminishing any positive clinical effect overexpressing FOXO3a could have (Maiese et al. 2008; L. Yang et al. 2005). Another difficulty is the unclear role of FOXOs in resistance to chemotherapy. For instance, an active form of FOXO3a sensitizes ovarian cancer cells for cisplatin, whereas FOXO1 contributes to drug-resistance in ovarian cancer (Arimoto-Ishida et al. 2004; Goto et al. 2008; Maiese et al. 2008). Furthermore activation of FOXO3a in association with SIRT1 promotes cell cycle arrest, but inhibits apoptosis. This may be beneficial for blocking degenerative disorders, but in the field of cancer treatment clinical utility would be severely limited (Brunet et al. 2004; Maiese et al. 2009). These findings reinforce the necessity of further studies to be conducted in order to evaluate FOXOs as potential therapeutic targets in cancer therapy.

1.3 microRNA

MicroRNAs (miRNAs) are a type of rather recently discovered small (approx. 22-nucleotide) noncoding RNA molecules that regulate the translation of messenger RNA and therefore regulate gene expression (Boyd 2008). Many microRNAs are well conserved in different organisms, whereas some are human-specific, suggesting a role as additional regulators resulting from selection pressure during evolution and necessary for the increased complexity of humans (Farh et al. 2005; Hertel et al. 2006; Lee et al. 2007; Sempere et al. 2006). Moreover, many miRNAs display high sequence similarity although residing at different chromosomal loci, leading to the hypothesis that miRNAs exhibit redundancy in one way or another (Boyd 2008; Griffiths-Jones et al. 2006). About one third of miRNAs show tissue specificity, which has already been taken advantage of, as poorly differentiated metastases of unknown primary cancers could be identified with the help of miRNA analysis (Landgraf et al. 2007; Lu et al. 2005).

The first discovery of miRNA was made in 1993 when Ambros and Ruvkun studied larval development in the nematode worm *Caenorhabditis elegans*. They discovered a gene, *lin-4*, encoding not a protein but instead giving rise to small noncoding RNA that is able to bind to the 3' untranslated region (3´UTR) of another gene, *lin-14*, thereby inhibiting translation of its messenger RNA (Boyd 2008; Lee et al. 1993; van Rooij 2011; Wightman et al. 1993). For the following 7 years, lin-4 was considered not more than an anomaly in *C. elegans*, until a second miRNA, *let-7*, was discovered yet again in *C. elegans* (Reinhart et al. 2000; van Rooij 2011). Further studies identified let-7 homologs in other species including humans, initiating a wave of subsequent research (Bentwich et al. 2005; Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001; van Rooij 2011). The steps of discoveries involving microRNA are chronologically displayed in Figure 7.

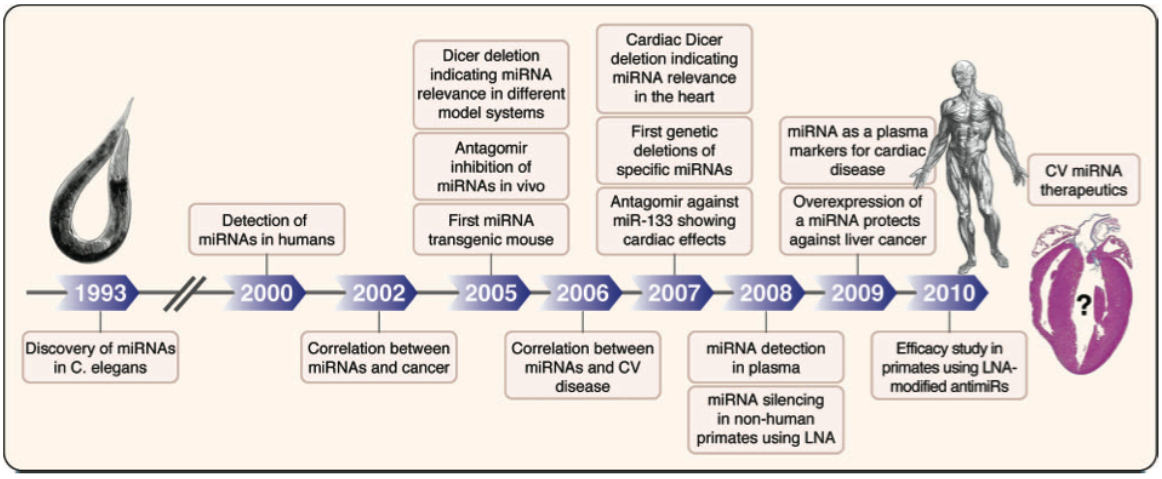

Figure 7 | Discoveries surrounding microRNA (miRNA, miR). It took 7 years to discover miRNAs in humans, after they were first discovered in worms. Another 2 years later, miRNAs were linked to cancer for the first time. From then on, miRNA research in humans flourished exponentially and might soon be culminating in the licensing of the first anti-miR-drug. CV (cardiovascular), LNA (locked nucleic acid). E. van Rooij, "The Art of microRNA Research." *Circulation research* 108 (2): 219–34. Copyright Clearance Center License Number: 3791930931834.

Until today, the role of miRNAs has been further characterized. Besides indispensable functions in normal physiology and most critical biological events such as stem cell renewal, cardiac development, hematopoiesis, angiogenesis, immunity, host cell defense against viruses, neuronal differentiation and many more, unsurprisingly microRNAs have been attributed versatile roles in disease such as heart disease, infectious disease and cancer, to mention only a few (Boyd 2008; Huang et al. 2011). The latter implication will be further addressed in chapter 1.3.4.

At present there are 1881 potential miRNAs documented in miRBase (miRBase [Internet] 2015), a database of published miRNAs, their sequences, confidences and annotations (Griffiths-Jones 2006; Griffiths-Jones et al. 2006).

1.3.1 RNA interference

RNA interference (RNAi) is a term used to describe the sequence-specific gene-silencing mechanism of small double-stranded RNA (dsRNA) (He and Hannon 2004; Kim and Rossi 2008; Mello and Conte 2004). Although many types of non-coding RNAs have emerged, the three main categories should be mentioned: microRNAs (miRNAs), short interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNAs). piRNAs are mainly found in germline cells, appear to be single-stranded, processed by Zucchini and bind to PIWI proteins for target gene regulation, whereas miRNAs and siRNA share very similar properties. They are broadly distributed, have double-stranded precursors, share the same biogenesis machinery (processing by Dicer) and associate with the same Argonaute proteins in RISC formation for target gene regulation (at least in humans) (Ha and Kim 2014). Yet, there are also unique features of miRNA and siRNAs. MicroRNAs differ from siRNAs in terms of their molecular origin and target recognition. They are encoded in the genome and have hairpin-structured precursors and often show imperfect complementarity to their targets, resulting in translational repression (Figure 8). In contrast, siRNAs are produced from long dsRNAs that are either introduced exogenously or exist as endogenous annealed dsRNA that was bi-directionally transcribed. They most often show perfect complementarity to their target, leading to its cleavage (Figure 8) (Hannon 2002; He and Hannon 2004). Interestingly, when siRNAs bind to their target in an imperfect manner, repression of transcription of the target is induced, rather than its cleavage (Doench et al. 2003; He and Hannon 2004). The biological function of siRNAs might reflect a defense mechanism against viral infections or parasitic nucleic acids which is supported by the finding that mutations of RNAi components severely limit virus resistance in plants (Baulcombe 1999; Hannon 2002; He and Hannon 2004). It follows from the foregoing that siRNAs, or RNAi in general, can be used to exogenously knock down specific genes in research and therefore potentially open a path into the development of novel therapeutic approaches (see chapter 1.3.5). Yet remains the question whether miRNAs and siRNAs truly differ from each other or whether our current knowledge fails to distinguish between those two species of small noncoding RNAs (He and Hannon 2004).

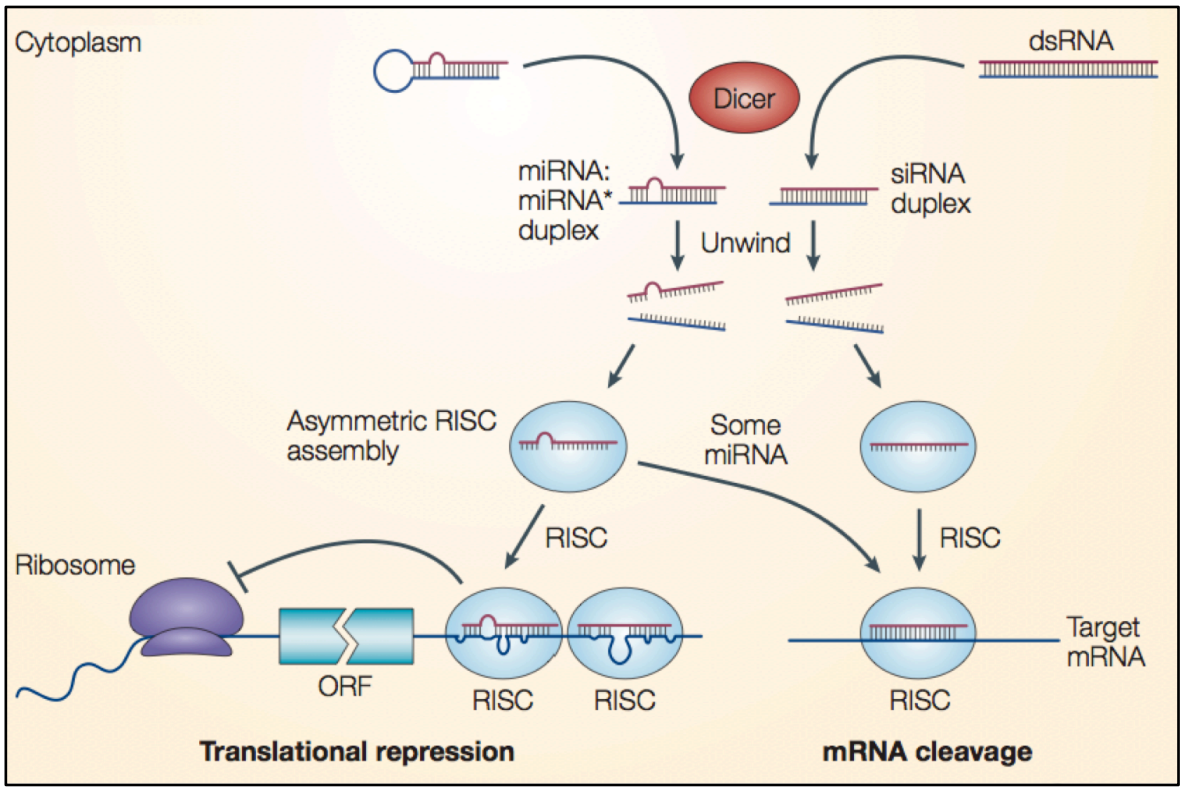

Figure 8 | miRNA (microRNA) and siRNA (short interfering RNA) processing and mechanisms of action. miRNAs tend to be imperfectly complementary to their target mRNAs (messenger RNAs) and lead to mainly translational repression. siRNAs are often perfectly complementary to their target mRNAs and initiate their cleavage. However, exceptions exist. dsRNA (double stranded RNA), miRNA* (passenger strand of the miRNA duplex, not incorporated into RISC), RISC (RNA induced silencing complex). Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (He and Hannon 2004), copyright (2004). Copyright Clearance Center License Number: 3791931223750.

1.3.2 MicroRNA biogenesis

MircoRNA genes are transcribed into primary miRNA transcripts (pri-miRNA) by either RNA polymerase II or III (Figure 9) (Borchert et al. 2006; Lee et al. 2004; Winter et al. 2009). Many primary microRNAs are capped and polyadenylated, which are unique properties of polymerase II transcription (Cai et al. 2004). The genes endcoding miRNAs are located either within annotated genes (introns of both coding and noncoding genes, or exons of noncoding genes) or intergenic. They can exist as single genes or are clustered polycistronically, suggesting that these miRNAs are regulated coordinately (Boyd 2008; He and Hannon 2004; Lagos-Quintana et al. 2003; Rodriguez et al. 2004; van Rooij 2011). However, it

has also been shown that miRNAs of the same cluster can be transcribed and regulated independently (G. Song and Wang 2008; Winter et al. 2009). The primary transcript, usually over 1kb long, has a local hairpin structure (containing the mature miRNA sequences), which is characterized by a 33 - 35 bp stem, a terminal loop and unpaired (single stranded) RNA segments at both the 5' and 3' end (Ha and Kim 2014).

This imperfectly base-paired stem-loop structure is subsequently processed by a nuclear protein complex called 'Microprocessor' consisting of the RNAse type III endonuclease Drosha, its partner protein DGCR8 (DiGeorge critical region 8, also known as Pasha, Partner of Drosha, in *D. melanogaster*) and other proteins leading to a 60 – 100-nt hairpin structure called pre-miRNA (precursor miRNA) (Boyd 2008; Denli et al. 2004; Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004; Lee et al. 2003; Winter et al. 2009). Both Drosha and DGCR8 seem to be critical for development as murine knockout experiments showed lethality (*Drosha* k.o.) or development arrest (*Dgcr8* k.o.) during embryogenesis (Chong et al. 2010; Ha and Kim 2014; Pusl and Beuers 2006; Wang et al. 2007).

The cleavage step by Drosha is critical for the export of pre-miRNA into the cytoplasm by the Ran-GTP dependent nucleo-cytoplasmic cargo transporter Exportin-5 (Exp5) (Bohnsack et al. 2004; E. Lund et al. 2004; Yi et al. 2003). Of note, knockdown of Exportin-5, as expected, leads to a reduced level of mature miRNA. However, no accumulation of nuclear pre-miRNA could be observed in the same setting. Therefore it was hypothesized that Exp5 not only ensures nucleo-cytoplasmic transport, but also protects pre-miRNA from exonucleolytic digestion (Winter et al. 2009; Yi et al. 2003). Recognition of pre-miRNA by Exp5 involves a defined length of the double-stranded stem and a 3' overhang, whereas 5' overhangs seem to be inhibitory (Zeng and Cullen 2004).

Further maturation occurs in the cytoplasm where the loop of the pre-miRNA is cleaved by Dicer type III RNAse to produce an imperfect 21 – 25-nt dsRNA duplex consisting of a mature miRNA strand and its complementary strand, termed miRNA* (Grishok et al. 2001; Hutvágner et al. 2001; Ketting et al. 2001; van Rooij 2011). This cleavage enzyme is of particular importance, as it was shown that deletion of *Dicer1* in mice, just like deletion of *Drosha*, leads to early embryonic death (Bernstein et al. 2003; Ha and Kim 2014; W J Yang et al. 2005). In humans, Dicer is assembled with its cofactors TRBP and PACT, as well as a core

component Argonaute protein (AGO) to form a so-called 'RISC loading complex' (RLC) that mediates miRNA maturation and RNA-induced silencing complex (RISC) assembly (Gregory et al. 2005; Haase et al. 2005; Lee et al. 2006; MacRae et al. 2008; Winter et al. 2009). However, Dicer cofactors, TRBP and PACT, do not seem to be essential for pre-miRNA processing, but they facilitate it and TRBP stabilizes Dicer (Chendrimada et al. 2005; Haase et al. 2005; Lee et al. 2006). The RLC is necessary for loading small double-stranded RNA onto an Argonaute protein (Ha and Kim 2014). Dicer, TRBP and PACT then dissociate from the miRNA duplex (Winter et al. 2009).

The formation of the active RISC, that performs gene silencing, involves two steps: firstly, the loading of the RNA duplex and secondly, RNA duplex unwinding. Loading of the RNA duplex occurs by association with Argonaute proteins (in humans: AGO1-4) (Azuma-Mukai et al. 2008; Dueck et al. 2012; Liu et al. 2004; Meister et al. 2004; Su et al. 2009). Human Argonaute proteins can incorporate miRNA as well as siRNA, whereas in flies AGO1 preferably binds to miRNA and AGO2 favors siRNAs (Förstemann et al. 2007; Liu et al. 2004; Meister et al. 2004; Okamura et al. 2004; Tomari et al. 2007; Yoda et al. 2010). Following the loading process, also known as formation of the pre-RISC, AGO2 cleaves the passenger strand (miRNA*) thereby releasing the guide strand (miRNA) as a single strand (Leuschner et al. 2006; Matranga et al. 2005), although diverse Helicases have been linked to the unwinding process as well (Robb and Rana 2007; Salzman et al. 2007; Winter et al. 2009). AGO2 is the only Argonaute protein in humans that exhibits slicer activities in contrast to the other AGOs (Leuschner et al. 2006; Liu et al. 2004; Meister et al. 2004). Selection of the guide strand (mature miRNA) usually depends on thermodynamic stability. The miRNA strand having a less stable base pair at the 5' end in the duplex is loaded into the mature RISC (Khvorova et al. 2003; Schwarz et al. 2003).

In the following chapter (1.3.3) the function of the active microRNA induced silencing complex (miRISC) will be explained.

