

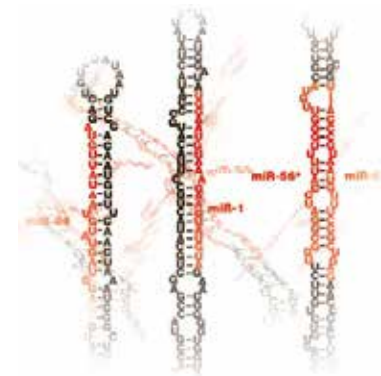
Epigenetics in Hodgkin lymphoma: a non-coding RNA point of view

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1. General epigenetic features of cHL
2. Overview of non-coding RNAs
3. microRNAs regulating crucial pathways of cHL
4. microRNAs regulated by methylation in cHL
5. Conclusions

- 1. General epigenetics features of HL**
2. Generalities of Non-coding RNAs
3. microRNAs regulating HRS cells crucial pathways
4. microRNAs regulated by methylation in HL
5. Conclusions

Classic genetics alone cannot explain the diversity of phenotypes within a population.

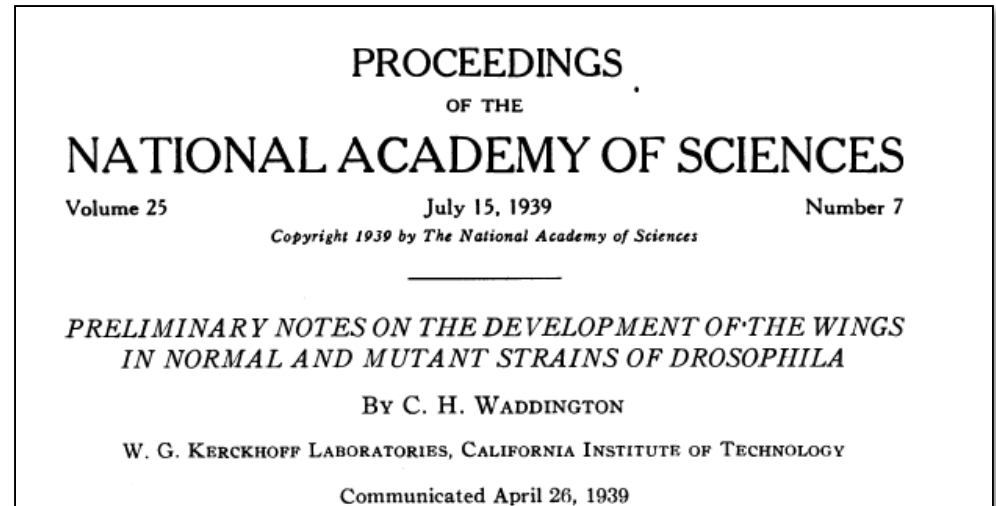


John Cloud, Time, January 06, 2010

1939 à C.H. Waddington

“the causal interactions between genes and their products, which bring the phenotype into being”