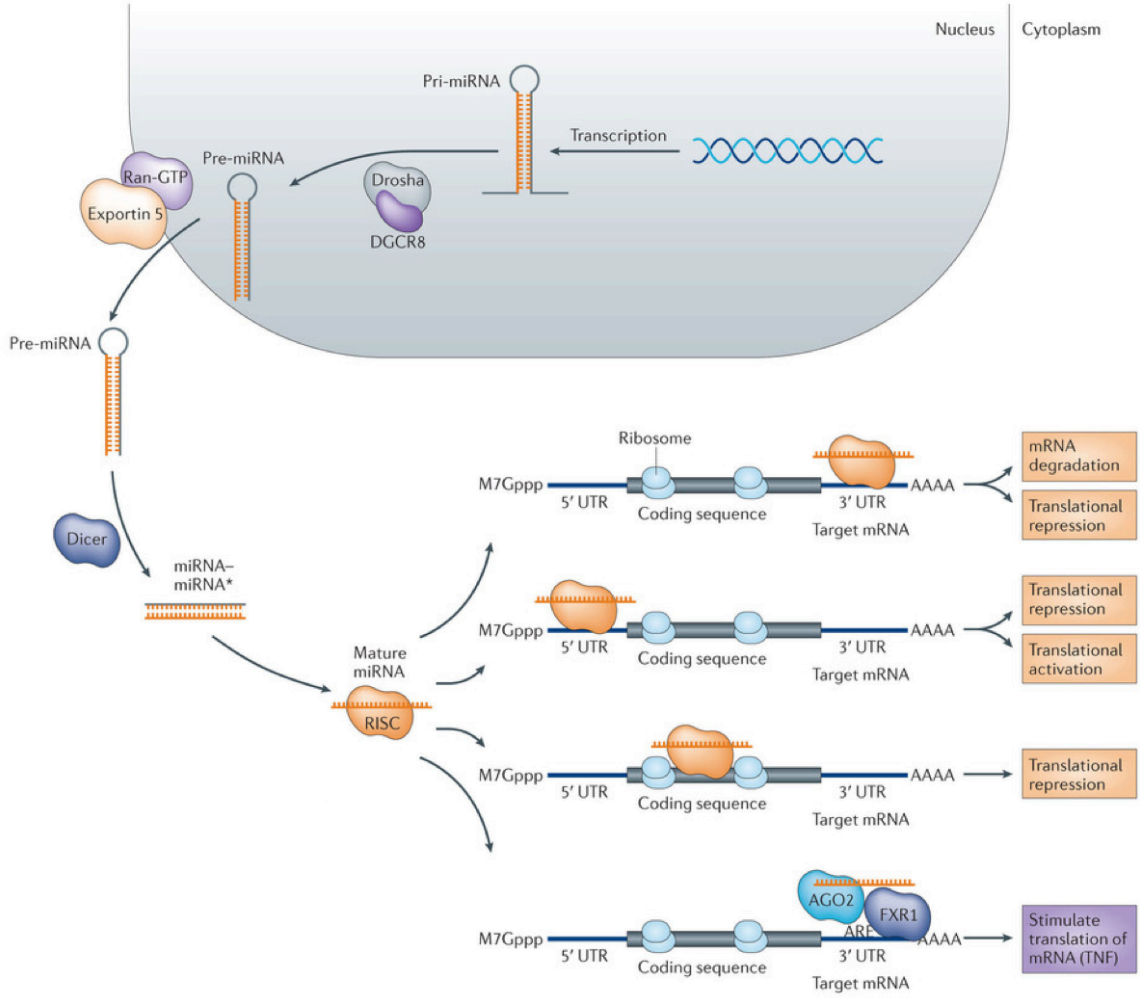

Figure 9 | Schematic illustration of microRNA biogenesis and mechanisms of action. MicroRNAs are transcribed and processed in the nucleus, exported and then further processed to a mature microRNA that assembles with RISC. The most common mechanism of action seems to be mRNA degradation or translational repression when miRNA loaded RISC binds to the 3´UTR of its target mRNA. However, further mechanisms have been identified, also leading to increased translational activity. AAAA (polyadenylate-tail), AGO2 (Argonaute 2), ARE (adenylate-uridylate-rich elements), DGCR8 (DiGeorge critical region 8), FXR1 (fragile X mental retardation-related protein 1), M7Gppp (7-methyl-guanosine-containing cap), pre-miRNA (precursor microRNA), pri-miRNA (primary microRNA), Ran-GTP (Ras-related nuclear protein-guanosine triphosphate), RISC (RNA induced silencing complex), TNF (tumor necrosis factor), 3´UTR (3´ untranslated region), 5´UTR (5´ untranslated region). Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery] (Ling et al. 2013), copyright (2013). Copyright Clearance Center License Number: 3791931421878.

1.3.3 Functions of the microRNA induced silencing complex (miRISC)

Originally it was believed that the mature miRNAs in association with the RNA induced silencing complex (RISC) generally regulate gene expression by sequence-specific binding of its so-called seed region (nucleotides 2 - 8 of the 5' end) to the 3´ untranslated region (3´UTR) of a target mRNA, thereby decreasing expression of protein-coding genes (Carthew and Sontheimer 2009; Ling et al. 2013). This mechanism of action has already been described 1993 when the first discovery of miRNA in *C. elegans* was made (Lee and Ambros 2001; Wightman et al. 1993). Later, its applicability in humans was confirmed, indicating that perfect homology to the target mRNA leads to its cleavage, whereas imperfect homology (resulting in a central bulge of the miRNA) induces translational inhibition (Figure 8) (Zeng et al. 2003). This view, however, has been substantially expanded as further investigations showed a far more extensive mode of gene regulation by microRNAs. Other binding sites at target mRNAs have been identified, such as coding regions or the 5´UTR (Figure 9) (Lytle et al. 2007; Tay et al. 2008). Traditionally it was thought that upon miRISC-mRNA interaction the translation of the mRNA is usually repressed and/or mRNA is cleaved which reduces the final protein levels of the target (Ling et al. 2013). Yet, this understanding has to be reconsidered as recent studies showed that the mechanism of action not only includes translational repression and mRNA cleavage, but also translational activation (Eiring et al. 2010; Vasudevan et al. 2007; Ørom et al. 2008). It was shown that miR-10a can bind to the 5´UTR of ribosomal protein mRNAs and enhances their translation, which in turn leads to global protein synthesis due to enhanced ribosome biogenesis (Ørom et al. 2008). Furthermore, miR-369-3 can increase transcriptional activity by recruiting protein complexes, namely Argonaute 2 (AGO2) and fragile X mental retardation-related protein 1 (FXR1), to AU-rich elements of the target (tumor necrosis factor α, TNFα) mRNA (Vasudevan et al. 2007). A well-known microRNA, let-7, can induce up-regulation of translation of target mRNAs on cell cycle arrest, whereas in proliferating cells it induces translational repression (Vasudevan et al. 2007). Other investigations showed that microRNA can indirectly increase target protein levels by de-repressing target mRNA translation by interfering with proteins that block target gene translation (Eiring et al. 2010). These findings suggest that we have not yet reached the final

understanding of how miRNA (among other non-coding RNA) operate in detail. Figure 9 visualizes different interactions of miRNA and their target mRNA known so far.

1.3.4 MicroRNA in cancer

MicroRNA has been linked to literally every known physiological and pathological process, including cancer (Lujambio and Lowe 2012). Since the first demonstrated involvement of miRNA in cancer 2002 (Calin et al. 2002), a wave of further investigations on the role of miRNA in cancer has been initiated. Meanwhile, it has become clear that miRNAs have implications in all of the hallmarks attributed to cancer by Hanahan and Weinberg, and have important roles in the clinical management of cancer (Hanahan and Weinberg 2011; Hayes et al. 2014). As mentioned before, a specific miRNA profile allows for the identification of cancers of unknown primary (CUPs) (Lu et al. 2005), but there are far more benefits to be derived from miRNA investigations in cancer. They may also help to classify tumors when other techniques fail to correctly classify specific subtypes of a type of cancer (Andorfer et al. 2011). Additionally, they could be used as predictors of prognosis, metastasis and drug efficacy, as they have been shown to have a role in cancer progression and chemosensitivity, respectively (Pencheva and Tavazoie 2013; Png et al. 2012; Ren et al. 2014; van Jaarsveld et al. 2015; Wynendaele et al. 2010; Zhao et al. 2015). Moreover, miRNAs comprise the potential of being markers for cancer susceptibility (Ziebarth et al. 2012) and non-invasive biomarkers, as they are secreted by tumor cells and show high stability in blood, urine and saliva, due to being incorporated into microvesicles (Mitchell et al. 2008; Schwarzenbach et al. 2014). Nevertheless, a cautionary note should be taken when analyzing microRNAs in bodily fluids: many factors may influence microRNA levels such as diet, treatment, blood cells and even a traumatic venipuncture (Hayes et al. 2014; Pritchard et al. 2012). Furthermore, it is difficult to find suitable endogenous controls for normalization purposes, because, unlike within tissue samples, messenger RNA and ribosomal RNA are absent in blood due to high levels of RNAses (Li and Kowdley 2012).

The ways cancer uses miRNAs to evolve are manifold. Genetic alterations are frequently observed, for example when microRNAs were first linked to cancer, it was found that miR-15 and miR-16 were deleted in more than half of B cell chronic lymphocytic leukemias (Calin et al. 2002). But also variation of the 3´UTR binding sites of target mRNAs is a common feature of cancer (Ziebarth et al. 2012), as well as single nucleotide polymorphisms in miRNAs that thereby alter their biological function (Sun et al. 2009). Alterations of the miRNA processing machinery can lead to the global reduction of microRNAs; a well-known feature observed in cancer (Allegra et al. 2014; Lu et al. 2005; Melo et al. 2010; Wegert et al. 2015). Interestingly, the repression of Dicer is also associated with an invasive phenotype and drug resistance in ovarian cancer (Kuang et al. 2013). A very striking way of altering miRNA action in cancer is by redirecting the RISC with so-called competing endogenous RNAs. These RNAs display multiple miRNA binding sites that act competitively in order to block specific miRNA action (Hayes et al. 2014). As an example, circular CDR1 (cerebellar degeneration-related protein 1) RNA contains more than 60 binding sites for a miR-7, a microRNA that acts as a tumor suppressor (Hansen et al. 2013; Memczak et al. 2013). Basically, alterations of genes, RNA or proteins can occur on any level in cancer and we have, by far, not yet reached an integral understanding of its complexity. There are many more mechanisms of miRNA alterations in cancer, but here is to mention only some.
FOXO1 has been identified as a tumor suppressor in breast cancer and subsequent miRNA studies were conducted, identifying 3 distinct miRNAs, miR-27a, miR-96 and miR-182, that led to a decrease in FOXO1 mRNA (Guttilla and White 2009). Since FOXO1 has also been identified to be downregulated in cHL by our group (Xie et al. 2012), it was of particular interest to investigate whether miRNAs contribute to FOXO1 repression in this lymphoma entity.

1.3.5 Therapeutic implications

As explained in the previous section, cancer and other diseases show a specific miRNA profile with alterations in regard to the normal cellular miRNA profile that facilitate the disease. For that reason, therapeutic approaches follow two basic considerations: firstly, inhibition of pro-oncogenic miRNAs may decrease the

tumorigenic function of a specific miRNA. Secondly, reintroduction of tumor suppressive miRNAs may restore protective miRNA functions.

Inhibition of oncogenic miRNAs can be performed by using antisense oligonucleotides, antagomiRs, sponges, locked nucleid acid (LNA) constructs or antisense peptide nucleic acids encapsulated in polymer nanoparticles (Babar et al. 2012; Garzon et al. 2010; Lujambio and Lowe 2012). A Danish group showed the involvement of miR-9 in Hodgkin Lymphoma pathogenesis and used a systemically delivered LNA-derived inhibitor of miR-9 in a xenocraft mouse model that lead to a decreased tumor outgrowth (Jansson and Lund 2012; Leucci et al. 2012). One of the most advanced trials in the area of miRNA-blockade involves Miravirsen (anti-miR-122), a locked nucleic acid-modified DNA phosphorothioate antisense oligonucleotide that leads to the reduction of Hepatitis C virus (HCV) RNA levels without inducing chemotherapy-resistance (Hayes et al. 2014; Janssen et al. 2013). Miravirsen inhibits the liver-specific miR-122, which plays an important role in HCV replication. Owing to its structure, it shows high target affinity and high resistance to degradation. Interestingly, the substance not only works via blocking miR-122 / HCV mRNA interaction, but also suppresses miR-122 biogenesis because of its affinity to the precursors pri- and pre-miR-122 (Gebert et al. 2014). Up until now, numerous clinical trials have been conducted, that showed high therapy effectiveness for Miravirsen (Miravirsen Clinical Trials [Internet] 2015; van der Ree et al. 2014; 2015; Vere Hodge 2013).

Reintroduction of relevant miRNAs can be achieved by using synthetic miRNA mimics or viral constructs (Garzon et al. 2010; Thorsen et al. 2012). Mimics are constructed with the original miRNA guide strand, whereas the passenger strand is chemically modified to prevent Argonaute association in the miRISC and to ensure its degradation. Advantages of a viral system are that these constructs are efficiently transduced to target cells and adenoassociated virus (AAV) based constructs do not integrate into the host genome (Thorsen et al. 2012). The first miRNA based cancer therapy follows the approach of reintroducing miRNA. A synthetic miR-34 mimic embedded in liposomal nanoparticles (“Smarticles”), reffered to as MRX34, is currently in a multicenter phase I clinical trial for patients with primary liver cancer, lymphoma, melanoma, multiple myeloma, renal cell carcinoma as well as lung cancer and expected to complete at the end of 2016 (Hayes et al. 2014; MRX34 Phase I Clinical Trial [Internet] 2015). Uptake of these

Smarticles into the tumor is, at least partially, ensured by its cationic charge at lower pH, which tumors tend to have. MRX34 restores miRNA-34a function, a miRNA that targets at least 24 known oncogenes (Bouchie 2013). The other miRNA mimics that have currently reached phase I clinical trial are “TargomiRs” (miR-16 mimics) for the 2nd or 3rd line treatment of patients with recurrent malignant pleural mesothelioma and non-small cell lung cancer (NSCLC) (TargomiRs Phase I Clinical Trial [Internet] 2015). As a reminder, miR-16 was one of the first miRNAs found to be a tumor suppressor in cancer (Calin et al. 2002).

A very interesting and alerting example for a tumor-suppressive miRNA is miR-26a. This miRNA was found to be reduced in hepatocellular carcinoma and AAV-based reintroduction in a mouse model led to apoptosis and reduced proliferation of the cancer cells (Jansson and Lund 2012; Kota et al. 2009). However, miR-26a has also been found to increase metastasis in lung cancer, to facilitate the development of acute T-cell lymphoblastic leukemia (T-ALL) and is frequently amplified in gliomas (Huse et al. 2009; Jansson and Lund 2012; B. Liu et al. 2012; Mavrakis et al. 2011). This shows the pleiotropic effects of miRNAs due to their multiple targets and should emphasize the cautiousness that has to be taken when assessing potential therapeutic targets as well as the safety and efficacy of miRNA based drugs (Jansson and Lund 2012).

Personalized medicine becomes more and more relevant and miRNA-based therapies will certainly be one column to individualize medicine. Yet, our knowledge of how miRNAs work has not reached its completion and many clinical trials have to be performed in order to assess miRNA function and miRNA-based drugs in vivo. The clinical implementation will require further improvement in off-target toxicity, but despite these challenges miRNAs offer a whole new view into our very own biology, as well as a novel field for disease control.

1.4 Aims of this study

Our group recently discovered that FOXO1 is repressed in classical Hodgkin Lymphoma, whereas in normal B cells and Non-Hodgkin Lymphomas it is highly expressed. Re-expressing FOXO1 in cHL leads to growth arrest and apoptosis. Other studies have shown that FOXO1 can be repressed by miRNA. Therefore this study aims to investigate whether miRNAs are responsible for FOXO1 repression in cHL and if so, which miRNAs are responsible and how they are influenced.

Potential miRNAs responsible for FOXO1 repression have been shown to be miR-27a, miR-96 and miR-182 (Guttilla and White 2009). We also aimed to investigate the role of miR-183 in FOXO1 repression, as we noticed, that this miRNA is part of a polycistronically transcribed cluster of miRNAs containing miR-183, miR-96 and miR-182 (5´ to 3´). Another highly expressed miRNA in HL is the well known oncomiR, miR-155. We therefore asked whether this miRNA could have an effect on the tumor suppressor FOXO1. In this study we concentrate on the five miRNAs, mir-27a, miR-96, miR-155, miR-182 and miR-183.

2 Material and methods

2.1 Materials

Reagents and chemicals

Agar Agar	GIBCO Life Technologies
agarose powder	Lonza
Ampicillin	AppliChem
Ampuwa water	Fresenius Kabi
APS	AppliChem
BSA	PAA
Butanol	AppliChem
DMSO	Sigma-Aldrich
Doxycycline (0.5 µg/mL)	Sigma Aldrich
EDTA	Sigma-Aldrich
ethidium bromide	AppliChem
HCl	Sigma-Aldrich
Hygromycin B 10µg/mL	InviviGen
KP3721 AKT-Inhibitor	Sigma-Aldrich
NaCl	AppliChem
NaH_2PO_4	AppliChem
Peptone	AppliChem
recombinant RNasin® Ribonuclease Inhibitor	Promega
SDS	AppliChem
SuperSignal West Dura Extended Duration Substrate	Thermo Scientific
SYBR Green	QIAGEN
TEMED	AppliChem
TG101348 JAK2-Inhibitor	Axon Medchem
Tris	AppliChem
Tween 20	AppliChem
U0126 MEK1/2 Inhibitor	Cell signaling
Yeast extract	Applichem
β-Mercaptoethanol	AppliChem

Gels

10% polyacrylamide gel	Polyacrylamid 2,8ml, Tris –HCl 1.5M pH8.8 2,8ml, 5,4ml, ddH_2O, SDS 20% 112,5µl, APS 10% 100µl, TEMED 7,5 µl
1,5% Agarose gel	1,8g Agarose, 120ml 1xTAE, 8µl ethidium bromide
Ampicillin Agar plates	10g NaCl, 10g Peptone, 5g yeast extract ad 1l H_20, 15g Agar Agar autoclave, 100µg/ml Ampicillin
Ampicillin Agar medium	10g NaCl, 10g Peptone, 5g yeast extract ad 1l H_2O, autoclave, 100µg/ml Ampicillin

Buffers

1x Passive Lysis Buffer	Promega
1x buffer H	Roche Applied Science
M-MLV RT 5x Reaction buffer	Promega
10x Annealing buffer	100mM KCH_3COO, 30mM HEPES/KOH pH 7.4, 2mM $Mg(CH_3COO)_2$
2x Lämmli Buffer	62,5 mM Tris-HCl (pH6,8); 10% Glycerol; 2% SDS, 6M urea; 0,00125% BPhB and 5% vol/vol beta-mercaptoethanol
50x TAE	968g Tris base, 228.4ml acetic acid, 148.8g EDTA ad 4l H_2O
10x PBS	87,65g NaCl, 2g KCl, 11,7g Na2HPO4, 2,4g NaH_2PO_4xH20 pH 6,9
1x PBST	500µl Tween 20 ad 1l 1x PBS
10x TBS	25mM Tris/HCl pH 7.5, 150mM NaCl
1x TBS	500µl Tween 20 ad 1l 1x TBS
5% BSA in TBS	5g BSA ad 100ml 1x TBS

phosphate buffer	Tris/HCl 50mM, pH 7.4, EDTA 1mM, NaCl 150mM, NP40 1%, NaF 5mM, Sodium Deoxycholate 0.25%, $NaVO_3$ 2mM, Protease Inhibitors cOmplete mini (Roche)
Antibody dilution buffer for phosphoprotein measurement	10ml TBST, 0.05g BSA, 50µl 20% NaN_3

Cell culture materials

RPMI	Gibco, Life Technologies
IMDM	PAN Biotech
FBS	PAA
L-Glutamine 200mM	Gibco, Life Technologies
Penicillin / Streptomycin (10,000 units/ml Penicillin, 10,000 µg/ml Streptomycin)	Gibco, Life Technologies
Ciprofloxacin 2mg/ml	Fresenius Kabi
Monothioglycerol 50mM	Sigma-Aldrich

U-HO1 medium:	400ml IMDM, 100 ml RPMI, 110ml h.i. FBS, 6ml Glutamine 200mM, 6ml Penicillin/Streptomycin, 2,5ml Ciprofloxacin 200mM, 500µl MTG 50mM
L428, L1236, KM-H2 medium:	500 ml RPMI, 55 ml h.i. FBS, 6ml Glutamine 200mM, 6ml Penicillin/Streptomycin, 2,5ml Ciprofloxacin 200mM, 500µl MTG 50mM
SUP-HD medium:	500 ml RPMI, 110ml h.i. FBS, 6ml Glutamine 200mM, 6ml Penicillin/Streptomycin, 2,5ml Ciprofloxacin 200mM, 500µl MTG 50mM

Cell lines

cHL: L428, KM-H2, L1236, U-HO1, SUP-HD1

NHL: Namalwa, Ramos, Raji, Daudi, DOHH-2, WSU-NHL, SU-DHL-5, Reh

Bacteria

E. coli strain XL1-Blue	Agilent Technologies

Restriction Enzymes

XhoI	New England Biolabs
NotI	New England Biolabs

Enzymes

M-MLV Reverse Transcriptase	Promega

Kits

PureLink™ miRNA Isolation Kit	Invitrogen
High Pure RNA Isolation Kit	Roche Applied Science
Rapid DNA Dephos & Ligation Kit	Roche Applied Science
miScript Reverse Transcription Kit	QIAGEN
miScript Primer Assay	QIAGEN
miScript SYBR® Green PCR Kit	QIAGEN
QIAquick Gel Extraction Kit	QIAGEN
QIAprep Spin Miniprep Kit	QIAGEN
EndoFree Plasmid Maxi Kit	QIAGEN
Dual-Luciferase® Reporter Assay System	Promega
Amaxa™ Cell Line Nucleofector™ Kit V	Lonza

Vectors and Oligonucleotides

psiCHECK™-2 vector	Promega
sequencing primer for psi-CHECK™-2 vector	5'–AGAAGTTCCCTAACACCG–3'

Table 2 | Primer for microRNA (miR) qRT-PCR (quantitative reverse transcription polymerase chain reaction), miScript Primer Assay, QIAGEN.