Greek, epi = above, upon - genetics

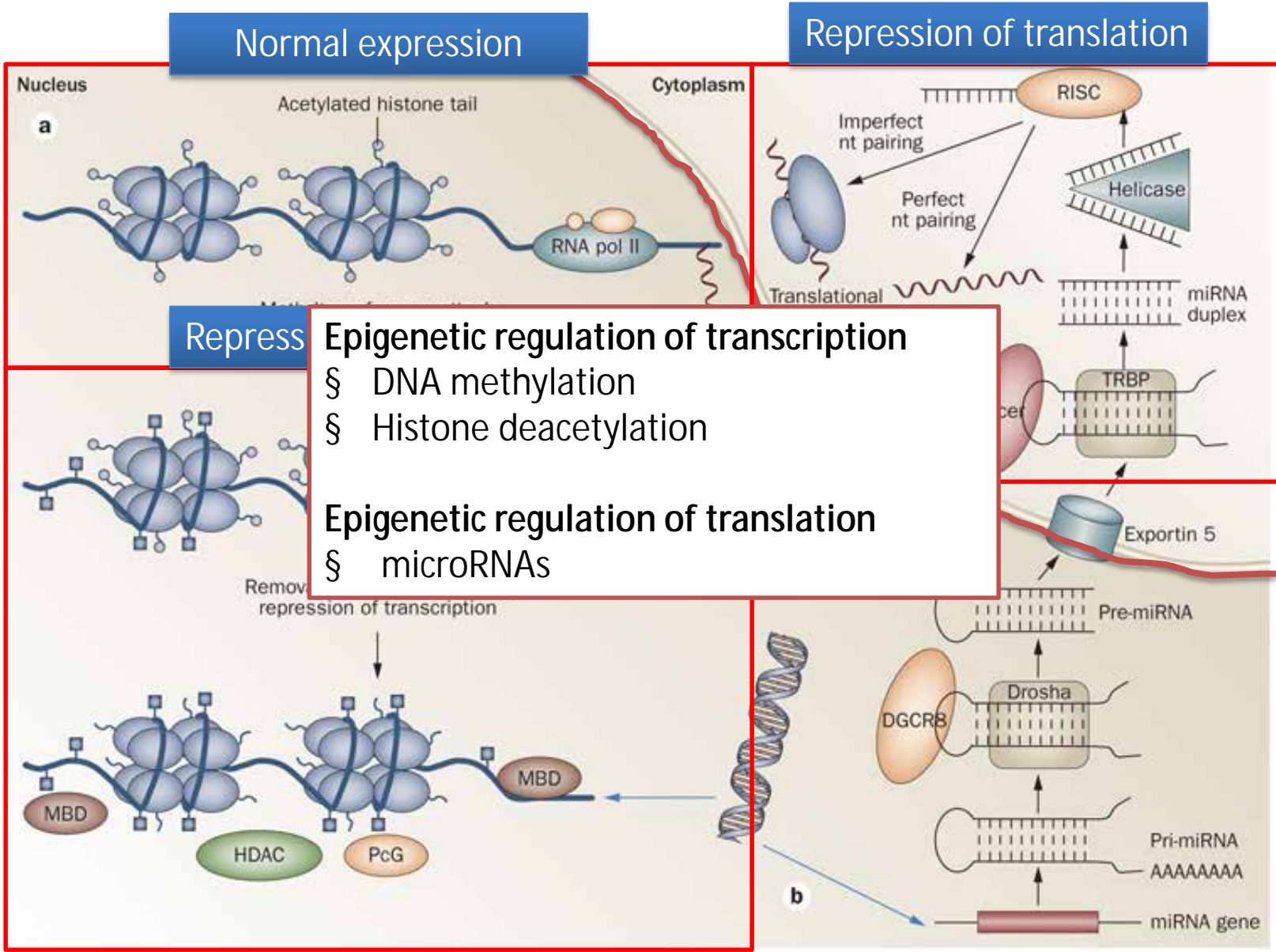


1987 à R. Holliday

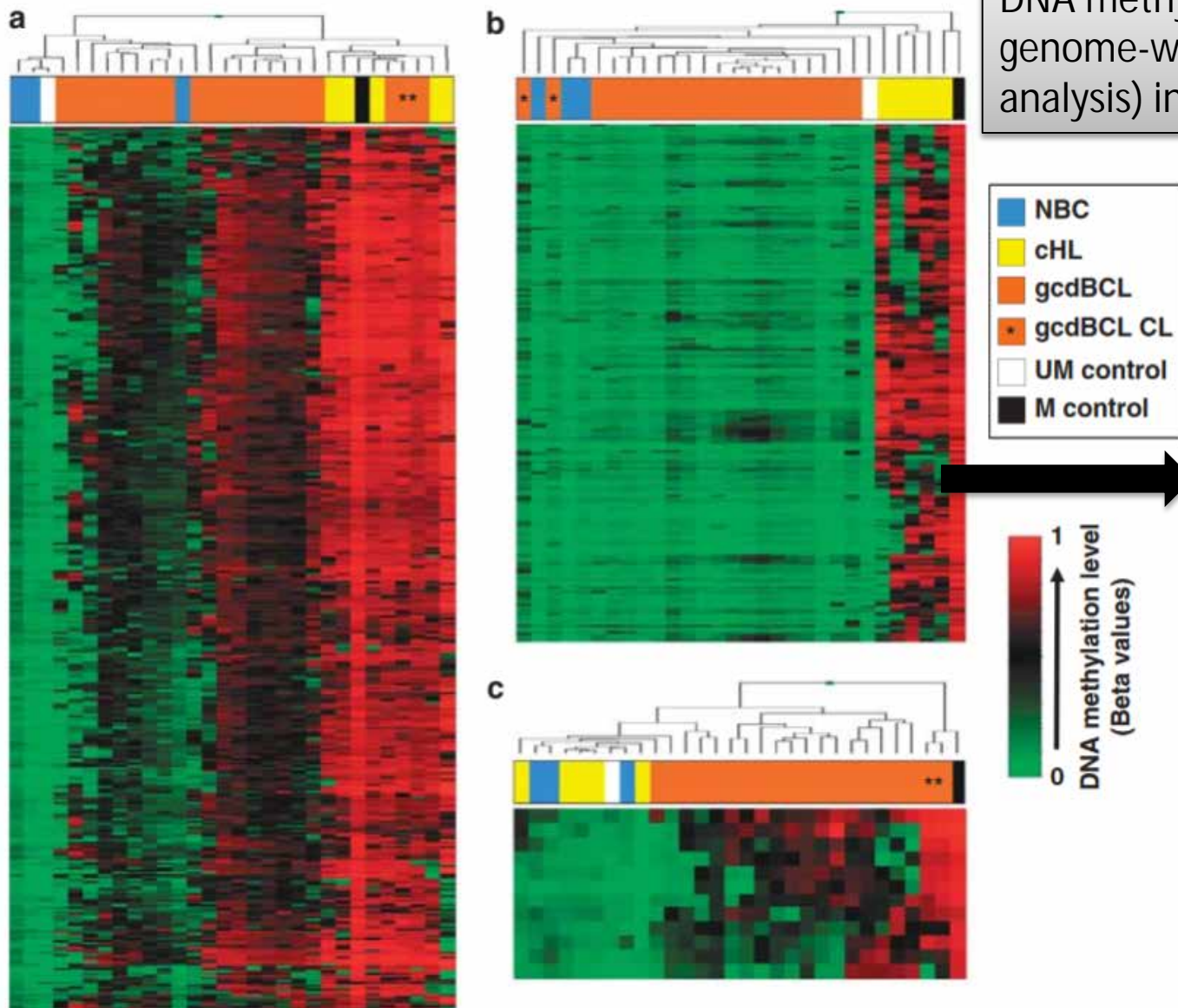
Holliday R. *The inheritance of epigenetic defects.*
Science 1987; 238:163-70

“Heritable changes in gene expression that are not due to any alteration in the DNA sequence.”