	Forward (5'-3')	Reverse (5'-3')
miR-27a	UUCACAGUGGCUAAGUUCCGC	miScript universal primer (QIAGEN)
miR-96	UUUGGCACUAGCACAUUUUUGCU	miScript universal primer (QIAGEN)
miR-155	UUAAUGCUAAUCGUGAUAGGGGU	miScript universal primer (QIAGEN)
miR-182	UUUGGCAAUGGUAGAACUCACACU	miScript universal primer (QIAGEN)
miR-183	UAUGGCACUGGUAGAAUUCACU	miScript universal primer (QIAGEN)
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT

Table 3 | Oligonucleotides to be inserted into psiCHECK™-2 vector. Shown are only the upper strand sequences. The corresponding complementary oligonucleotides were designed in a way that when annealed, the double stranded sequence has sticky ends according to Table 4. The nucleotides complementary to the microRNAs are shown in red; the mutated nucleotides are shown in blue; sequences for sticky ends of restriction enzymes are shown in green. miR (microRNA), MUT (mutated), WT (wild type).

FOXO1 binding site	Upper strand sequence (5´-3´)
miR-96/182 site 1 WT	TCGAGTGTGCCATTGGAAATTTCATTACAATGAAGTGCCAAACTCACTACACCATATAGC
miR-96/182 site 1 MUT	TCGAGTGTGCCATTGGAAATTTCATTACAATGAAGACGGTAACTCACTACACCATATAGC
miR-183 site 2 WT	TCGAGTCTATTACTACGTACTGTCACTTTCCTCCCGTGCCATTACTGCATCATAATACGC
miR-183 site 2 MUT	TCGAGTCTATTACTACGTACTGTCACTAAGCTGGCCACGGATTACTGCATCATAATACGC
miR-155 WT	TCGAGTATTGTGGTTTATGCGAACAGACCAACCTGGCATTACAGTTGGCCTCTCCTTGGC
miR-155 MUT	TCGAGTATTGTGGTTTATGCGAACAGACCAACCTGCGTAAACAGTTGGCCTCTCCTTGGC

Table 4 | Restriction enzymes for psiCHECK™-2 digestion, their cutting sequences and the according sticky ends of the FOXO1 3´UTR (3´ untranslated region) part to be inserted (see Table 3). Sequences for sticky ends of restriction enzymes are shown in green. Cutting sites shown as red triangles. Cutting sequences adapted from https://www.neb.com/products/r0146-xhoi and https://www.neb.com/products/r0189-noti, last accessed on Dember 7, 2015.

Restriction enzyme	Cutting Sequence	Sequence for psiCHECK™-2
XhoI	5'...CTCGAG...3' 3'...GAGCTC...5'	5' TCGAG 3' 3' C 5'
NotI	5'...GCGGCCGC...3' 3'...CGCCGGCG...5'	5' GC 3' 3' CGCCGG 5'

Table 5 | Primers for qRT-PCR (quantitative reverse transcription polymerase chain reaction). Primer design with the help of www.genescript.com. c-Myc (V-Myc Myelocytomatosis viral oncogene homolog), CCL5 (CC-motif chemokine ligand 5), RPL13A (Ribosomal protein L13A).

	Forward (5´-3´)	Reverse (5´-3´)
c-Myc	TCGGATTCTCTGCTCTCCTC	TGTTCCTCCTCAGAGTCGCT
CCL5	GGTTCTGAGCTCTGGCTTTG	GCCAGTAAGCTCCTGTGAGG
RPL13A	CGGACCGTGCGAGGTAT	CACCATCCGCTTTTTCTTGTC

Oligo(dT)18 Primer: TTTTTTTTTTTTTTTTT

All Oligonucleotides purchased from Biomers.net.

Anti-miRs

Anti-hsa-miR-96 miScript miRNA Inhibitor	QIAGEN
Anti-hsa-miR-182 miScript miRNA Inhibitor	QIAGEN
Anti-hsa-miR-183 miScript miRNA Inhibitor	QIAGEN
miScript inhibitor negative control	QIAGEN

Antibodies

Table 6 | Antibodies for immunoblot. AKT (protein kinase B), BSA (bovine serum albumin), ERK (extracellular signal-regulated kinase), FOXO1 (forhead box O1), HRP (horseradish peroxidase), IgG (immunoglobulin G), IκBα (nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha), NaN_3 (sodium azide), p- (phospho-), PBS (phosphate-buffered saline), STAT (signal transducer and activator of transcription), TBST (tris-buffered saline with tween).

Primary Antibody	**Origin**	**Company**	**Cat. No.**	**Dilution**
FOXO1	rabbit	Cell signaling	#2880	1:10000 in milk (5% in PBS)
β-Actin	rabbit	Cell signaling	#4970	1:10000 in milk (5% in PBS)
STAT3	rabbit	Cell signaling	#9132	1:5000 in 5% BSA in TBST, 0,1% NaN_3
p-STAT3 pY705	rabbit	Epitomics	#2236-1	1:200 in 5% BSA in TBST, 0,1% NaN_3
STAT5	rabbit	Cell signaling	#9363	1:1000 in 5% BSA in TBST, 0,1% NaN_3
p-STAT5 pY694	rabbit	Epitomics	#ab32364	1:1000 in 5% BSA in TBST, 0,1% NaN_3
AKT	rabbit	Cell signaling	#9272	1:1000 5% BSA in TBST, 0,1% NaN_3
p-AKT pT308	Rabbit	Cell signaling	#9275	1:1000 in 5% BSA in TBST, 0,1% NaN_3
ERK2	rabbit	Santa cruz	Sc-154	1:200 in 5% BSA in TBST, 0,1% NaN_3
p-ERK1/2 pT202 pY204	Rabbit	Cell signaling	#4377	1:1000 5% BSA in TBST, 0,1% NaN_3
IκBα	Goat	Santa cruz	#sc-371G	1:500 in milk (5% in PBS, 0,1% NaN_3)
Secondary Antibody	**Origin**	**Company**	**Cat. No.**	**Dilution**
Anti-Rabbit- IgG-HRP	goat	Thermo Scientific	#31460	1:10000 in milk (5% in PBS)
Anti-Goat-IgG HRP	donkey	Santa Cruz	#sc-2020	1:5000 in milk (5% in PBS)

Plastic ware

6-well non-tissue culture plates	Greiner Bio-One
10-cm non-tissue culture dishes	Greiner Bio-One
Cell culture flasks (25 cm^2, 75 cm^2)	Greiner Bio-One
96-well q-PCR wells	Roche
luminometer PS tubes	Greiner Bio-One

Instruments

AMAXA Nucleofector	Lonza
Gene Pulser II	Bio-Rad
NanoDrop 1000 Spectrophotometer	Thermo Scientific
Light Cycler 480	Roche Diagnostics
Lumat LB 9507 tube luminometer	Berthold Technologies
Vi-CELL XR cell counter	Beckman Coulter
Mini-PROTEAN® Tetra System	Bio-Rad
EBU-204	C.B.S. Scientific
Polymax 1040	Heidolph
Blockthermostat TCR 100	Roth
Heraeus Pico 17 Centrifuge	Thermo Scientific
Genosmart	VWR
Transilluminator BIO view UV light	biostep
Video graphic printer UP-890CE	Sony
Curix 60 developing machine	Agfa
ArtixScan F1	Microtek
FACSCalibur	Becton Dickinson

Software

GenePalette version 2.0 software (www.genepalette.org)

miRanda (www.microrna.org/microrna/home.do).

Genescript online software (www.genescript.com)

Image J 64 Version 1.48 (http://imagej.nih.gov/ij/)

ModFit LT version 2.0 software (http://www.vsh.com/products/mflt/index.asp)

Miscellaneous

Fuji medical X-ray film Fuji, Japan

PageRuler prestained protein ladder, Fermentas Life Sciences, USA

1kb plus DNA ladder Invitrogen, Life Technologies, USA

Chromatography Paper 3mm Chr (Whatman)

0.45µm protran BA85 nitrocellulose membrane (Whatman)

dNTPs 2mM Genaxxon

Syringe filter, 0.2 µm, FP 30/0,2 CA-S Whatman

2.2 Molecular biological methods

2.2.1 Cloning of FoxO1 3´UTR sequences into psiCHECK™-2 vector

For miRNA activity measurements psiCHECK™-2 vector (Promega) was used. In order to insert the desired sequences, the vector was treated with XhoI and NotI restriction enzymes in 1x buffer H (Roche) for 1h at 37°C, according to the manufacturer's protocol for psiCHECK™-2, in which XhoI and NotI are listed as restriction enzymes cutting only 1 site, respectively, at the multiple cloning region downstream of the *Renilla* luciferase reporter gene (Figure 10). The digested vector backbone was isolated using 1.5% agarose ethidium bromide gel electrophoresis and purified using QIAquick Gel Extraction Kit (QIAGEN).

The target sequences were located using GenePalette software (www.genepalette.org) and miRanda (www.microrna.org). Mutations were performed at the seed sequences. The oligomers were ordered in a way to produce the 5' XhoI sticky ends and the 3' NotI sticky ends when annealed. The sense and antisense strands were annealed by diluting them to 3µg/µl and adding 1µl of each to 48µl 1x annealing buffer. The mixture was incubated for 3min at 95°C, 10min at 70°C and subsequently cooled down slowly to 10°C.

The annealed oligonucleotides and the digested psiCHECK-2 vector, both containing sticky ends, were ligated by using Rapid DNA Dephos & Ligation Kit (Roche). 50µl *E.coli* XL1-Blue were brought together with 0.8µl β-Mercaptoethanol and were transformed with 5µl of ligated vector solution (30min on ice, 1 min 42°C waterbath „heat shock"). The transformed *E.coli* were spread on Ampicillin agar plates, because the vector contained an Ampicillin resistance gene (see Figure 10), and incubated at 37°C overnight for selection purposes. Single colonies were picked for MiniPrep using QIAprep Spin Miniprep Kit (QIAGEN). The extracted plasmids were sequenced by GATC (www.gatc-biotech.com/de/index.html) and when correct, MaxiPrep was carried out, using EndoFree Plasmid Maxi Kit (QIAGEN). DNA concentration was then measured using NanoDrop 1000 Spectrophotometer (Thermo Scientific).

The psiCHECK™-2 vector works by producing a fusion transcript consisting of the *Renilla* luciferase gene and the gene of interest. Upon specific miRNA targeting,

we expected a decreased *Renilla* luciferase protein expression and therefore a decrease in luminescence by RNA interference (Figure 11).

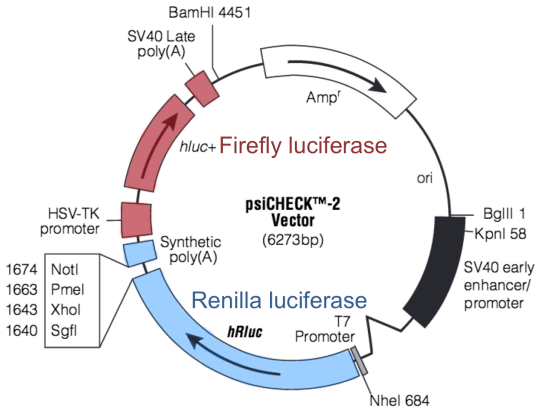

Figure 10 | Schematic illustration of psiCHECK™-2 vector. *Renilla* luciferase (hRluc) reporter gene shown in blue, Firefly luciferase (hluc+) control gene shown in red. Potential restriction enzyme cutting sites for insertion of the target gene shown in the box (cutting locations displayed on the left). Amp^r (Ampicillin resistence gene, β-lactamase gene), bp (base pairs), HSV-TK (Herpes simplex virus thymidine kinase), ori (origin of replication), poly(A) (polyadenylate), SV40 (Simian virus 40). Modified from https://www.promega.de/~/media/files/resources/protocols/technical%20bulletins/101/sicheck%20vectors%20protocol.pdf, last accessed on December 7, 2015. Reprint permisson kindly granted by Promega Corp.

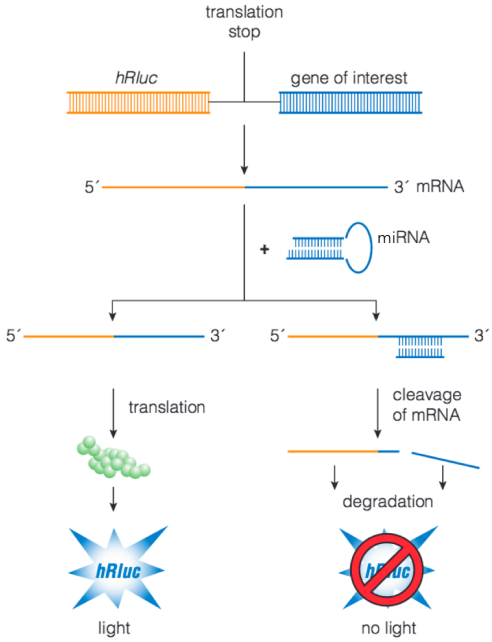

Figure 11 | Schematic illustration of psiCHECK™-2 vector mechanism of action. Upon specific miRNA (microRNA) action at the inserted sequence, *Renilla* mRNA (messenger RNA) is degraded and therefore fewer light emission can be detected. hRluc (*Renilla* luciferase reporter gene). Modified from https://www.promega.de/~/media/files/resources/protocols/technical%20bulletins/101/sicheck%20vectors%20protocol.pdf, last accessed on December 7, 2015. Reprint permisson kindly granted by Promega Corp.

2.3 Cell culture methods

2.3.1 Cell lines, culture conditions and treatments

Table 7 | cHL cell lines and their culture mediums, doubling time (DT), incubation conditions (Inc.) and clinical data. All cell lines are EBV (Epstein Barr Virus) negative. °C (degrees Celsius), cHL (classical Hodgkin Lymphoma), CO_2 (carbon dioxide), FBS (fetal bovine serum), h (hours), h.i. (heat inactivated), IMDM (Iscove's Modified Dulbecco's Medium), RPMI (Cell culture medium developed by Roswell Park Memorial Institute), SCT (stem cell transplantation). Adapted from (DSMZ [Internet] 2016).

Cell line	Culture medium	DT	Inc.	Clinical data
U-HO1	80% (IMDM + RPMI 1640 4:1) + 20% h.i.FBS + supplements	100h	37°C with 5% CO_2	- pleural effusion - 23 year old male - nodular sclerosis type cHL - 2nd relapse post-SCT - established 2005
L1236	90% RPMI 1640 + 10% h.i. FBS + supplements	48h	37°C with 5% CO_2	- peripheral blood - 34 year-old male - mixed cellularity type cHL - stage IV, refractory, terminal, 3rd relapse - established 1994
L428	90% RPMI 1640 + 10% h.i. FBS + supplements	35h	37°C with 5% CO_2	- pleural effusion - 37-year-old female - nodular sclerosis type cHL - stage IVB, refractory, terminal - established 1978
KM-H2	90% RPMI 1640 + 10% h.i. FBS + supplements	48h	37°C with 5% CO_2	- pleural effusion - 37-year-old male - mixed cellularity type cHL progressing to lymphocyte depletion - stage IV at relapse - established 1974
SUP-HD1	80% RPMI + 20% h.i. FBS + supplements	40-80 h	37°C with 5% CO_2	- 37-year-old male - nodular sclerosis type cHL progressing to lymphocyte depletion - stage IV, 2nd relapse, refractory, terminal - established 1987

Cell count analysis

The assessment of the amount of viable cells was performed using Vi-CELL XR cell counter (Beckman Coulter).

Treatment with JAK2 Inhibitor TG101348

Cell culture medium was supplemented with TG101348 to give a final concentration of 2 µM. As control we used the same volume of vehicle (DMSO). After 48 hours of incubation the cells were harvested for immunoblot (phosphoprotein extraction) and RT-qPCR. The JAK/STAT pathway is consitutively active in cHL (Figure 12).