Epigenetic modifications that regulate transcription and translation



The role of methylation in HL

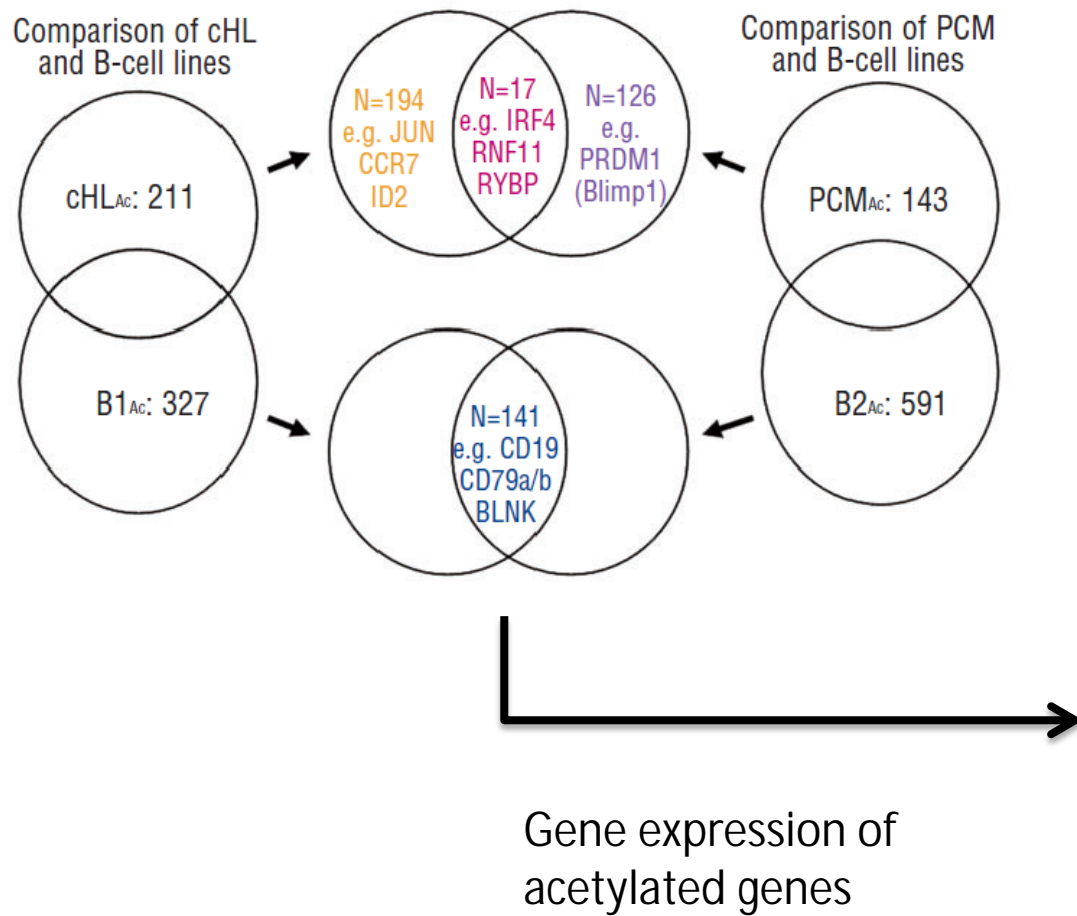


DNA methylation patterns on a genome-wide level (27K BeadArray analysis) in 5 cHL cell lines.

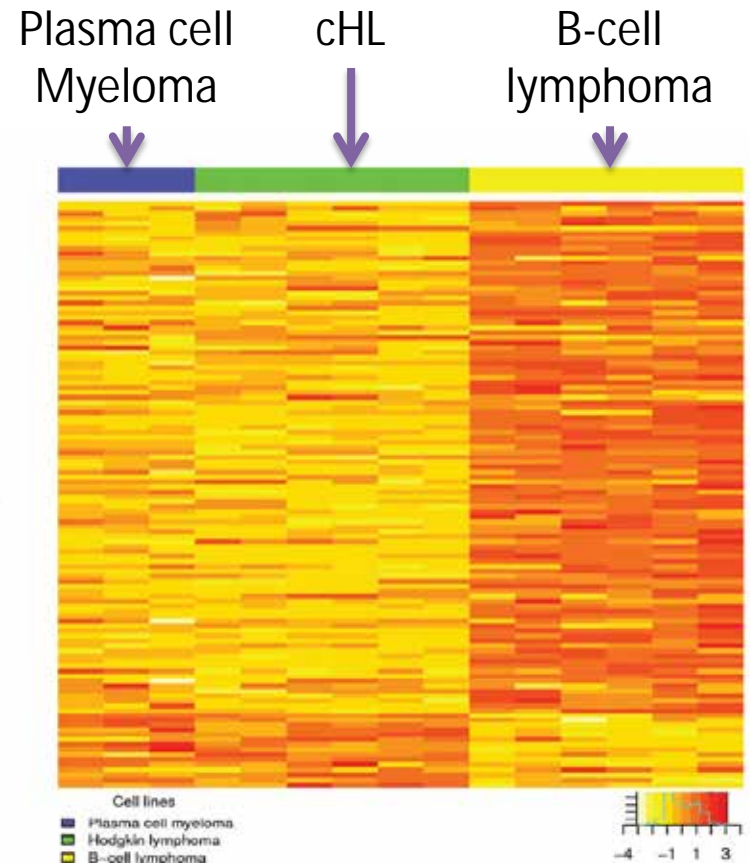
Genes hypermethylated only in cHL are enriched for functions silenced in cHL, like regulation of B-cell or T-cell activation.