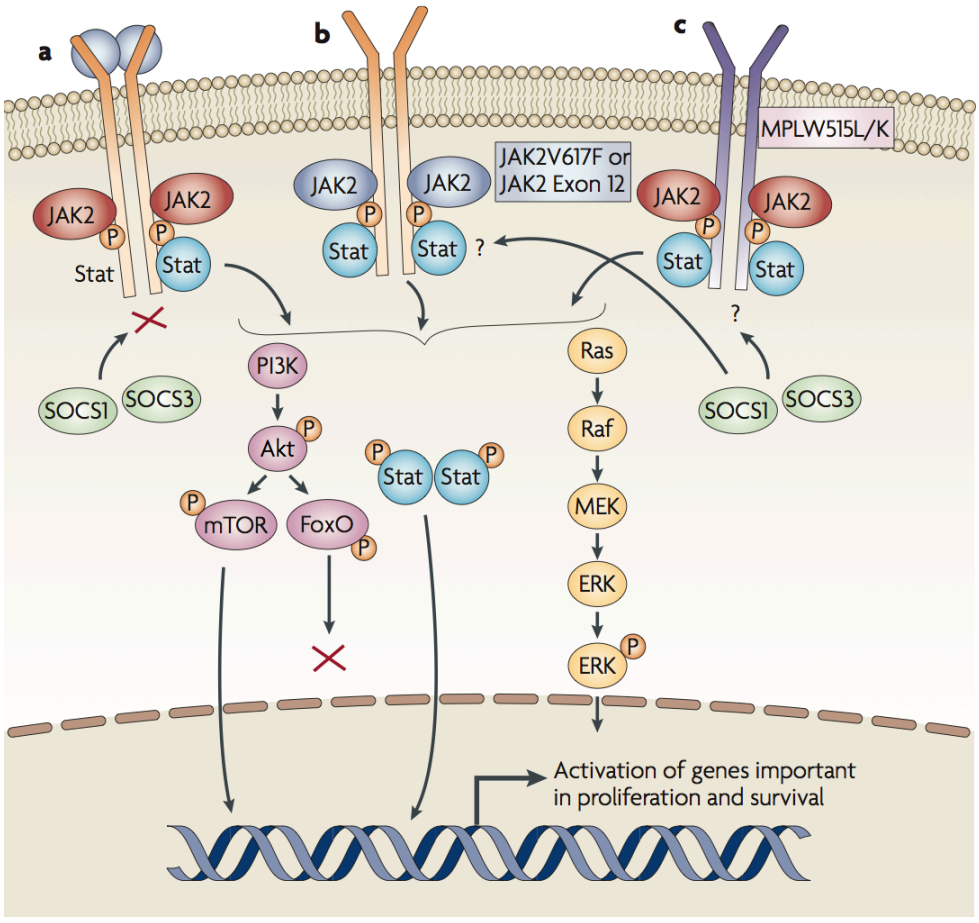

Figure 12 | JAK2 (janus kinase 2) signaling and its connections to other cellular signaling pathways. JAK2 activation leads, among others, to the recruitment of STAT (signal transducers and activators of transcription) proteins, their phosphorylation and further downstream signaling. Akt (protein kinase B), ERK (extracellular signal-regulated kinase), FoxO (forkhead box O), MEK (mitogen-activated protein kinase kinase), mTOR (mechanistic target of rapamycin), P

(phosphorylation site), PI3K (Phosphoinositide 3 kinase), Raf (rapidly accelerated fibrosarcoma), Ras (rat sarcoma), SOCS (suppressor of cytokine signaling). Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Levine et al. 2007), copyright (2007). Copyright Clearance Center License Number: 3791940538244.

Treatment with AKT/PDK1 Inhibitor KP372-1

L428 cells were treated with KP372-1 at a final concentration of 0.4 µM. As control we used the same volume of DMSO, in which KP372-1 was dissolved. Cells were harvested after 24 hours. KP372-1 inhibits the kinase activities of AKT, PDK1 and FLT3. The mechanism of action of KP372-1 is visualized in Figure 13.

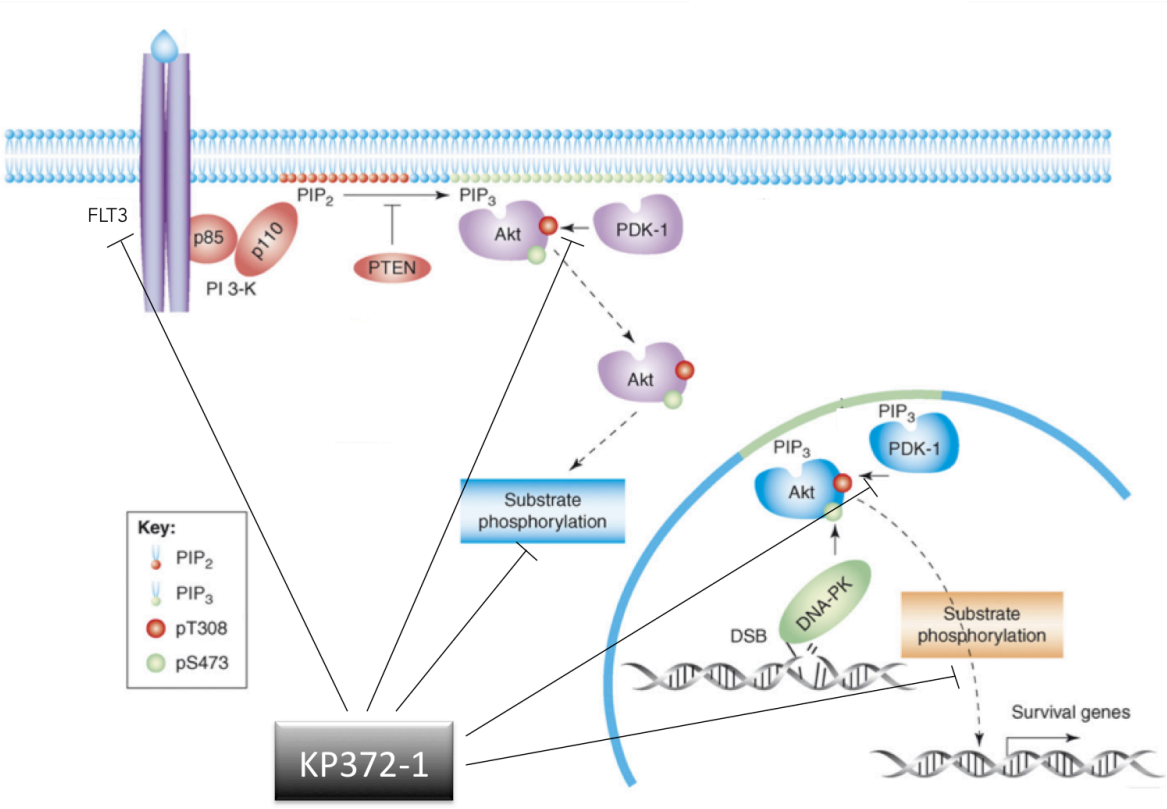

Figure 13 | Visualized model of the inhibition of FLT3 (Fms-like tyrosine kinase 3), PDK-1 (phosphoinositide-dependent kinase-1) and AKT (protein kinase B) by KP372-1. DNA-PK (DNA dependent protein kinase), DSB (double strand breaks), PI3K (Phosphoinositide 3 kinase), PIP_2 (phosphatidylinositol-4,5-bisphosphate) PIP_3 (phosphatidylinositol-3,4,5-triphosphate) PTEN (phosphatase and tensin homolog), Adapted with permission from Toker, A. (2008). Akt signaling: a damaging interaction makes good. *Trends in Biochemical Sciences*, *33*(8), 356–359, with permission from Elsevier. Copyright Clearance Center License Number: 3791940659815.

Treatment with MEK1/2 Inhibitor U0126

L428 cells were treated with U0126 at a final concentration of 18 µM. As control we used the same volume of DMSO in which U0126 was dissolved. Cells were harvested after 24 hours. The mechanism of action of U0126 is illustrated in Figure 14.

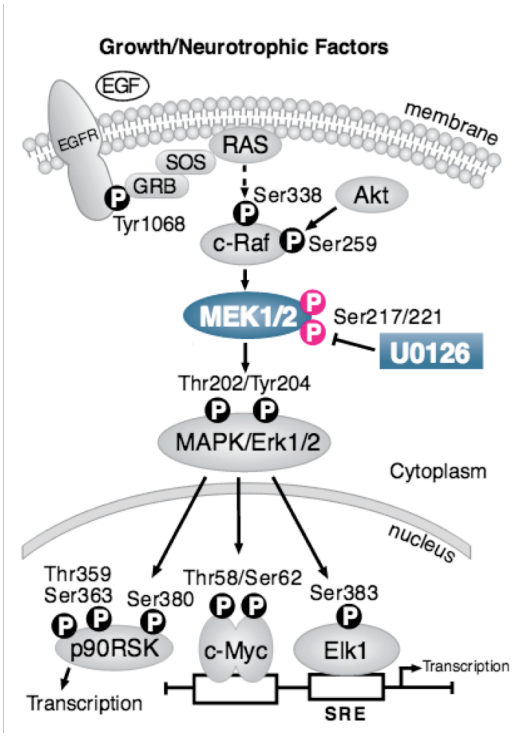

Figure 14 | Inhibition of MEK1/2 (mitogen activated protein kinase kinase 1/2) by U0126. No further cascade members are inhibited - the inhibitor is highly selective. By inhibiting MEK 1/2 it prevents MAPK/Erk1/2 (mitogen-activated protein kinase / extracellular signal-regulated kinase 1/2) from being phosphorylated and thereby being activated. Akt (protein kinase B), c-Myc (myelocytomatosis viral oncogene homolog), c-Raf (rapidly accelerated fibrosarcoma), EGF (epidermal growth factor), EGFR (epidermal growth factor receptor), Elk1 (E26 transformation-specific domain-containing protein 1), GRB (growth factor receptor-bound protein), P (phosphorylation site), Ras (rat sarcoma), RSK (Ribosomal S6 kinase), Ser (Serine), SOS (son of sevenless), SRE (serum response element), Thr (Threonine), Tyr (Tyrosine). Illustration reproduced courtesy of Cell Signaling Technology, Inc. (wwww.cellsignal.com). Modified from http://media.cellsignal.com/pdf/9903.pdf, last accessed on January 11, 2016.

IkBα expressing cells

KM-H2 cells stably transfected with a pRTS-1 vector expressing a mutant variant of IkBα as well as green fluorescent protein (GFP) were kindly provided by K. Klapproth, a former member of our research unit. The conditional transgene IkBα was under the control of a tet-on promoter (vector design see (Klapproth et al. 2009). Upon treatment with doxycycline (0.5 µg/mL) IkBα and GFP were expressed. After 96h cells were harvested for further analysis (immunoblot and RT-qPCR) if GFP expression could be shown in >95% of cells. Activation efficiency was validated directly by measuring GFP expression using flow cytometry and indirectly by measuring NF-kB target gene expression using quantitative RT-PCR. As control, KM-H2 cells transfected with the pRTS-1 empty vector were used. Stable transfection was ensued by hygromycin B selection (10µg/mL) due to a resistance gene included in the pRTS-1 vector (Bornkamm et al. 2005). IkBα is an inhibitor of NF-kB and therefore interferes with the transcriptional activity of NF-kB (Figure 15).

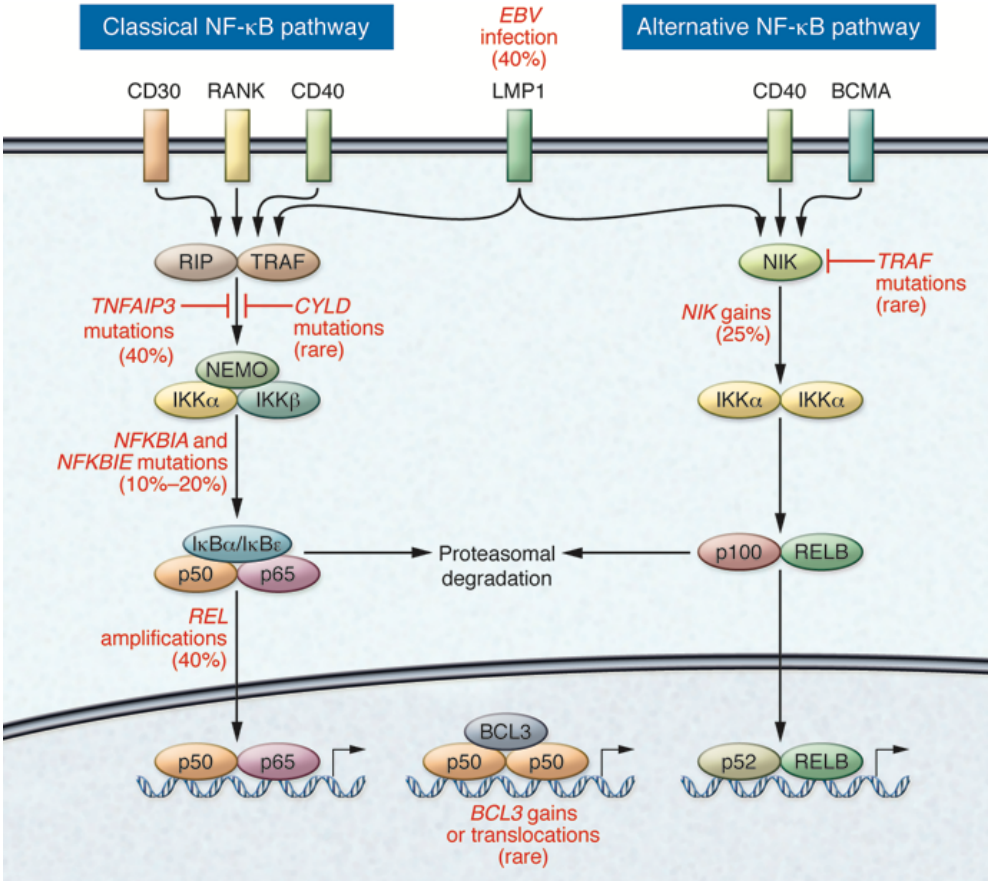

Figure 15 | Constitutively active NF-kB signaling in cHL. In the classical pathway, IkBα inhibits p50 and p65 from acting as transcription factor by masking their nuclear localization signals. Genetic

lesions or viral infections that are present in cHL are marked in red. BCL3 (B-cell lymphoma 3), BCMA (B-cell maturation antigen), CD30 (cluster of differentiation 30), CD40 (cluster of differentiation 40), CYLD (CYLD Lysine 83 deubiquitinase), EBV (epstein barr virus), IκBα/ε (inhibitor of NF-κB α/ε), IKKα/β (IκB kinase α/β), LMP1 (Latent membrane protein 1), NEMO (NF-κB essential modulator), NFκB (nuclear factor kappa-light-chain-enhancer of activated B-cells), NFKBIA/E (NF-κB inhibitor α/ε gene), NIK (NF-κB inducing kinase gene), p50 (NF-κB1), p65 (RelA, V-Rel avian reticuloendotheliosis viral oncogene homolog A), RANK (receptor activator of NFκB), REL (V-Rel avian reticuloendotheliosis viral oncogene homolog, c-Rel), RelB (V-Rel avian reticuloendotheliosis viral oncogene homolog B), RIP (receptor interacting protein), TNFAIP3 (tumor necrosis factor alpha-induced protein 3 gene), TRAF (tumor necrosis factor alpha receptor associated factor gene). Republished (modified) with permission of the American Society For Clinical Iinvestigation, from Hodgkin Lymphoma, Küppers et al., volume 122, edition number 10, 2012; permission conveyed through Copyright Clearance Center, Inc. License ID: 3950180147539.

GFP expression analysis using flow cytometry

GPF expression of stably transfected pRTS-1 IkBα bearing KM-H2 cells was measured using FACSCalibur (Becton Dickinson). The data was analyzed using ModFit LT Version 2.0 software. If GFP expression of viable cells after 96h of doxycycline treatment was >95%, the cells were approved for harvesting.

2.3.2 Transfection

Gene pulser Electroporation

For transfection of psiCHECK™-2, $10x10^6$ L428 cells were brought together with 10µg of the appropriate vector in fresh medium adding up to 300µl. Transfection was carried out by using electroporation method using Gene Pulser II (Bio-Rad, 250kV, 975,000 µF, high capacity). The luciferase activity was measured 24 hours later.

Nucleofection

$4 x10^6$ L428 cells were transfected with the adequate miScript miRNA inhibitor (QIAGEN) using AMAXA Nucleofector™ (Lonza). The provided Nucleofector™ Solution (95µl) was mixed with 5µl of the 20µM Anti-miR solution and program X-

01. As negative control miScript Inhibitor Neg. Control (20µM, QIAGEN) was used. The transfected cells were cultivated for 48 hours at standard cultivation conditions for L428.

2.4 Analysis of nucleic acids

2.4.1 Isolation of miRNA and total RNA with cDNA preparation

MicroRNA and total RNA was isolated from 1 x 10^6 cells by using PureLink™ miRNA Isolation Kit (Invitrogen) and High Pure RNA Isolation Kit (Roche), respectively, according to the manufacturer's instructions. Preparation of cDNA was performed using miScript Reverse Rranscription Kit (QIAGEN) for miRNA (diluted to 1:100) and according to Table 8 for total RNA.

Table 8 | cDNA (complementary DNA) preparation for total RNA. °C (degrees celsius), dNTPs (deoxynucleotides), min (minutes), M-MLV RT(Moloney Murine Leukemia Virus Reverse Transcriptase), mM (millimolar), µg (micrograms), µl (microliters), Oligo(dT)18 Primer (synthetic single-stranded 18-mer deoxythimidine primer).

Amount	Substance	Action
2µg	RNA	5 min, 70°C,
1µl	Oligo(dT)18 Primer (biomers.net)	subsequently on ice
5µl	M-MLV RT 5x Reaction buffer (Promega)	1 hour, 42°C,
5µl	2mM dNTPs	subsequently diluted
1µl	recombinant RNasin® Ribonuclease Inhibitor (Promega)	1:10
1µl	M-MLV Reverse Transcriptase (Promega)	

2.4.2 quantitative RT-PCR

Quantification of miRNA

The expression of specific miRNAs was detected with the help of the specific miScript Primer Assays (QIAGEN) and miScript SYBR Green PCR kit (QIAGEN) as a 10µl mix each (cDNA 1:100), in a Light Cycler 480 (Roche Diagnostics) (settings see Table 9). U6 RNA was used as reference gene (qRT-PCR reactants composition see Table 10). The data was analyzed by comparative C_t method (Schmittgen and Livak 2008). The experiments were performed at least in triplicates.

Table 9 | LightCycler Settings for miRNA (microRNA) quantification. °C (degrees Celsius), min (minutes), s (seconds), x (time[s]).

Step	Temperature (°C)	Duration	Repeats of sets
Heating	95	15 min	1x
Amplification	94	15 s	40x
	55	30 s	
	70	30s	
Melting	95	1 s	1x
	65	15 s	
	95	continuous, ramp rate 0,19°C/s, 3 acquisitions per °C	
Cooling	40	30 s	1x

Table 10 | U6, RPL13a (ribosomal protein L13a), c-Myc (V-Myc Myelocytomatosis viral oncogene homolog) and CCL5 (C-C-motif ligand 5) RT-qPCR (reverse transcription quantitative polymerase chain reaction) reactants composition (per well). cDNA (complementary DNA), H_2O (water), µl (microliters).

Amount	Substance
3,6µl	RNAse free H_2O
0,2µl	forward primer
0,2µl	reverse primer
5µl	SYBR Green (QIAGEN)
1µl	cDNA (1:100 for U6, 1:10 for RPL13a, c-Myc, CCL5)

Quantification of mRNA

PCR primers were designed by Genescript online software (www.genescript.com) and synthetized by biomers.net. Reactants composition was chosen according to Table 10. Light cycler settings were made according to Table 11.

Table 11 | LightCycler Settings for mRNA (messenger RNA) Quantification. °C (degrees Celsius), min (minutes), s (seconds), x (time[s]).