The role of acetylation in HL

HL shows epigenetic features of abortive plasma cell differentiation



Expression analysis of 158 genes differentially acetylated in cHL and PCM cell lines (17 hyperacetylated and 141 hypoacetylated genes)



1. General epigenetics features of HL
- 2. Generalities of Non-coding RNAs**
3. microRNAs regulating HRS cells crucial pathways
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Classes and functions of non-coding RNAs

Epigenetic
Modifications



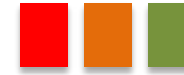
Genome Integrity/
transposon defence



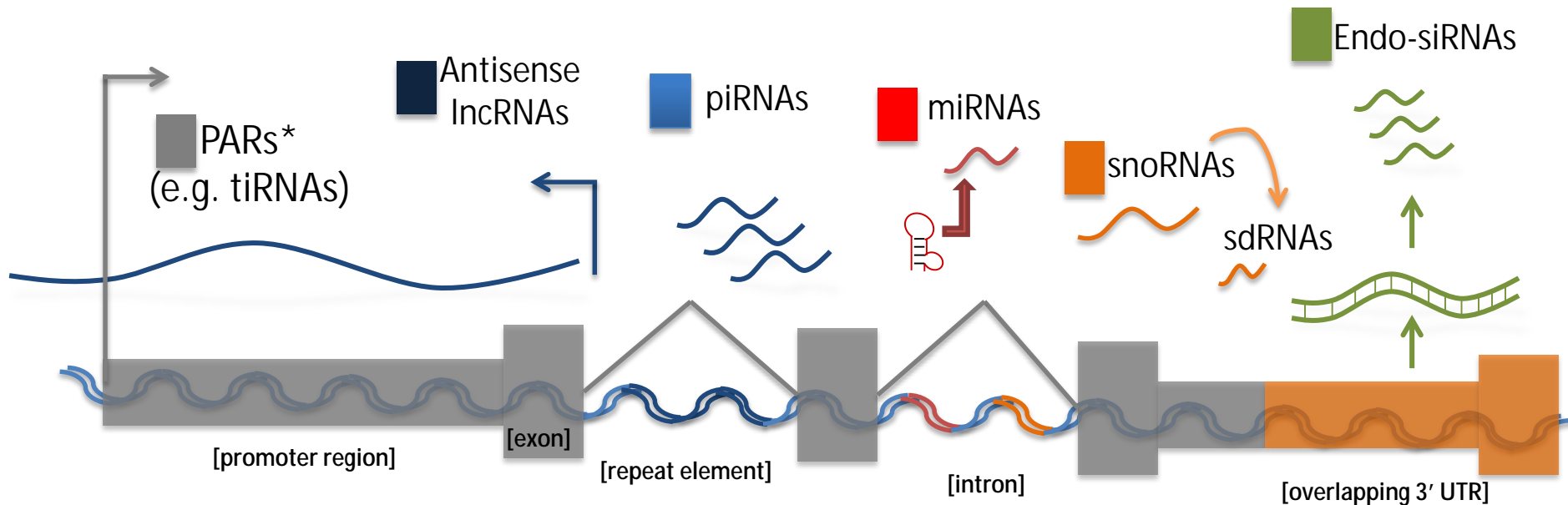
Transcriptional
regulation



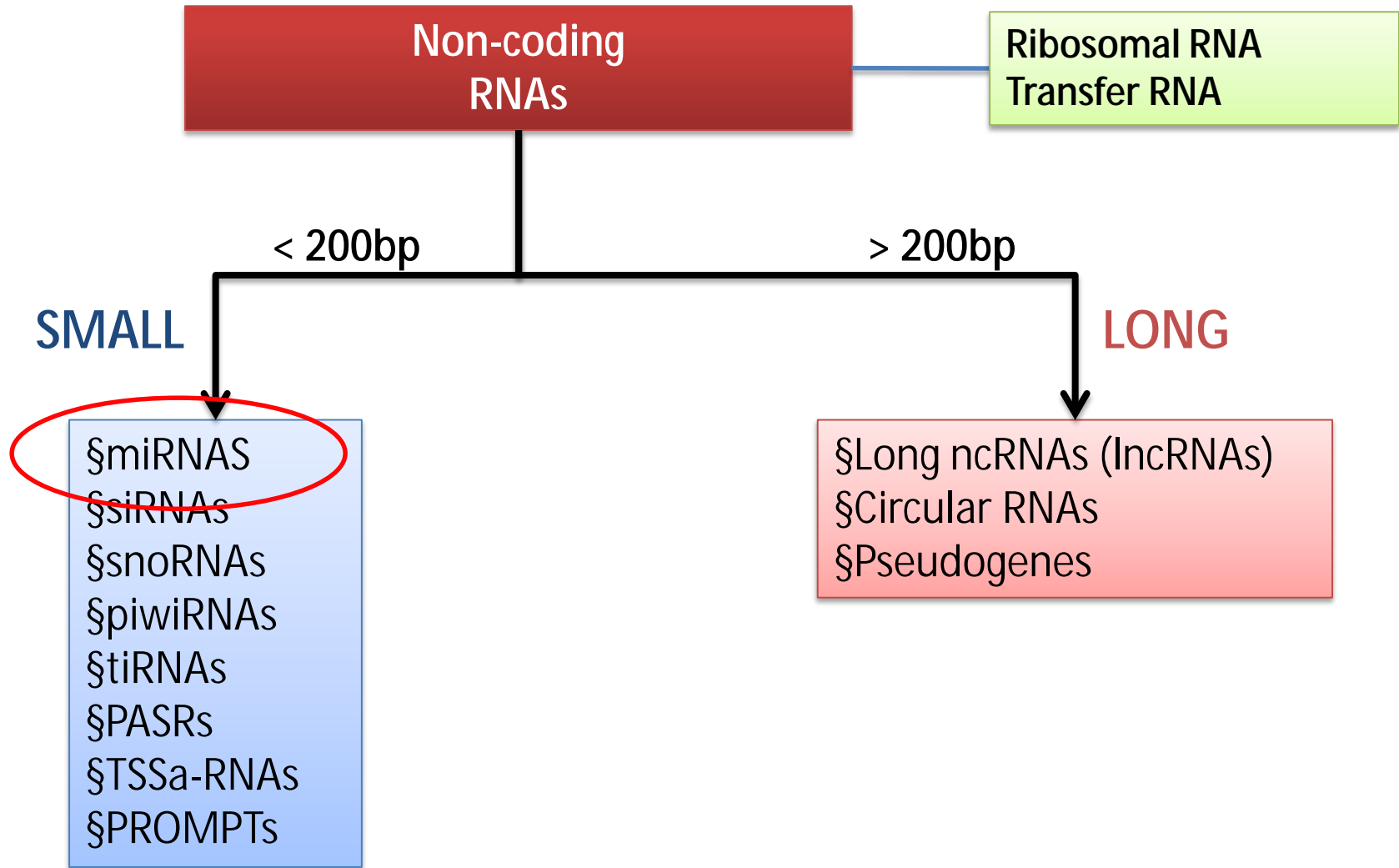
Post-transcriptional
regulation



Viral defense

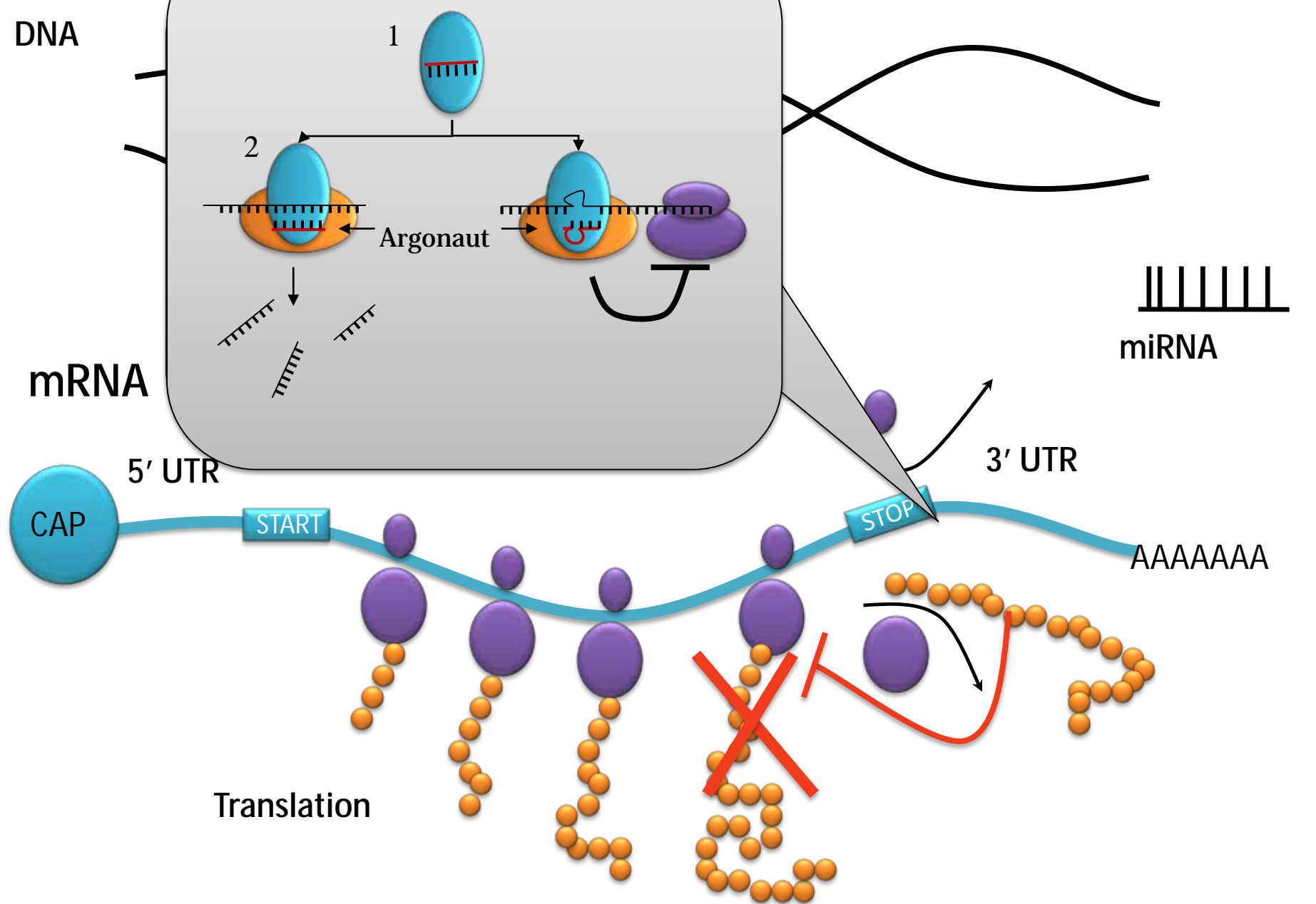


Classification of non-coding RNAs

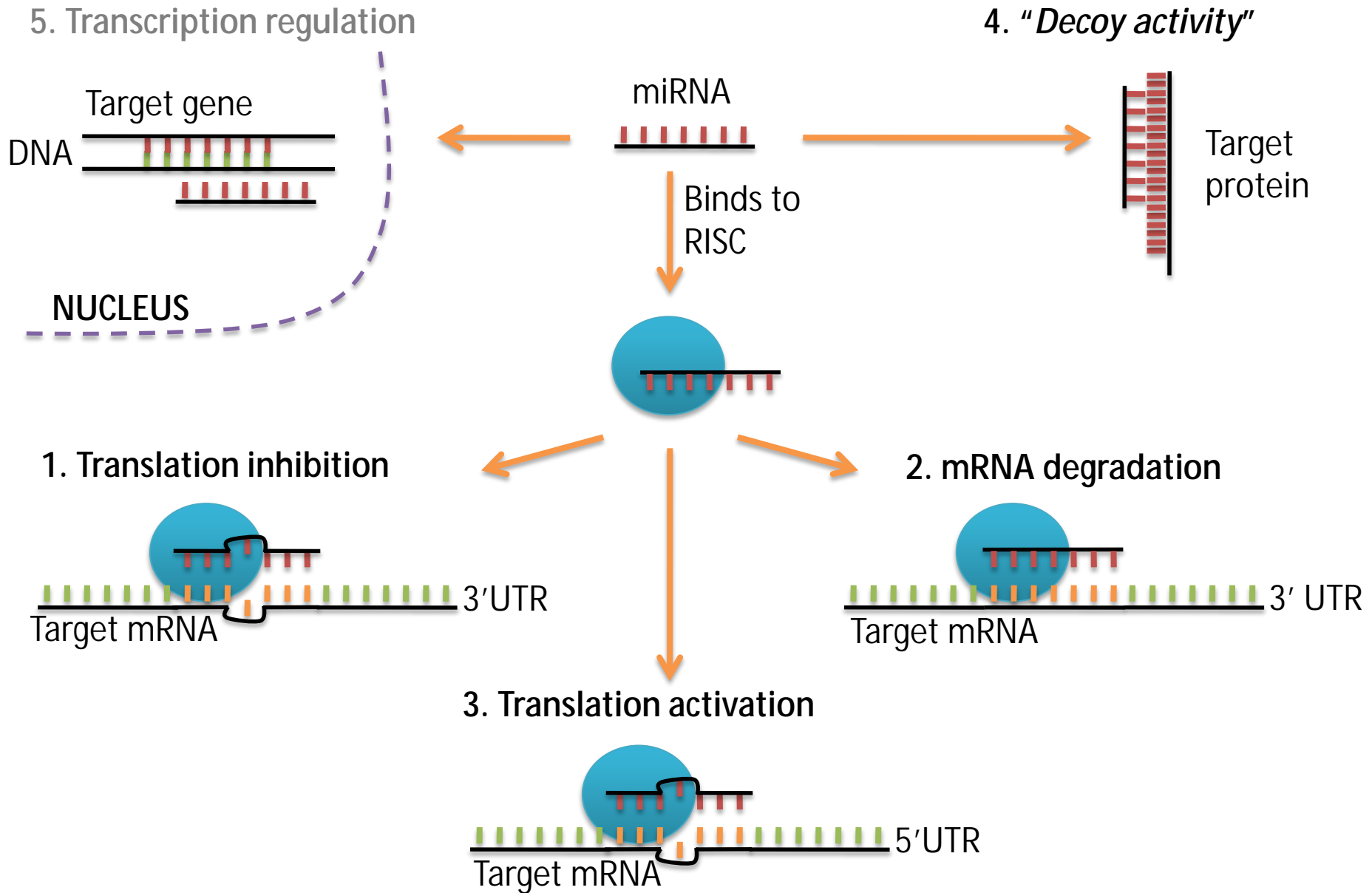