Step	Temperature (°C)	Duration	Repeats of sets
Heating	95	15 min	1x
Amplification	94	15 s	40x
	60	30 s	
	72	30s	
Melting	95	1 s	1x
	65	15 s	
	95	continuous, ramp rate 0,19°C/s, 3 acquisitions per °C	
Cooling	40	30 s	1x

2.5 Protein analysis

2.5.1 Immunoblot

Regular Immunoblot

For Immunoblot (Western Blot) analysis $1x10^6$ cells were washed with phosphate-buffered saline (PBS), lysed with 100µl 1x Lämmli Buffer and boiled for 10 min. 20µl of lysates were separated on a 10% polyacrylamide gel using a BIORAD Mini-PROTEAN® Tetra System (90V, 100mA) and electroblotted onto a 0.45µm protran BA85 nitrocellulose membrane (Whatman) using a EBU-204 (C.B.S. Scientific) blotting system (65 min, 80V, 350mA). Membranes were blocked 15 min at 37°C with 5% skimmed milk powder in PBS. Primary antibodies were brought together with the membrane for 1 hour at room temperature on a Polymax 1040

(Heidolph) motion platform. Alternatively, primary antibodies were incubated overnight at 4°C on a Polymax 1040 (Heidolph) motion platform. The washing step was performed by rinsing the membrane with 1x PBS, then 3 x 5 minutes with 1x PBST and another 5 min PBS. The secondary antibody was incubated at the same conditions. Another washing step was performed as mentioned before. Signals were visualized using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific), supplying a substrate for the horseradish peroxidase coupled secondary antibodies. We used an Agfa Curix 60 developer and medical X-ray film (Fuji). Films were scanned on an ArtixScan F1 (Microtek) scanner and semi-quantitatively analyzed using Image J 64 software.

Immunoblot of Phosphoproteins

$1x10^6$ cells were washed with phosphate-buffered saline (PBS), lysed with 20µl phosphate buffer and 40µl 1x Lämmli Buffer and boiled for 10 min. 15µl of lysates were separated by SDS PAGE and electroblotted at the same conditions as described for regular immunoblotting. Membranes were blocked 15 min at 37°C with 5% BSA in TBS. Primary antibodies (diluted in a TBST-BSA-NaN_3 buffer) were brought together with the membrane for 1 hour at room temperature on a Polymax 1040 (Heidolph) motion platform. Alternatively, primary antibodies were incubated overnight at 4°C on a Polymax 1040 (Heidolph) motion platform. The washing step was performed by rinsing the membrane with 1x TBS, then 3x 5 minutes with 1x TBST and another 2x 5 min TBS. The secondary antibody (anti-rabbit in milk) was incubated at the same conditions. The following washing step was performed as mentioned before. Further analysis was carried out as described for regular immunoblotting.

2.5.2 Luciferase reporter assay

After 24h 5ml of the transfected L428 cell solution was centrifuged and the pellet was washed with phosphate buffered saline (PBS). Dual-Luciferase® Reporter Assay System (Promega) was used for luciferase activity analyses. For that purpose, the pellet was resuspended in 500µl of 1x Passive Lysis Buffer, left to

lyse for 10 minutes. Subsequently in each case 5µl of the suspension was analyzed in a luminometer PS tube (Greiner Bio-One) using the Lumat LB 9507 tube luminometer (Berthold Technologies). Firstly, 50µl of Luciferase Assay Reagent II (LAR II) was added to the tube in order to measure firefly luminescence. Secondly, Stop & Glo® Reagent was added, stopping the firefly luminescence and initiating *Renilla* luminescence, and the second luminescence measurements were conducted (Figure 16). Measurements were performed in duplicates, the experiment was performed in triplicates.

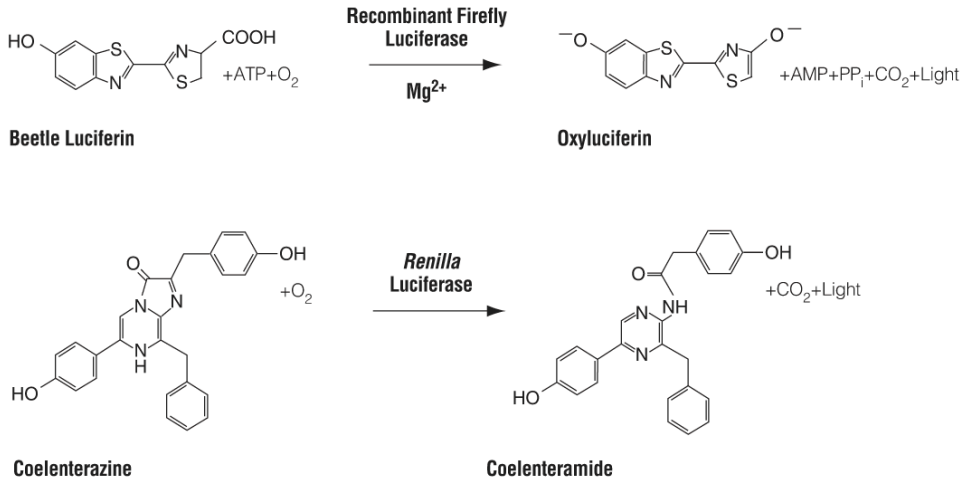

Figure 16 | Illustration of how psiCHECK™-2 luciferases produce light. (psiCHECK^{Tm} Vectors [Internet] 2015). AMP (Adenosine monophosphate), ATP (Adenosine triphosphate), CO_2 (Carbon dioxide), Mg^{2+} (Magnesium), O_2 (Oxygen), PP_i (Pyrophosphate). Modified from https://www.promega.de/~/media/files/resources/protocols/technical%20bulletins/101/sicheck%20vectors%20protocol.pdf, last accessed on December 7, 2015. Reprint permisson kindly granted by Promega Corp.

3 Results

3.1 *In silico* prediction of miRNA potentially targeting FOXO1 mRNA

While analyzing possible miRNAs targeting FOXO1 using miRanda (miRanda FOXO1 Target mRNA [Internet] 2015), we identified the following: miR-27a, miR-96, miR-155, miR-182 and miR-183 (among others). MicroRNA-27a/96/182 were previously found to regulate FOXO1 in breast cancer cells (Guttilla and White 2009). MicroRNA-155 was chosen because of its previously described upregulation in cHL and its attributed role as being an oncomiR (Czyzyk-Krzeska and Zhang 2014; Gibcus et al. 2009). The miRNAs miR-96, miR-182 and miR-183 can be found in a cluster on chromosome 7q32.2, which is transcribed polycistronically (Xu et al. 2007). Because of multiple potential target sites at the 3´UTR of FOXO1 mRNA, those locations were chosen for Luciferase reporter assay that had the best mirSVR score. The mirSVR algorithm aims to predict target mRNA downregulation of miRanda-predicted miRNA target sites according to their duplex features, local context features and global context features (Betel et al. 2010). Gene Palette software was used to visualize the positions of the FoxO1 target sites (Figure 17). Interestingly, miR-96 and miR-182 have the same target sites (Figure 18), but according to their mirSVR score, miR-96 presumably is the more efficient miRNA. All five miRNAs chosen were tested for upregulation in classical Hodgkin Lymphoma compared to normal B cells and NHL cell lines.

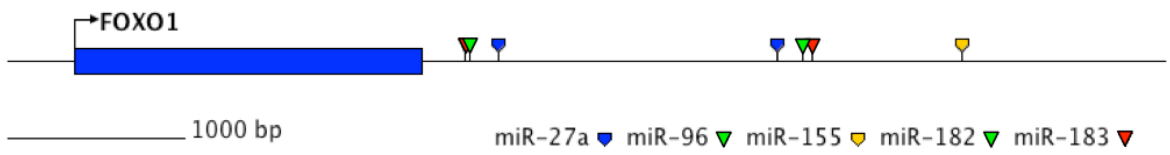

Figure 17 | Illustration of FOXO1 mRNA using Gene Palette software. The blue bar represents the coding region. The line behind the bar represents FOXO1 3´UTR (3´ untranslated region). The positions of miR-27a (blue), miR-96 (green), miR-155 (yellow), miR-182 (green) and miR-183 (red) target sites at the FOXO1 3´UTR are marked. Interestingly, miR-96 and miR-182 share the same target sites. bp (base pairs), miR (microRNA).

3' ucACUUAAGAUGGUCACGGUAu 5' hsa-miR-183 | :|| : | ||||||| 222:5' ugUAGAUAAGGACUGUGCCAUu 3' FOXO1	mirSVR score: -0.4815 PhastCons score: 0.7869
3' ucacacucaagaugguaACGGUUu 5' hsa-miR-182 |||||| 248:5' auuucauuacaaugaagUGCCAAa 3' FOXO1	mirSVR score: -0.0603 PhastCons score: 0.8056
3' ucguUUUUACACGAUCACGGUUu 5' hsa-miR-96 | |||| |||||||| 252:5' cauuACAAUG---AAGUGCCAAa 3' FOXO1	mirSVR score: -1.0683 PhastCons score: 0.8056
3' cgccuuGAAUCG--GUGACACUu 5' hsa-miR-27a :|| |: :||||||| 411:5' uacucuUUUUGUAAUACUGUGAu 3' FOXO1	mirSVR score: -0.5576 PhastCons score: 0.6832
3' cgccUUGAAUCGGUGACACUu 5' hsa-miR-27a |:||| |||||||| 1988:5' uguaAGCUU-CCCACUGUGAu 3' FOXO1	mirSVR score: -0.0524 PhastCons score: 0.5593
3' ucguuUUUACACG-AUCACGGUUu 5' hsa-miR-96 || || | |:||||||| 2123:5' uauguAACUGAACUUGGUGCCAAa 3' FOXO1	mirSVR score: -0.2119 PhastCons score: 0.5196
3' ucacacucaagaugguaACGGUUu 5' hsa-miR-182 |||||| 2123:5' uauguaacugaacuuggUGCCAAa 3' FOXO1	mirSVR score: -0.0056 PhastCons score: 0.5196
3' ucacuuAAGAUGGUCACGGUAu 5' hsa-miR-183 ||| || |||||| 2180:5' gucacuUUCCUCCCGUGCCAUu 3' FOXO1	mirSVR score: -0.0395 PhastCons score: 0.4866
3' uggggauagugcuaAUCGUAAUu 5' hsa-miR-155 |:||||| 3025:5' cgaacagaccaaccUGGCAUUAc 3' FOXO1	mirSVR score: -0.0019 PhastCons score: 0.5811

Figure 18 | FOXO1 mRNA (messenger RNA) 3´UTR (3´ untranslated region) target sites of miR-27a, miR-96, miR-155, miR-182 and miR-183. The data was mined with the help of miRanda online software (www.microrna.org). Order according to their position 5' to 3'. miR-96 and miR-182 share the same target sites. miR-182 seed sequences are 6 bases long, miR-96 seed sequences are 13 bases long, although not contiguously. miR (microRNA), mirSVR score (microRNA support vector regression, score to predict target downregulation by miRNAs), PhastCons score (target site conservation score, part of software "Phylogenetic Analysis mit Space/Time models" designed to measure conservation of nucleotide positions among vertebrates). Data provided by http://www.microrna.org/microrna/getMrna.do?gene=2308&utr=29019&organism=9606, last accessed on December 11, 2015.

3.2 miR-96, -155, -182 and -183 are upregulated in classical Hodgkin Lymphoma

As previously described by Guttilla and White (Guttilla and White 2009), FOXO1 can be repressed by miR-27a, miR-96 and miR-182. For this reason, and also due to *in silico* FOXO1-miRNA-target prediction, expression of these three miRNAs and additionally miR-155 and miR-183 (the latter located in the same cluster as miR-96 and miR-182) was tested in $CD19^+$ tonsillar B cells of 5 different patients, 5 cHL (L428, KM-H2, L1236, U-HO1, SUP-HD1) and 8 NHL cell lines (Namalwa, Ramos, Raji, Daudi, DOHH-2, WSU-NHL, SU-DHL-5, Reh) by quantitative RT-PCR. U6 mRNA was used as reference. Interestingly, miR-27a was only significantly higher expressed in U-HO1 compared to tonsillar B cells and NHL cell lines, but not in other cHL cell lines. By contrast, miR-96, miR-182 and miR-183 showed a significantly higher expression in all cHL cell lines compared to tonsillar B cells. The highest expression levels were found in cell lines L428, L1236 and U-HO1 and exceeded those in the compared NHL cell lines markedly. MicroRNA-155 was elevated in all tested cHL cell lines, except L1236. Therefore, further investigations were carried out concentrating on these four elevated miRNAs (miR-96, miR-155, miR-182, miR-183).

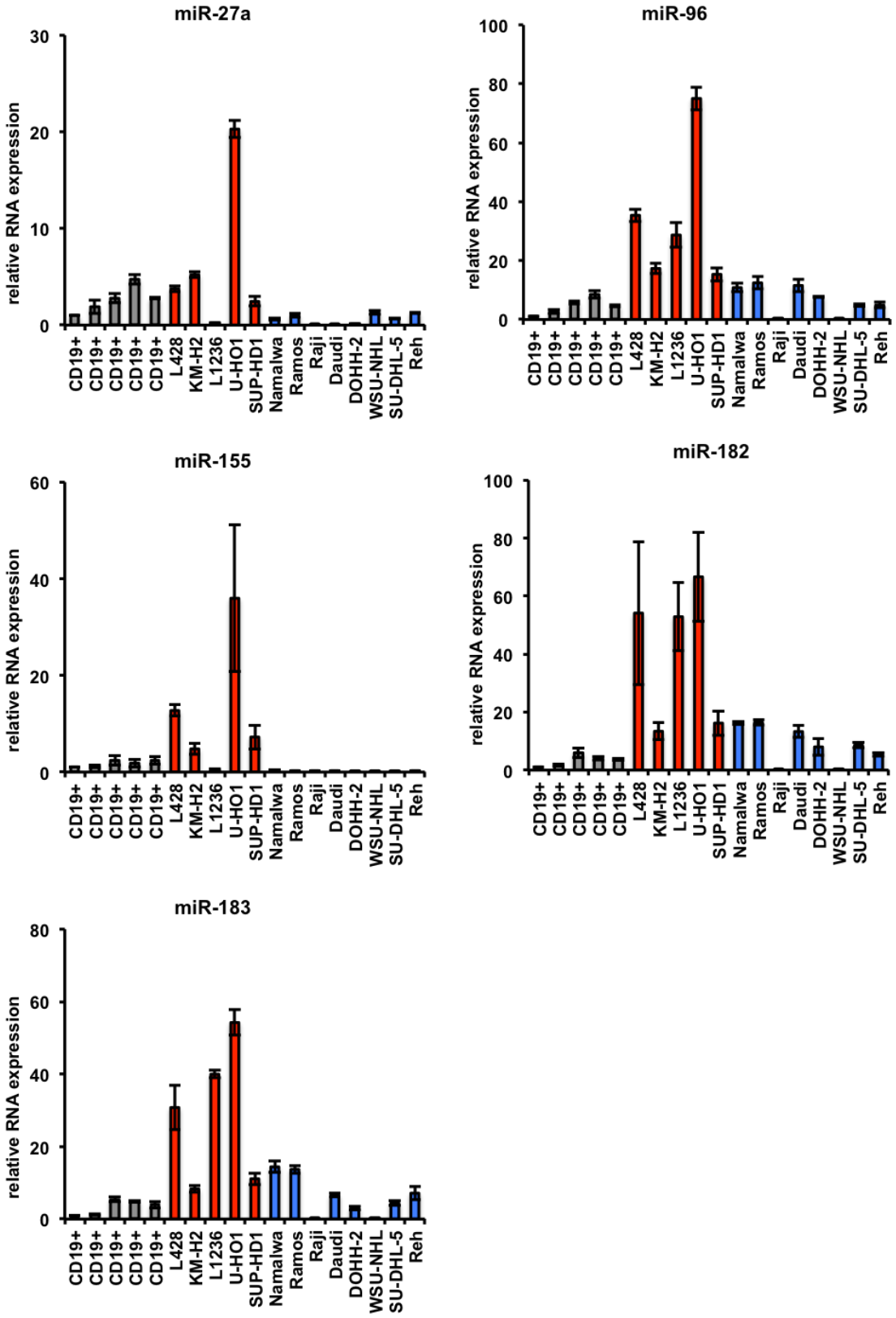

Figure 19 | quantitative RT-PCR (reverse transcription polymerase chain reaction) of miR-27a, miR-96, miR-155, miR-182 and miR-183 extracted from $CD19^+$ tonsillar B cells from 5 different patients (grey bars), cHL cell lines (red bars) and NHL cell lines (blue bars). U6 RNA served as reference. All experiments were performed in at least triplicates. Analysis of the data was

performed using comparative C_t method. The bars represent mean ± SD (standard deviation). miR-27a was only elevated in U-HO1 compared to tonsillar B cells. miR-96, miR-182 and miR-183 were found to be elevated in all cHL cell lines compared to tonsillar B cells with the highest expression levels to be found in L428, L1236 and U-HO1. Interestingly, miR-155 was elevated in most of the cHL cell lines, except L1236. $CD19^+$ (cluster of differentiaton 19 positive), cHL (classical Hodgkin Lymphoma), NHL (Non-Hodgkin Lymphoma), miR (microRNA). Partially republished (modified) with permission of the American Society of Hematology from L Xie, A Ushmorov, F Leithäuser, H Guan, C Steidl, J Färbinger, C Pelzer, M J Vogel, H J Maier, R D Gascoyne, P Möller, and T Wirth. (2012). FOXO1 is a tumor suppressor in classical Hodgkin lymphoma. *Blood*, *119*(15), 3503–3511.; permission conveyed through Copyright Clearance Center, Inc. License Number: 3950240393740.

3.3 The miR-183/96/182 cluster regulates FoxO1 mRNA translation

Since we were able to show that miR-96, miR-155, miR-182 and miR-183 are overexpressed in cHL, we initiated the functional analysis of FOXO1 repression due to these miRNAs by Luciferase Reporter Assay. Vectors were created bearing those segments of FoxO1 3´UTR targeted by the miRNA to be evaluated, coupled to the *Renilla* luciferase sequence (reporter gene). As an internal control, Firefly luciferase is part of the psiCHECK™-2 vector that was used. Upon miRNA action, *Renilla* luciferase mRNA is repressed by the RISC, therefore showing fewer light emission compared to the stable Firefly luciferase light emission, respectively. As control, we used vectors bearing the mutant FOXO1 seed sequences.

The relative luminescence of lysed L428 cells bearing constructs with wild type binding sites for miR-96/182 and miR-183 was significantly lower compared to their mutated equivalents, pointing to an involvement of these miRNAs in FOXO1 regulation (Figure 20). By contrast, the miR-155 binding site did not repress luciferase activity.

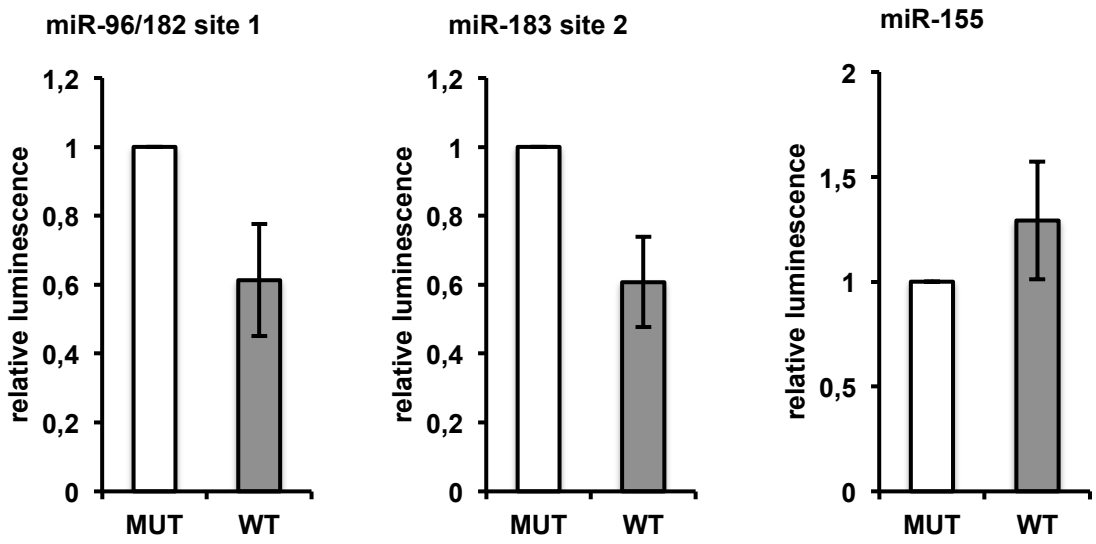

Figure 20 | Luciferase reporter assay for FOXO1 3´UTR (3´ untranslated region). Relative luminescence measured in lysed L428 cells bearing the psiCHECK™-2 vector with either a wild type (WT) or mutated (MUT) part of FOXO1 3´UTR cloned into the multiple cloning region downstream of the *Renilla* luciferase reporter gene 24 hours after transfection. The FOXO1 3´UTR parts were chosen according to the predicted miRNA (microRNA) target sites. Mutations were performed at the FOXO1 3´UTR seed regions of the miRNAs to be tested. Relative luminescence was measured as ratio of *Renilla* to Firefly luciferase luminescence and was normalized to the mutated control. All experiments were performed in at least triplicates. The bars represent mean ± SD (standard deviation). The relative luminescence in L428 cells bearing the construct with the WT binding site for both miR-96/182 and miR-183 was approximately 40% lower compared to the mutated version, whereas the miR-155 WT site did not lead to lower relative luminescence. In conclusion, miR-96/182 and miR-183 seem to be negatively affecting FOXO1 expression in L428 cells. Partially republished (modified) with permission of the American Society of Hematology from L Xie, A Ushmorov, F Leithäuser, H Guan, C Steidl, J Färbinger, C Pelzer, M J Vogel, H J Maier, R D Gascoyne, P Möller, and T Wirth. (2012). FOXO1 is a tumor suppressor in classical Hodgkin lymphoma. *Blood*, *119*(15), 3503–3511.; permission conveyed through Copyright Clearance Center, Inc. License Number: 3950240393740.

3.4 Anti-miRs against miR-183/96/182 increase FOXO1 protein levels

The functional effect of miRNAs on FOXO1 protein seen in the luciferase reporter assay needed to be validated. For this purpose, we transfected L428 cells with inhibitors of miR-96, miR-182 and miR-183 and investigated the effect on FOXO1 protein levels 48 hours later. The validation of anti-miR-performance was

established via quantitative RT-PCR, measuring miRNA levels of L428 cells transfected with specific anti-miRs compared to those transfected with negative control. U6 mRNA served as control. MicroRNA expression was reduced by approximately 70-80% in all three cases and therefore very effective (Figure 21).

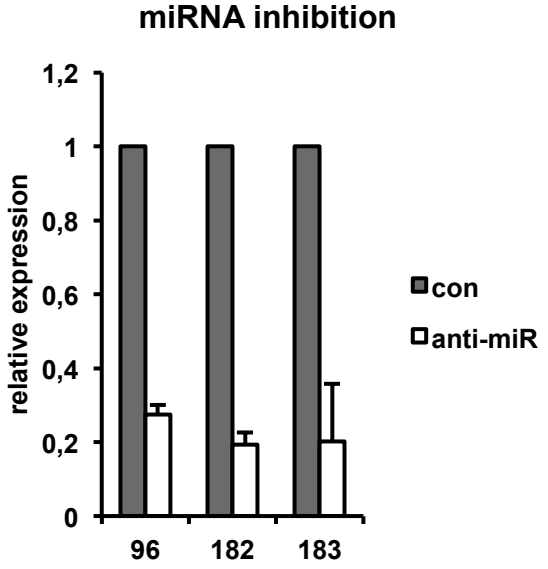

Figure 21 | qRT-PCR (quantitative reverse transcription polymerase chain reaction) of microRNAs in L428 cells transfected with either the specific anti-miR (anti-miR-96, anti-miR-182 or anti-miR-183, respectively) or negative control (con). Relative expression of miR-96, miR-182 and miR-183 48 hours after transfection. U6 RNA served as reference. All experiments were performed in at least triplicates. The data was analyzed using comparative C_t method. Expression was normalized to the negative control. The bars represent mean ± SD (standard deviation). In all three cases, miR expression levels were reduced by approximately 70-80%, which indicates that the anti-miRs used were very effective. miR (microRNA). Republished (modified) with permission of the American Society of Hematology from L Xie, A Ushmorov, F Leithäuser, H Guan, C Steidl, J Färbinger, C Pelzer, M J Vogel, H J Maier, R D Gascoyne, P Möller, and T Wirth. (2012). FOXO1 is a tumor suppressor in classical Hodgkin lymphoma. *Blood*, *119*(15), 3503–3511.; permission conveyed through Copyright Clearance Center, Inc. License Number: 3950240393740.

After 48 hours, transfected cells were harvested and processed for immunoblot. A 2-3-fold increase in FOXO1 protein upon anti-miR-transfection could be measured in comparison to control-transfection (Figure 22).

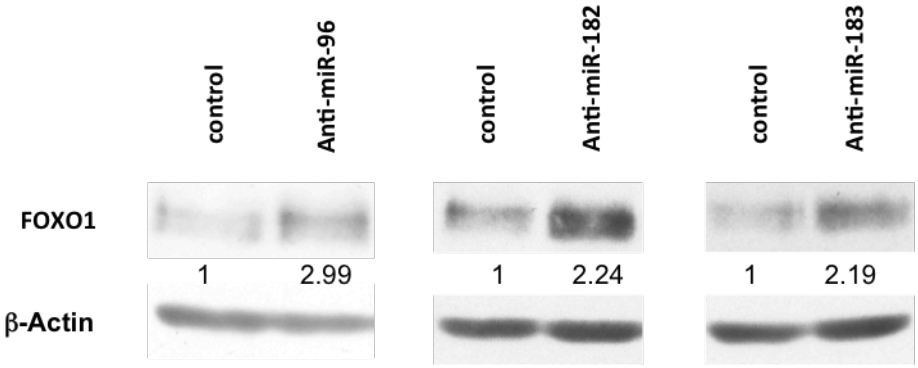

Figure 22 | Immunoblot of FOXO1 protein of L428 cells transfected with either an anti-miR (anti-micro-RNA) or negative control. Cells were harvested 48 hours after transfection. Image analysis was performed with the help of Image J 64 software (http://imagej.nih.gov/ij/). β-Actin was used as loading control. Upon treatment with one of the three anti-miRs levels of FOXO1 protein more than doubled in each case. In turn, miR-96, miR-182 and miR-183 seem to have a repressing effect on FOXO1 protein levels. Republished (modified) with permission of the American Society of Hematology from L Xie, A Ushmorov, F Leithäuser, H Guan, C Steidl, J Färbinger, C Pelzer, M J Vogel, H J Maier, R D Gascoyne, P Möller, and T Wirth. (2012). FOXO1 is a tumor suppressor in classical Hodgkin lymphoma. *Blood*, *119*(15), 3503–3511.; permission conveyed through Copyright Clearance Center, Inc. License Number: 3950240393740.

3.5 Inhibition of JAK/STAT, NF-κB, PI3K/AKT and MAPK/ERK signaling affects miR-183/96/182 expression

In the attempt of finding pathways influencing the miRNAs repressing FOXO1 in cHL, we looked at different, very important pathways in cHL. Constitutively active pathways are the JAK/STAT and NF-κB pathway (Küppers et al. 2012). Other deregulated pathways include the PI3K/AKT as well as the MAPK/ERK pathway (Dutton et al. 2005; B. Zheng et al. 2003)]. Therefore we investigated the effect of specific inhibitors interfering with JAK/STAT, NF-κB, PI3K/AKT and MAPK/ERK signaling on the expression of miR-96, miR-182 and miR-183, respectively.

3.5.1 Inhibiting JAK2 pathway increases miR-183/96/182 levels

Validation of the efficiency of JAK2 Inhibitor was performed indirectly by measuring protein levels of JAK2 targets (Figure 23). As expected, pSTAT3 and pSTAT5 were decreased upon treatment with TG101348, whereas their non-phosphorylated counterparts were not affected. This is due to the fact, that JAK2 Kinase phosphorylates STAT proteins, which in turn dimerize and activate transcription. Hence the name STAT, signal transducer and activator of transcription. When JAK2 is inhibited, fewer STAT proteins become phosphorylated, which can be observed in the immunoblots below.

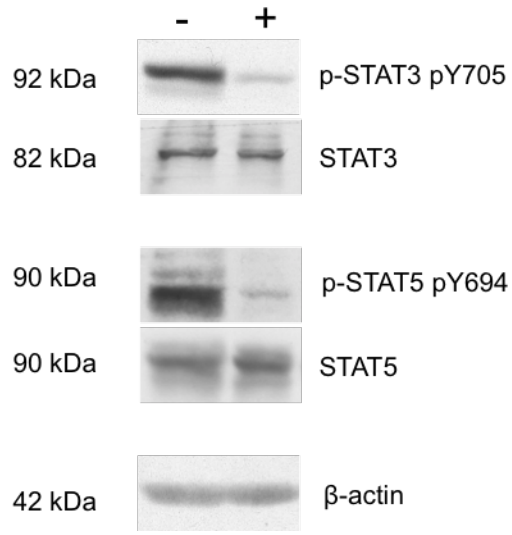

Figure 23 | Immunoblot of STAT3, p-STAT3, STAT5 and p-STAT5 protein of L428 cells incubated with the JAK2 (janus kinase 2) inhibitor TG101348 (2 µM) (+) for 48 hours. DMSO (Dimethyl sulfoxide) treatment was used as control (-). β-Actin was used as loading control. Treatment with TG101348 does not affect the levels of unphosphorylated STAT3/5 protein but reduces the protein levels of their phosphorylated counterparts. kDa (kilo-Dalton), µM (micromolar), STAT3 (signal transducer and activator of transcription 3), STAT5 (signal transducer and activator of transcription 5), p-STAT3 (phosphorylated signal transducer and activator of transcription 3), p-STAT5 (signal transducer and activator of transcription 5).

Interfering with the JAK2 pathway leads to an up to 3-fold increase of miR-96/183/182 in all 5 tested cell lines (Figure 24). The lowest effect was observed in the U-HO1 cell line. In this cell line inhibiting JAK2 does not have an effect on miR-96 expression and only leads to a slight increase in expression of miR-182 and miR-183.

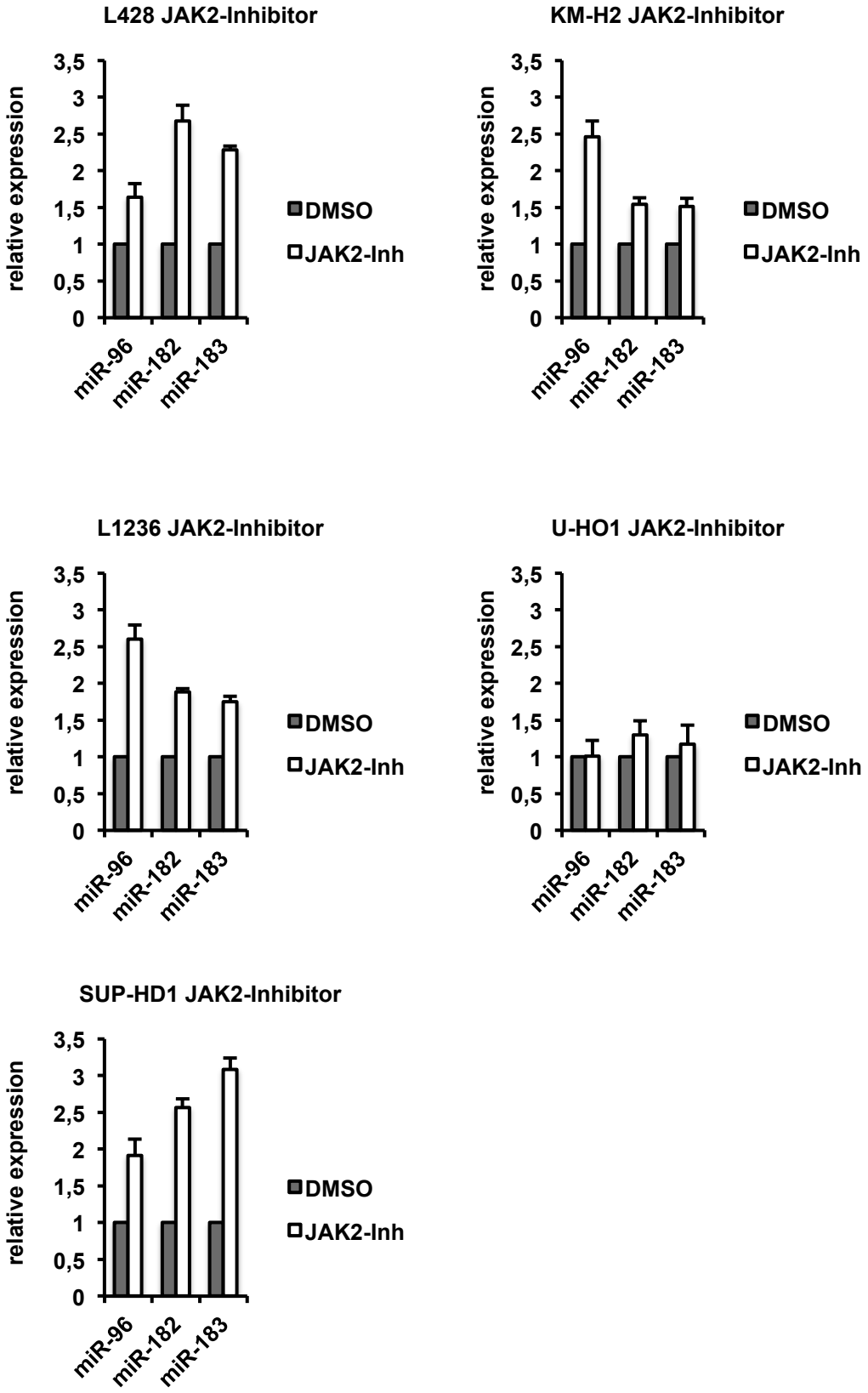

Figure 24 | qRT-PCR (quantitative reverse transcription polymerase chain reaction) of miR-96, miR-182 and miR-183 in 5 different cHL (classical Hodgkin Lymphoma) cell lines that were treated with the JAK2 inhibitor (JAK2-Inh, Janus kinase 2 inhibitor) TG101348 (2 µM) for 48 hours. DMSO

(Dimethyl sulfoxide) treatment was used as control. U6 RNA served as reference. All experiments were performed in at least triplicates. The data was analyzed using comparative C_t method. Expression was normalized to the DMSO control. The bars represent mean ± SD (standard deviation). Upon JAK2 inhibitor treatment levels of the 3 tested microRNAs miR-96, miR-182 and miR-183 increased in all 5 cell lines up to 3-fold. The lowest effect, however, was observed in U-HO1. In fact, in this cell line no significant effect was seen for miR-96. µM (micromolar).

3.5.2 NF-κB Inhibition might not affect the expression of miR-183/96/182

To investigate the role of NF-κB in the regulation of miRNA expression, we used a cHL cell line, KM-H2, stably transfected with a pRTS-1 vector, containing a conditionally regulated IκBα mutant. This cell line was kindly provided by K. Klapproth, a former member of our team. Upon stimulation with doxycycline (0.5 µg/mL), IκBα was expressed, inhibiting NF-κB.

We monitored these effects to validate the efficiency of the vector system by three different experiments. Firstly, we measured the expression of GPF, a direct marker of doxycycline stimulation efficiency by flow cytometry (data not shown). If more than 95% of cells showed GPF expression, the cells were harvested and further processed. Secondly, we analyzed IκBα protein expression using immunoblot experiments (Figure 25). We were able to show, that only the cell line with the vector containing the transgene showed high levels of IκBα, indicating that the system is working well. Thirdly, we investigated the expression of NF-κB target genes, namely c-Myc and CCL5 (Figure 26). When IκBα is induced, it is expected to inhibit NF-κB and therefore fewer NF-κB target genes would be expressed. We observed that upon IkBα activation the expression of c-Myc and CCL5 is in deed downregulated. However, even in cells expressing the empty vector, treatment with Doxycycline inhibited the expression of c-Myc and CCL5, therefore Doxycycline itself seems to have a negative effect on the expression of the two NF-κB target genes.

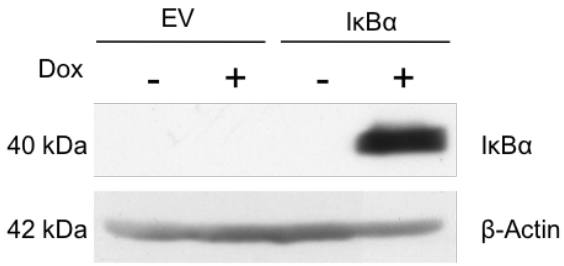

Figure 25 | Immunoblot of IκBα protein in unstimulated (-) and doxycycline-stimulated (0.5µg/ml) (+) pRTS-1 empty vector (EV) and pRTS-IκBα (IκBα) bearing KMH2 cells. β-Actin was used as loading control. Only the cells bearing the pRTS-IκBα vector treated with doxycycline showed high levels of IκBα protein. This shows that the conditional expression of IκBα is working well. Dox (Doxycycline), IκBα (inhibitor of nuclear factor kappa-light-chain-enhancer of activated B-cells α), kDA (kilo-Dalton), µg (microgram), ml (milliliter) pRTS-IκBα (pRTS-1 vector, containing a conditionally regulated IκBα mutant expressed upon treatment with doxycycline).