How do microRNAs work?

Mature miRNA in miRISC



microRNA functions



Mechanisms of deregulation of miRNA expression

- Chromosome alterations
- Regulator genes
- Methylation/acetylation
- Mutations/deletions/Polymorphism
- Regulation of the maturation process



miRNAs and cancer

ONCOGENES & TUMOR SUPPRESSOR GENES

Diagnosis

Prognosis

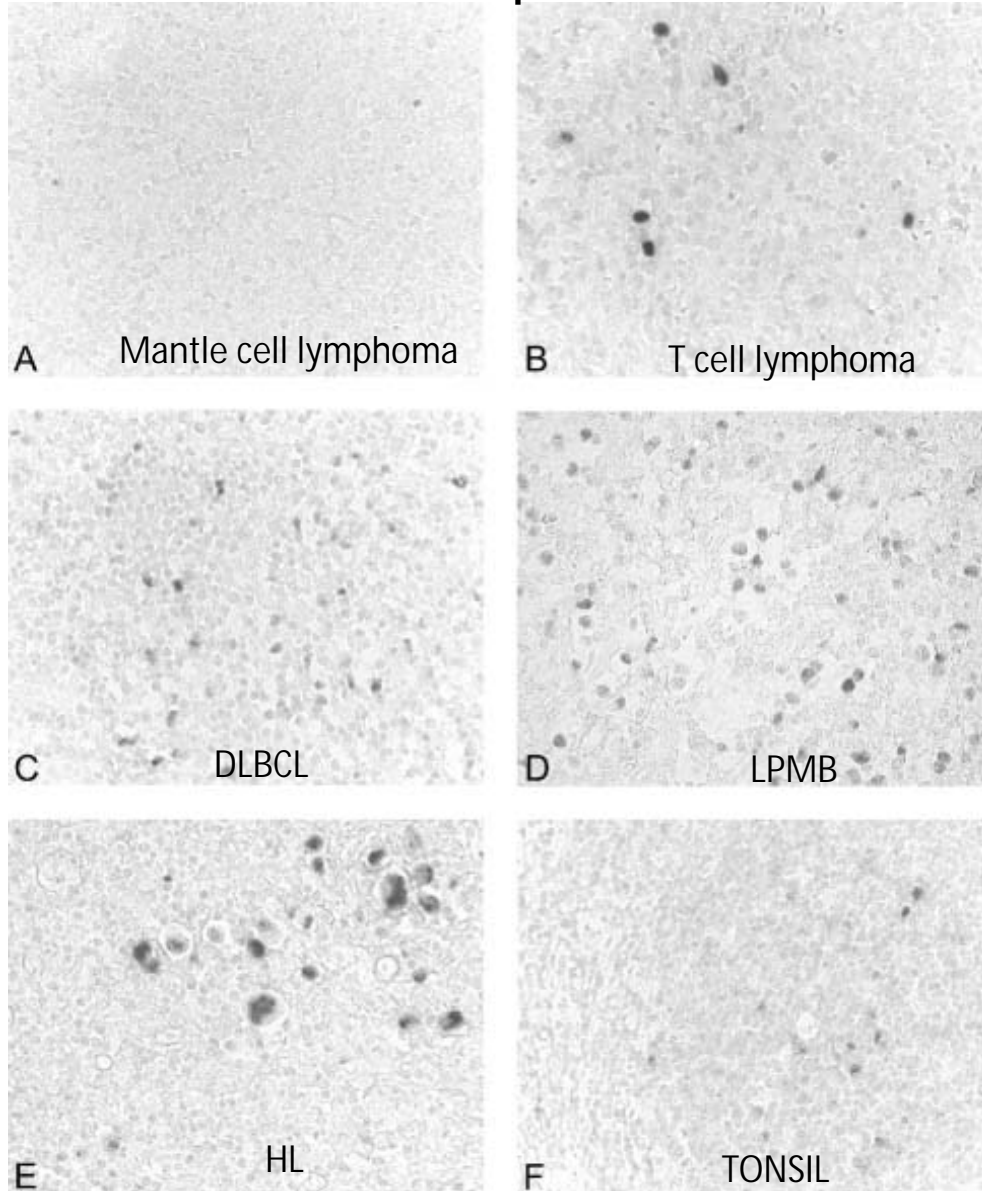
Therapeutic application?

1. General epigenetics features of HL
2. Generalities of Non-coding RNAs
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4. microRNAs regulated by methylation in HL
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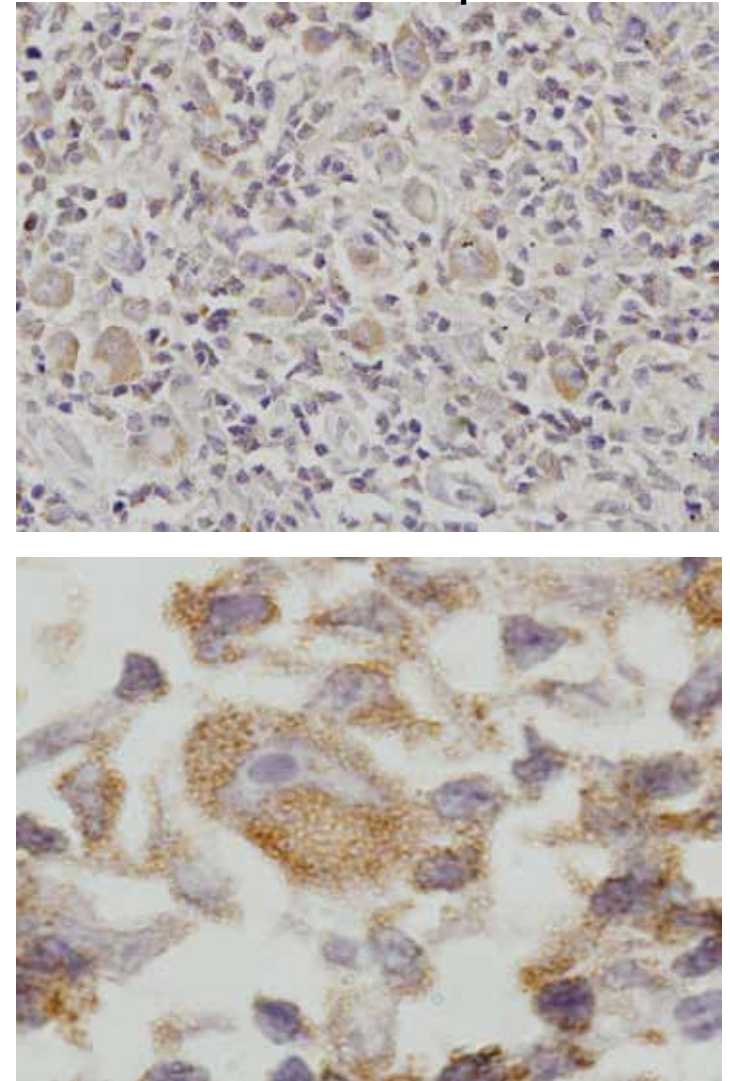
miR-155