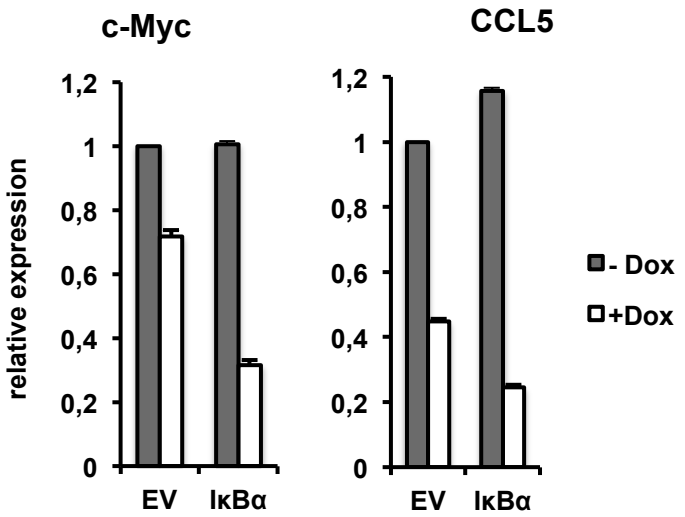

Figure 26 | qRT-PCR (quantitative reverse transcription polymerase chain reaction) showing the expression of NF-κB target genes c-Myc and CCL5. Unstimulated (-Dox) and doxycycline-stimulated (0.5 µg/mL) (+Dox) pRTS-1 empty vector (EV) and pRTS-IκBα (IκBα) bearing KMH2 cells were used. RPL13a mRNA (messenger RNA) served as reference. All experiments were performed in at least triplicates. The data was analyzed using comparative C_t method. Expression was normalized to the unstimulated EV control. The bars represent mean ± SD (standard deviation). When IκBα is induced, significantly less NF-κB target genes (c-Myc and CCL5) are expressed. However, a suppressive effect on NF-κB target gene expression can also be seen in the EV system upon Doxycycline treatment, however not as strong as when IκBα is induced. This shows that Doxycycline itself has a negative influence on the expression of c-Myc and CCL5. CCL5 (C-C-motif ligand 5), c-Myc (V-Myc Myelocytomatosis viral oncogene homolog), IκBα (inhibitor of NF-κB α), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells), RPL13a (ribosomal protein L13a), pRTS-IκBα (pRTS-1 vector, containing a conditionally regulated IκBα mutant expressed upon treatment with doxycycline).

Lastly, we monitored the expression of miR-96, miR-182 and miR-183 upon Doxycycline treatment (Figure 27). Intriguingly, the expression of these miRNAs is apparently not affected by inhibiting NF-κB, but seemingly Doxycycline has an elevating effect on their expression, showing approximately the same levels of miRNA expression upon Doxycycline treatment in both empty vector and pRTS-IκBα bearing cells. However, the expression of the 3 miRNAs was already lower in the cells bearing the pRTS-IκBα vector and therefore the relative increase in expression of all 3 miRNAs when IκBα was induced by Doxycycline was higher compared to the relative increase obtained by Doxycycline application in the EV system when no IκBα was induced.

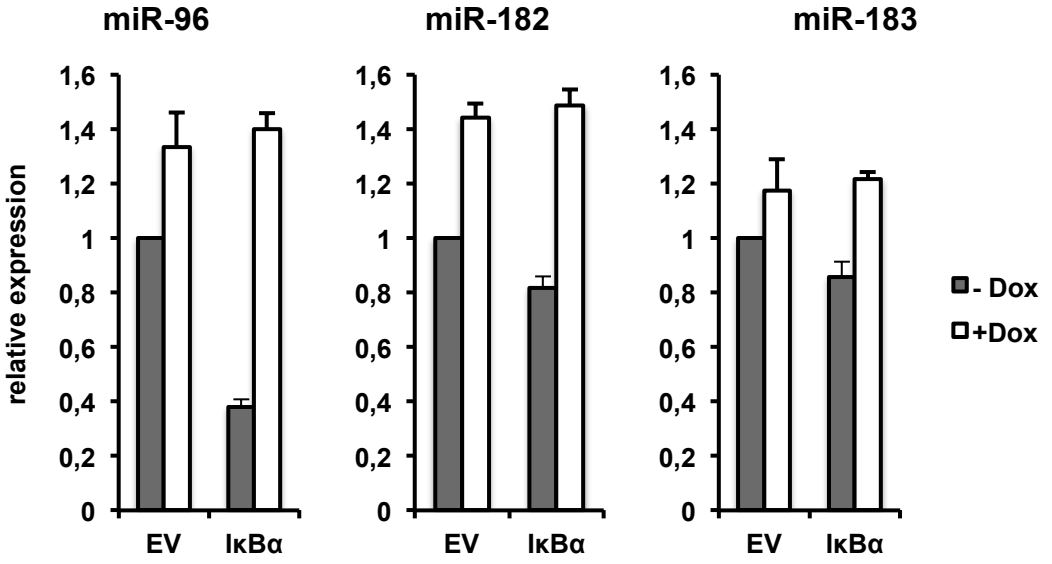

Figure 27 | qRT-PCR (quantitative reverse transcription polymerase chain reaction) showing the expression of miR-96, miR-182 and miR-183 upon IκBα expression. Unstimulated (-Dox) and doxycycline-stimulated (0.5 µg/mL) (+Dox) pRTS-1 empty vector (EV) and pRTS-IκBα (IκBα) bearing KMH2 cells were used. U6 RNA served as reference. All experiments were performed in at least triplicates. The data was analyzed using comparative C_t method. Expression was normalized to the unstimulated EV control. The bars represent mean ± SD (standard deviation). Interestingly, the expression levels of all 3 microRNAs are approximately the same in the EV and pRTS-IκBα system when Doxycycline was applied. However, since the expression levels were already lower in the pRTS-IκBα system, the relative increase in microRNA expression was actually higher when IκBα was induced by Doxycycline compared to the relative increase induced by Doxycycline in the EV system when no IκBα was induced. IκBα (inhibitor of NF-κB α), NF-κB (nuclear factor kappa-light-chain-enhancer of activated B-cells), pRTS-IκBα (pRTS-1 vector, containing a conditionally regulated IκBα mutant expressed upon treatment with Doxycycline).

3.5.3 Inhibiting the AKT pathway decreases miR-96/183/182 levels

For AKT inhibition we used an AKT small molecule inhibitor, KP372-1, that inhibits the kinase activities of AKT, PDK1 and FLT3. After the application time of 24 hours we harvested the cells. Immunoblot analysis shows that AKT itself is not reduced but in turn its phosphorylated (active) counterpart is (Figure 28). We used an antibody detecting the version of AKT that is phosphorylated at threonine 308, the amino acid that is phosphorylated by PDK1. This leads to the conclusion, that the AKT inhibitor is functional.

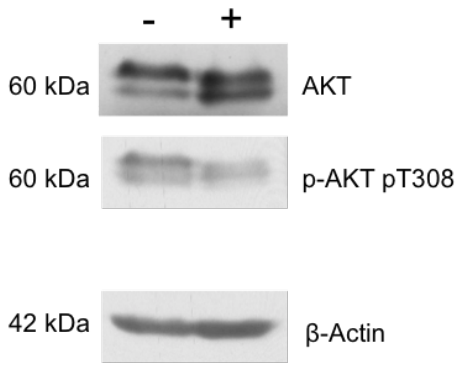

Figure 28 | Immunoblot of AKT (Protein kinase B) and p-AKT (phosphorylated Protein kinase B) protein in L428 cells after 24 hours of incubation with the AKT inhibitor KP372-1 (0.4 µM) (+). DMSO (Dimethyl sulfoxide) treatment was used as control (-). β-Actin was used as loading control. We observed a decrease in p-AKT protein, the active version of AKT, when KP372-1 was applied, so the AKT inhibitor is shown to be functional. kDA (kilo-Dalton), pT308 (phosphorylated threonine 308).

Subsequently, we investigated miR-96/183/182 expression while inhibiting AKT (Figure 29). Strikingly, levels of all three miRNA were reduced between approximately 60 - 80%, with the highest effect seen for miR-96.

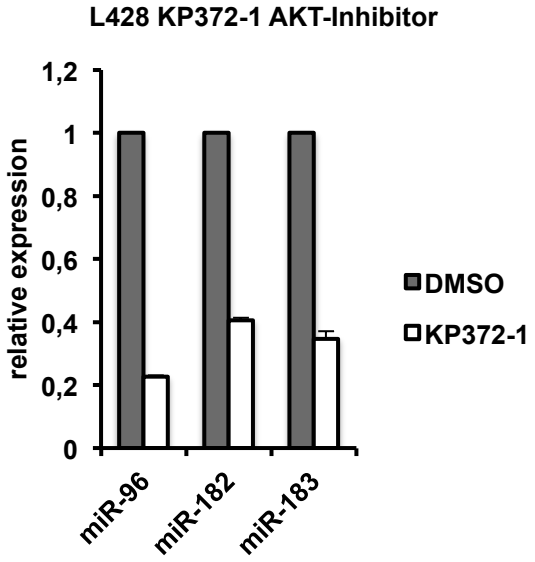

Figure 29 | qRT-PCR (quantitative reverse transcription polymerase chain reaction) showing the expression of miR-96, miR-182 and miR-183 in L428 cells upon 24 hours of treatment with the AKT (Protein kinase B) inhibitor KP372-1 (0.4 µM). DMSO (Dimethyl sulfoxide) treatment was used as control. U6 RNA served as reference. All experiments were performed in at least triplicates. The data was analyzed using comparative C_t method. Expression was normalized to the DMSO control. The bars represent mean ± SD (standard deviation). For all three microRNAs the relative expression decreased between approximately 60 and 80% when AKT was inhibited. miR (microRNA).

3.5.4 Inhibiting MEK pathway decreases miR-96/183/182 levels

We inhibited the MEK/ERK pathway by applying a MEK1/2 inhibitor, U0126, for 24 hours. The inhibition of MEK leads to a decrease in phosphorylated (functionally active) ERK1/2 (Figure 30). For immunoblotting, we used an antibody that can detect ERK1 and ERK2 phosphorylated at the MEK phosphorylation sites threonine 202 and tyrosine 204. We saw no signal at all after the application of U0126, while ERK2 was still expressed, suggesting that the inhibitor is highly effective.

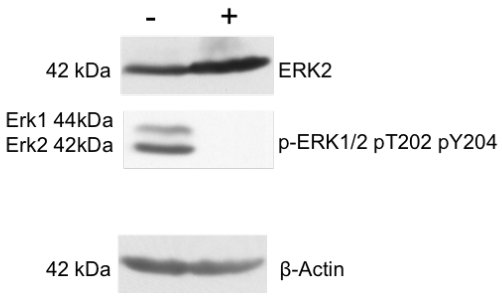

Figure 30 | Immunoblot of ERK2 and p-ERK1/2 protein in L428 cells after 24 hours of incubation with the MEK1/2 inhibitor U0126 (18 µM) (+). DMSO (Dimethyl sulfoxide) treatment was used as control (-). β-Actin was used as loading control. When MEK 1/2 was inhibited, the protein expression of p-ERK 1/2 expression vanished, while ERK2 was still expressed, suggesting that U0126 is highly effective. ERK (mitogen-activated protein kinase / extracellular signal-regulated kinase), kDa (kilo-Dalton), MEK (Mitogen-activated protein kinase kinase), pT202 (phosphorylated threonine 202), pY204 (phosphorylated tyrosine 204).

The examination of miRNA levels showed that inhibiting the MEK/ERK pathway also leads to a repression of miR-96, miR-182 and miR-183 of up to 84%. The strongest effect was seen for miR-96. (Figure 31).

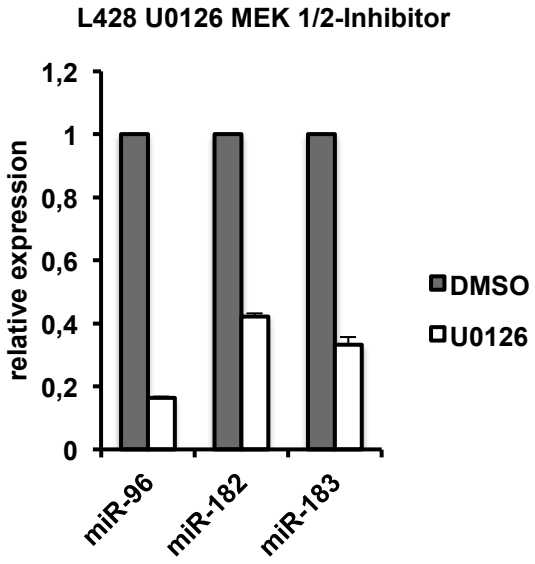

Figure 31 | qRT-PCR (quantitative reverse transcription polymerase chain reaction) showing the expression of miR-96, miR-182 and miR-183 in L428 cells after 24 hours of treatment with the MEK1/2 inhibitor U0126 (18 µM). DMSO (Dimethyl sulfoxide) treatment was used as control. U6 RNA served as reference. All experiments were performed in at least triplicates. The data was analyzed using comparative C_t method. Expression was normalized to the DMSO control. The bars represent mean ± SD (standard deviation). In all three cases the MEK1/2 inhibitor led to a notable decrease in the expression of the three miRNAs. MEK 1/2 (Mitogen-activated protein kinase kinase 1/2), miR (microRNA), µM (micromolar).

4 Discussion

Every type of cancer has its very own alterations from the normal cellular conditions ultimately leading to its oncogenic transformation (Croce 2008). In order to be able to treat cancer, it is essential to understand the oncogenic program of a particular tumor. This has been and probably will be a very challenging assignment for a long time, since studies have to be performed looking at each individual type of cancer as well as its subgroups. In other words, personalized medicine will become more and more relevant as we learn more about pathomechanisms and therefore provide increasingly more treatment options.
Despite the very high cure rates of classical Hodgkin Lymphoma, there are still 15% of patients that fail to respond to treatment so that searching for new therapeutic options for HL is still critical (Moskowitz 2012). Those that could be treated successfully, frequently suffer from secondary neoplasias as a result of the still very aggressive drugs that do not act in a very tumor specific manner and hence also harm normal cells (Eichenauer et al. 2013). Thus, investigating pathomechanisms in cHL is not only meant to find therapy options for the yet untreatable patients, but also to reduce chemotoxicity of the current therapies.
Our group identified FOXO1 as a tumor suppressor in classical Hodgkin Lymphoma (Xie et al. 2012). We were able to show that re-expressing FOXO1 leads to apoptosis and cell cycle arrest. FOXO1 was shown to play an essential role in B cell death and its inactivation by B cell receptor signaling was reported to be crucial for B cell survival (Srinivasan et al. 2009). FOXO1 repression hence contributes to the malignant transformation of B cells by promoting survival.

4.1 Differential expression of miR-96, -155, -182 and -183 in classical Hodgkin Lymphoma

We have shown that miR-96, miR-155, miR-182 and miR-183 are specifically upregulated in cHL. In case of miR-27a the differences of expression in normal B cells / NHL cell lines and cHL cell lines was not as conclusive as for the other three miRNAs. Only the cHL cell line U-HO1 showed increased levels of miR-27a.

Subsequently, no further functional experiments were performed concerning this microRNA. MicroRNA-155 was previously shown to be upregulated in cHL and has been termed OncomiR in the literature (Czyzyk-Krzeska and Zhang 2014; Gibcus et al. 2009). As FOXO1 3´UTR also shows a predicted target site for miR-155, we included this miRNA in our investigations on FOXO1 regulation, although this miRNA has not been linked to FOXO1 regulation before and does not display a good mirSVR score in the target prediction program miRanda. Given that miR-27a is not upregulated in cHL but has been reported to regulate FOXO1 expression in breast cancer cells (Guttilla and White 2009), these findings may indicate that FOXO1 is regulated by different miRNAs depending on the type of tumor and even its very specific subtype as different studies show different miRNA regulations in different (tumor) cell types. Also according to several prediction programs, there is a plethora of potential miRNAs possibly targeting FOXO1 3´UTR that have not yet been investigated. The common denominator, however, seems to be the cluster of miR-96, miR-182 and mir-183 (Guttilla and White 2009; McLoughlin et al. 2014; Myatt et al. 2010; Stittrich et al. 2010).

4.2 miR-96, -182 and -183 are involved in FOXO1 repression in classical Hodgkin Lymphoma cell lines

We were able to show that miR-96, miR-182 and miR-183 are able to repress FOXO1. Functional experiments using a Luciferase Reporter Assay showed that those three miRNAs, but not miR-155, were able to reduce the reporter activity by about 40%, indirectly showing that they act at the 3´UTR of FOXO1 mRNA and subsequently reduce its translation. Interestingly, miR-96 and miR-182 share the same target sites, with miR-96 having the better mirSVR scores (miRanda) and the larger seed sequences (Betel et al. 2010). The question therefore arises whether miR-96 or miR-182 are responsible for these effects. Knock-down experiments using artificial, commercially available anti-miRs showed that both miR-96 and miR-182 seem to be targeting the FOXO1 3´UTR. The same applies for miR-183. Yet, other, more indirect mechanism that lead to an elevation of FOXO1 protein upon miRNA-knock-down, cannot be excluded, as miRNAs have plentiful targets.

Recently it was reported that only one of the miR-183 sites in FOXO1 3´UTR is functional, specifically the second site, unique to humans, but not the other, conserved site (McLoughlin et al. 2014). As shown in Figure 32, the human specific miRNA recognition element (MRE) for miR-183 of the FOXO1 3´UTR differs from chimpanzee and murine sequences by only one nucleotide.

Figure 32 | Comparison of FOXO1 3´UTR (3´ untranslatedregion) in humans (hsa), chimpanzees (pan) and mice (mmu). Red nucleotides on the left indicate the conserved miR-183 miRNA recognition element (MRE) and their corresponding miR-183 sequence. Red nucleotides on the right indicate the human specific miR-183 MRE and their corresponding miR-183 sequence. The nucleotides differing between the species are shown in a grey box. Only the human specific MRE is functional. McLoughlin, H. S., Wan, J., Spengler, R. M., Xing, Y., & Davidson, B. L. (2014). Human-specific microRNA regulation of FOXO1: implications for microRNA recognition element evolution. *Human Molecular Genetics*, *23*(10), 2593–2603., by permission of Oxford University Press. Copyright Clearance Center License Number: 3791950029232.

4.3 JAK2 pathway may inhibit miR-96/182/183 expression

The JAK2 pathway is constitutively active in classical Hodgkin Lymphoma. Several mechanisms were identified such as JAK2 gene amplifications and stimulatory loops by Interleukin-13 (IL-13) and its receptor IL13Rα1 (Meier et al. 2009). In this study we show that the levels of miR-96, miR-182 and miR-183 increase when cells are treated with a JAK2 inhibitor. Interestingly, the effect was the lowest in U-HO1, the cell line in which the highest levels of miR-96/183/182 were detected among all cHL cell lines tested. This could possibly account for the low increase of the three miRNAs in this cell line upon JAK2 inhibitor treatment as these miRNAs might already be upregulated to a certain maximum. One could speculate, that therefore inhibiting the JAK2 pathway is not a suitable therapy option. Controversially, it was shown that JAK2 inhibition actually decreases proliferation of cHL cell lines and induces apoptosis (Hao et al. 2014). In a murine xenograft model of cHL JAK2 inhibition led to a decrease in tumor growth and prolonged

survival (Hao et al. 2014). Even a phase I study was published, demonstrating that the JAK2 inhibitor, SB1518, is able to decrease tumor size in relapsed or refractory cHL patients at a good tolerability (Younes et al. 2012). This emphasizes the complexity that has to be considered when it comes to choosing appropriate therapeutic targets. In this study, JAK2 inhibitors would have seemed to have a negative treatment effect when used as therapeutics by increasing those miRNAs responsible for lowering FOXO1 levels, a key regulator of apoptosis. The overall effect of such inhibitors, however, seems to be the opposite (growth inhibition).

Strikingly, as discussed in chapter 4.5, AKT and ERK pathways seem to stimulate miR-96/182/183 expression, although the JAK2 pathway also activates these two pathways (Figure 12). Hence, the effect of STAT signaling seems to overcome the signaling of the other two pathways, resulting in an overall increase in these miRNAs upon JAK2 inhibition.

4.4 The NF-κB pathway may or may not affect the expression of miR-96/182/183 in cHL

Multiple studies have shown the importance of NF-κB signaling for the maintenance of the HL phenotype (Bargou et al. 1996; 1997; Hinz et al. 2002; Krappmann et al. 1999). In fact, the consitutive NF-κB activation in HL was one of the first reports to link deregulated NF-κB activity to cancer (Bargou et al. 1996; Nagel et al. 2014). NFkB signaling in HL accounts for an anti-apoptotic and pro-proliferative genetic program, promoting tumor growth and cell survival (Nagel et al. 2014). For this reason, we investigated the effect of NF-κB signaling on miR-96/182/183 expression using a cHL cell line expressing the conditional transgene IkBα (inhibitor of NF-kB α). Intriguingly, miRNA expression was already lower in those cells bearing the vector with the transgene IkBα compared to the cells bearing the empty vector, even without stimulation. Treatment with Doxycycline, in order to activate transgene expression, led to an increase in expression of all three tested miRNAs to about the same expression level, regardless of the vector the cells contained. Therefore we propose a supportive effect of Doxycycline on the expression the miR-183/96/182 cluster, rather than by inhibiting the NFkB pathway. However, compared to the unstimulated vector bearing cells, the relative

increase in microRNA expression upon Doxycycline stimulation when IkBα was induced (pRTS- IkBα) was higher than when no IkBα was induced by Doxycycline stimulation (EV). This finding could also point to an involvement of NFkB in the regulation of the miR-183/96/182 cluster. Yet, a clear statement about the involvement of NFkB in miR-96/182/183 expression cannot be made, as this model was influenced by Doxycycline too much.

4.5 Constitutively active AKT and ERK pathways may promote miR-96/182/183 expression

The PI3K/AKT and MEK/ERK pathway are deregulated and consitutively active in HL (Dutton et al. 2005; Zheng et al. 2003). We investigated the effect of inhibiting those pathways on miR-183/96/182 expression in cHL cell lines. The inhibition of these pathways showed a decrease in miRNA expression up to roughly 80%, respectively. Thus, we propose that these pathways are supporting miR-183/96/182 expression in some way. However, only inhibiting experiments were performed, as those pathways are consitutively active in HL *a priori*. One could argue that inhibiting the AKT or ERK pathway leads to cell cycle arrest and apoptosis, thereby potentially generally decreasing gene expression. However, when inhibiting the JAK2 pathway, which also leads to growth arrest and apoptosis, miR-183/96/182 expression was even increased, suggesting that this theory does not apply.

The findings in our study could mean that both pathways are not only crucial for survival by FOXO1 phosphorylation (and thereby conducting its nuclear export), but also by miRNA-regulated FOXO1 repression (Asada et al. 2007; Brunet et al. 1999). When searching the literature for possible connections between the AKT pathway and miR-183/96/182 cluster expression, one comes across a publication reporting a functional p53 response element in the miR-183 promoter region (Chang et al. 2011). AKT negatively regulates p53, hence, AKT inhibition leads to increased p53 action. In this case, miR-183/96/182 expression should theoretically increase if the regulation by p53 applies for cHL cell lines. In our experiments we observed the opposite, suggesting another regulatory mechanism than p53. Albeit, several other transcriptional regulators have been identified, as well as epigenetic regulation by DNA methylation (Dambal et al. 2015). FOXP3, an AKT-mediated

transcription factor, has been reported to regulate miR-183 expression in human leukemia cells (Liu and Chang 2012). AKT inhibiton led to a downregulation of FOXP3, which in turn downregulated miR-183. These findings are in line with our study. The role of FOXP3 in transcriptional regulation of miR-183/96/182 in cHL, however, has not yet been elucidated. Interestingly, FOXP3 also mediates the expression of the oncomiR miR-155 (Kohlhaas et al. 2009; Lu et al. 2009).

The effect we see upon MEK/ERK inhibition is probably caused by another mechanism, because apparently inhibiting MEK/ERK inhibition leads to an upregulation of FOXP3, at least in T cells (Gabryšová et al. 2011). It has to be kept in mind, that we used a MEK1/2 inhibitor. The only known substrates of MEK1/2 are ERK1/2 and therefore this kind of inhibitor is suitable for ERK inhibition purposes. However, off target effects such as reduced calcium influx and inhibition of mitochondrial respiration have been reported for the MEK1/2 inhibitor U0126 (Ripple et al. 2013; Wauson et al. 2013). In case of the ERK pathway, a possible link to miR-183/96/182 transcriptional regulation could be SMAD2, as this protein has been shown to regulate miR-182 transcription (Song et al. 2012). The cross-talk between the ERK and SMAD pathway has been described repeatedly (Blanchette et al. 2001; Burch et al. 2010; Hayashida et al. 2003; Hough et al. 2012).

An overview of the transcriptional regulators of the miR-183/96/182 cluster known so far can be found in (Dambal et al. 2015).

As the effects seen upon AKT or MEK inhibition appear to be so similar, a common regulatory mechanism of miR-183/96/182 expression is also imaginable since the two pathways are crosstalking (Mendoza et al. 2011). The exact pathway, however, remains to be examined.

4.6 The EBV status might have an impact on miRNA expression in cHL

It must be considered that all five cHL cell lines that were used in this study had a negative EBV status. A French-German research group very recently (data published December 2015) discovered a link between EBV-expressed latent membrane protein 1 (LMP1) and the downregulation of the miR-183/96/182 cluster

in Burkitt Lymphoma (BL), involving the AKT pathway (Oussaief et al. 2015). EBV positive HRS cells display high levels of LMP1, suggesting an important role in pathogenesis (Flavell and Murray 2000). EBV positive cases can be found in about 40% of HL (Jarrett et al. 1996). EBV itself expresses miRNAs and is able to modulate cellular miRNA expression, therefore the data obtained from EBV negative cell lines has to be considered with caution and potentially separate from EBV positive cases. It is possible that FOXO1 repression by the miR-183/96/182 cluster might not apply for EBV positive HL cases and that in these cases other mechanism of FOXO1 repression could predominate.

The same study has also shown that in BL cell lines LMP1 induces miR-155, a well-known oncomiR, which we also investigated, but that had no relevance in FOXO1 repression (Oussaief et al. 2015). The connection between miR-155 and EBV has been made before in latently infected B lymphocytes (Yin et al. 2008). The mechanism was identified to be transcriptional activation of the miR-155 promoter by NF-κB, that in turn is activated by LMP1 (Gatto et al. 2008). It was also reported that miR-155 plays a key role in immortalization of B cells by EBV, as those cells lacking miR-155 undergo apoptosis (Linnstaedt et al. 2010). The oncogenic effects of miR-155 were discovered in Eμ-mmu-miR155 transgenic mice. Those mice developed lymphoproliferative disease indicating an important role of miR-155 in disease initiation and/or progression (Costinean et al. 2006).

The data discussed above was obtained in Burkitt Lymphoma cell lines and B lymphocytes. Its relevance in HL remains to be validated.

4.7 The miR-183/96/182 cluster: Oncogenes or Tumor suppressors?

Despite the role of the miR-183/96/182 cluster in normal development, especially in sensory organs such as eye and ear, as well as in the regulation of circadian rhythm, it has also been attributed roles in diseases such as autoimmunity, neuronal pathologies and cancer (Dambal et al. 2015). Here we focus on its role in human malignancies.

In the last couple of years the cluster of the miR-183/96/182 has been investigated extensively, producing an impressive heterogeneity of results. It appears that this

microRNA cluster is commonly upregulated in cancer, pointing to an oncogenic function (Table 12). However, multiple studies have also shown roles as tumor suppressors, even within the same cancer entity (Table 13).

One outstandig example is breast cancer. It was found that miR-96, and in case of FOXO1 also miR-182, provide a growth advantage by downregulation of FOXO3a and FOXO1 (Guttilla and White 2009; Lin et al. 2010). Furthermore, miR-182 targets MIM (Missing in Metastasis) thereby fostering metastasis (Lei et al. 2014). On the other hand, miR-183 hampers migration of breast cancer cells by targeting Ezrin (Lowery et al. 2010).

Another sweeping example is lung cancer. In most of the studies the function of the miR-183/96/182 cluster was oncomiRic by promoting growth, motility, chemoresistance and invasion (Guo et al. 2014; Ning et al. 2014; M Wang et al. 2014). Additionally, miR-183 was even found to be a risk factor for lung cancer (Zhu et al. 2011). Yet, there have also been reports about tumor suppressive functions of the cluster. MicroRNA-183 was identified as being a potential metastasis inhibitor by targeting Ezrin and miR-182 inhibits cell proliferation and cell growth in lung cancer cell lines by targeting RGS17 (Y. Sun et al. 2010; G. Wang et al. 2008). Conversely, one study showed that the downregulation of miR-182 actually promoted migration and invasion via an increase in N-cadherin expression, although it inhibited cell growth at the same time by decreased targeting of FOXO3 (Yang et al. 2014). These findings could even be correlated to the stages of lung cancer, when in early stages there are high levels of miR-182 expression stimulating tumor growth and proliferation, and in late stages the levels of miR-182 decline, leading to metastasis (Yang et al. 2014).

In conclusion, the diverse effects of the miR-183/96/182 cluster emphasize the target heterogeneity of miRNAs depending on cell type and transcriptional features. A general statement about the oncogenic or tumor suppressive effect cannot be made, although several findings point to a rather oncogenic role of the cluster, as it can be observed in cHL, at least in EBV negative cases.

Table 12 | Relative Expression of the miR-183/96/182 cluster in human cancer compared to healthy tissue. Modified from (Dambal et al. 2015). Dambal, S., Shah, M., Mihelich, B., & Nonn, L. (2015). The microRNA-183 cluster: the family that plays together stays together. *Nucleic Acids Research*, *43*(15), 7173–7188. By permission of Oxford University Press. Copyright Clearance Center License Number: 3791950144795.

miR	Prostate	Breast	Lung	Liver	Brain	Bladder	Retinoblastoma	Colorectal	Pancreatic	Ovarian	Endometrial	Gastric	Thyroid
183	↑	↕	↕	↑	↑	↑	↕	↑	↑	↕	↑	↑	↑
182	↑	↑	↕	↑	↑	↑	↑	↑	↑	↑	↑	↕	↑
96	↑	↑	↑	↑	↑	↑	↑	↑	↓	↑	↑	↑	↑

Table 13 | The role of microRNAs of the miR-183/96/182 cluster in cancer based on publications from 2009-2013. CYLD (CYLD Lysine 83 deubiquitinase), EGR1 (Early growth response protein 1), Ezrin (Cytovillin, Villin-2), FOXO (forkhead box O), KRAS (Kirsten rat sarcoma viral oncogene homolog), PDCD4 (Programmed Cell Death 4 [Neoplastic Transformation Inhibitor]), PTEN (Phosphatase and tensin homolog), RGS17 (Regulator Of G-Protein Signalling 17), ZIP (Zrt- And Irt-Like Protein 1, Zinc/Iron Regulated Transporter-Like), ZnT (Solute Carrier Family 30 Zinc Transporter). Modified from (Li et al. 2014). Open Access CC BY 4.0. https://creativecommons.org/licenses/by/4.0/

miRNA	Oncogene/tumor suppressor	Cancer type	Function	Target genes
miR-96	Oncogene	Hepatocellular carcinoma	Increases proliferation and colony formation	*FOXO1, FOXO3a*
miR-96	Oncogene	Prostate cancer	Inhibits zinc uptake	*ZIP1, ZIP3, ZIP7, ZIP9, ZnT1, ZnT7*
miR-182				
miR-183				
miR-96	Oncogene	Medullo-blastoma	Inhibits apoptosis, destroys DNA repair, promotes cell migration	*See reference*
miR-182				
miR-183				
miR-96	Oncogene	Breast cancer	Induces proliferation	*FOXO3a*
miR-96	Oncogene	Breast cancer	Increases cell number	*FOXO1*
miR-182				
miR-182	Oncogene	Glioma	Promotes glioma cell aggression	*CYLD*
miR-182	Oncogene	Melanoma	Promotes cell migration and survival	*FOXO3*
miR-183	Oncogene	Synovial sarcoma	Promotes tumor cell migration	*EGR1*
				PTEN
miR-183	Oncogene	Hepatocellular carcinoma	Iinhibits TGF-beta1-induced apoptosis	*PDCD4*
miR-96	Tumor suppressor	Pancreatic cancer	Decreases cell invasion, migration and tumor growth	*KRAS*
miR-183	Tumor suppressor	Breast cancer	Inhibits migration	*Ezrin*
miR-183	Tumor suppressor	Osteosarcoma	Inhibits migration and invasion	*Ezrin*
miR-182	Tumor suppressor	Lung cancer	Inhibits cancer cell proliferation	*RGS17*

4.8 Conclusions

FOXO1 is a tumor suppressor in classical Hodgkin Lymphoma. In its function as transcription factor it regulates the transcription of genes involved in cell cycle regulation and apoptosis. Levels of FOXO1 in cHL are low, whereas high levels of FOXO1 could be found in B cells and NHL cell lines. The same was observed in HRS cells of histological specimens. Re-introduction of FOXO1 in cHL cell lines results in cell cycle arrest and apoptosis. This downregulation of FOXO1 in cHL is achieved by several mechanisms such as chromosomal aberrations and constitutively active AKT and ERK pathways.

As a newly emerging group of translational regulators, miRNAs have repeatedly been linked to cancer and other diseases. The regulation of FOXO1 by miRNAs has previously been reported. In this study we show that the miRNAs of the miR-183/96/182 cluster regulate FOXO1 expression in classical Hodgkin Lymphoma. All three miRNAs are upregulated in cHL cell lines. Inhibition of these miRNAs in cHL cell lines increases FOXO1 protein levels. Our data suggests that this cluster is negatively regulated by the JAK/STAT pathway and positively regulated by the AKT and ERK pathway. The latter regulations possibly share a common mechanism. However, it must be considered that the cHL cell lines used in this study were all EBV negative. Another study showed that LMP1, a transmembrane protein induced by EBV, can downregulate the expression of the miR-183/96/182 cluster and therefore our data might only apply for EBV negative cases, which account for approximately 60% of cases.

MicroRNAs have also been reported to be predictors of prognosis, metastasis and drug efficacy, while being measurable in bodily fluids. In case of the miR-183/96/182 cluster in cHL, it is imaginable that at least in their role as negative regulators of FOXO1 expression, they could potentially also have an implication in prognosis. With novel miRNA-targeting drugs on the rise, it is conceivable that targeting the oncomiRic miR-183/96/182 cluster in (EBV-negative) cHL could be beneficial.

5 Summary

Hodgkin Lymphoma (HL) is a B cell derived type of lymphoma with age-peaks in the 3rd and 7th decade of life. Despite its high cure rate of approximately 85%, further research is necessary in order to find new treatment options offering lower toxicity at consistent or improved efficacy. Current treatment approaches are very toxic and lead to secondary neoplasias and other adverse treatment effects. Moreover, there are still 15% of patients that will die from Hodgkin Lymphoma. A very tragic finding when considering that HL is also a disease of the young.

Recently, our team identified FOXO1 to be a tumor suppressor in classical Hodgkin Lymphoma (cHL). Its tumor suppressive activities arise from its function as transcription factor regulating the expression of various genes involved in cell cycle and apoptosis. Our previous studies showed that FOXO1 is downregulated in cHL by diverse mechanisms including dysregulated AKT and ERK signaling, as well as chromosomal aberrations. Re-introduction of FOXO1 leads to cell cycle arrest and apoptosis in cHL cell lines. Within the last couple of years increasing evidence suggested that altered microRNA expression is associated with various disease states, such as cancer. In this study, we asked whether miRNAs are also involved in FOXO1 downregulation in classical Hodgkin Lymphoma and if so, which particular miRNAs are responsible.

We performed *in silico* target prediction for miRNAs targeting FOXO1 3´UTR. In line with the literature, we chose miR-27a, miR-96, miR-182 and miR-183 for further investigation. Additionally, we also found a potential target site for miR-155, a well-known oncomiR, reported to be highly expressed in HL. For this reason, we also investigated this miRNA. Quantitative RT-PCR showed high expression of miR-96, miR-155, miR-182 and miR-183 compared to normal B cells and NHL cell lines. We then performed functional experiments using a luciferase reporter assay to study the effects of those four miRNAs on FOXO1 3´UTR. Our findings suggest the targeting of FOXO1 3´UTR by miR-96/182, sharing the same target site, and miR-183 but not miR-155. To support this finding, we used artificial antimiRs to block miRNA action of miR-96, miR-182 and miR-183, and observed an increase in FOXO1 protein in all three cases. Lastly, we studied potential influences on the expression of these three miRNAs by inhibiting four of the constitutively active

pathways in cHL: the JAK/STAT, NF-κB, PI3K/AKT and ERK pathway. Inhibiting the JAK/STAT pathway led to an increase in miR-96/182/183. For NF-κB inhibition we used an inducible system expressing IκBα upon Doxycycline application. However, doxycycline itself seems to increase miR-96/182/183 expression and no further conclusion could be drawn for NF-κB. Inhibiting the AKT or ERK pathway led to a downregulation of miR-96/182/183 expression by up to 80%, suggesting that these pathways may support the expression of the three miRNAs.

Our findings proof that FOXO1 is repressed by miR-96, miR-182 and 183 in classical Hodgkin Lymphoma. These three miRNAs are located in very close genomic proximity to each other, sharing the same transcription start site and thereby leading to a cooperative repression of the tumor suppressive transcription factor FOXO1. However, it must be kept in mind that our data was obtained from only EBV negative cHL cell lines. Another study has shown that the EBV derived protein LMP1 downregulates the miR-183/96/182 cluster, which in turn could mean that our findings might only apply for EBV negative cases (approx. 60%).

Novel therapies pursue the goal of targeting oncogenic miRNAs. Therefore, the miR-183/96/182 cluster could possibly be a suitable target for new therapy approaches in cHL. Furthermore, miRNAs from bodily fluids seem to bear the potential of being prognostic markers and as the miR-183/96/182 cluster is targeting a tumor suppressor in cHL, it might be possible to serve as such in the future. Nevertheless, further studies are needed to determine the exact implication of these miRNAs in cHL before they can be used as potential therapy targets.

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