1st miRNA described in HL

BIC Overexpression



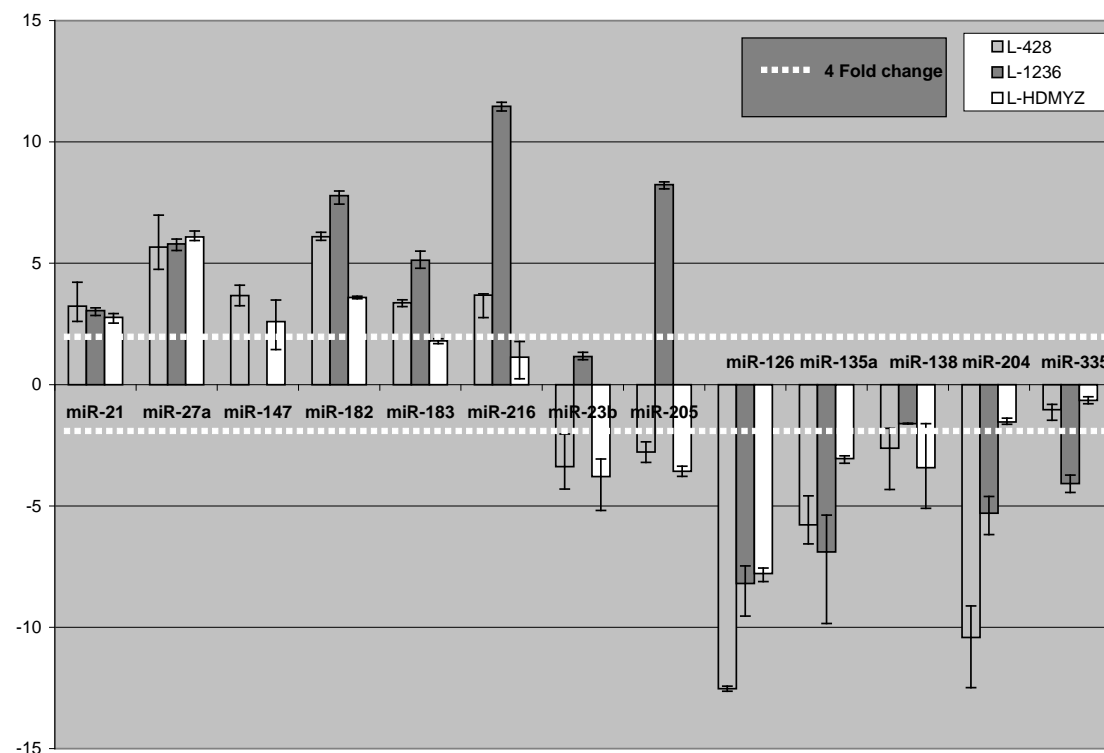
miR-155 Overexpression



Navarro *et al.* 2008

Kluiver J. *et al.* J Pathol 2005

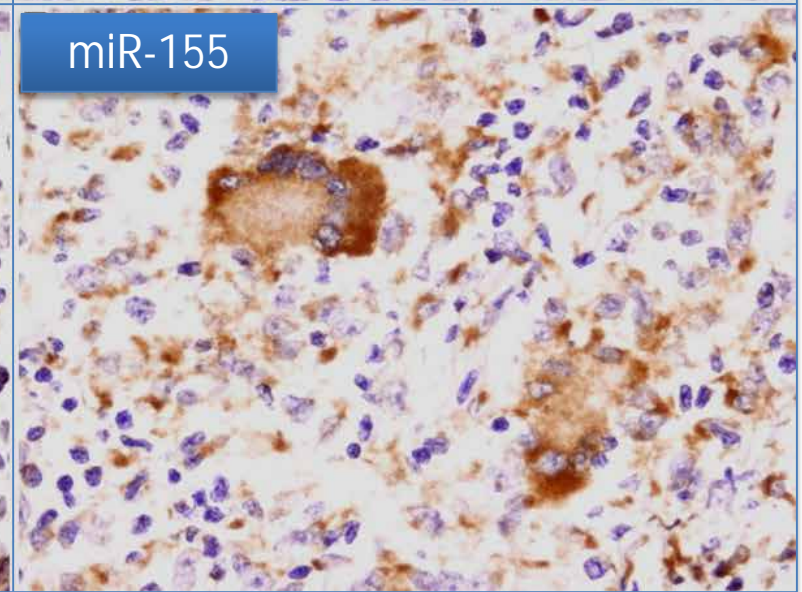
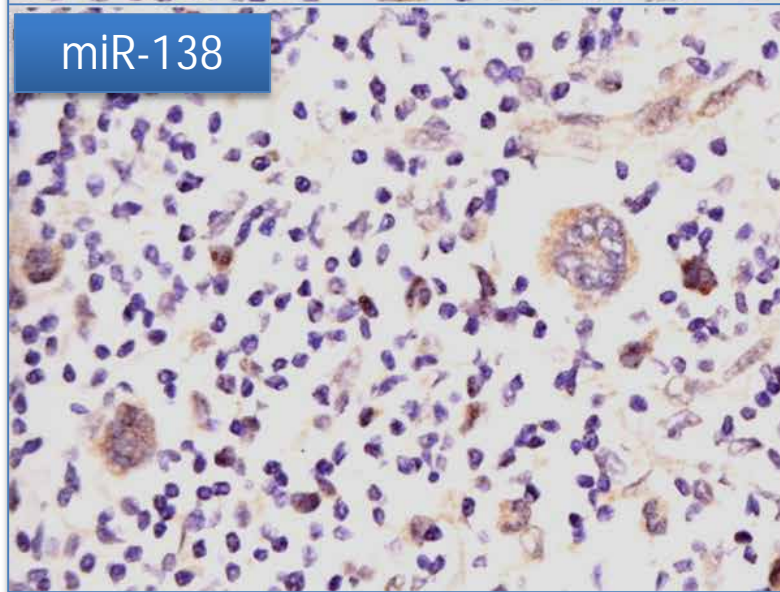
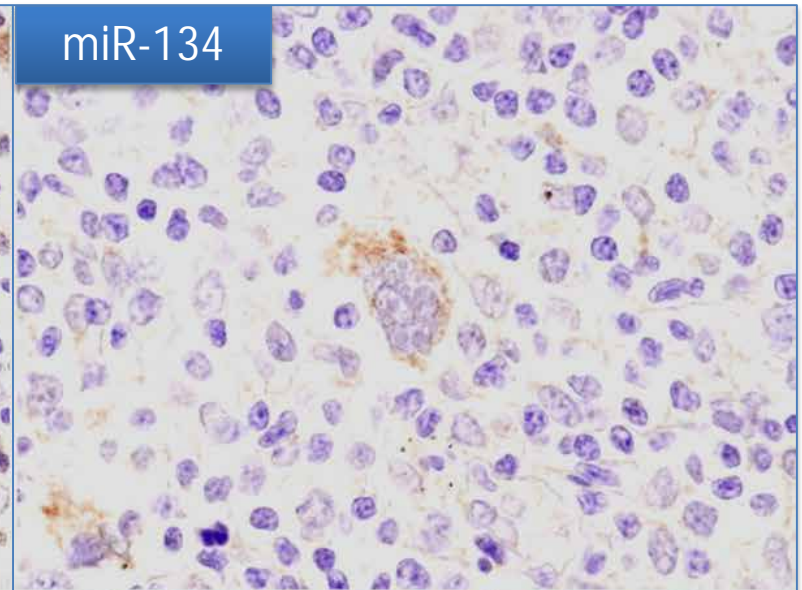
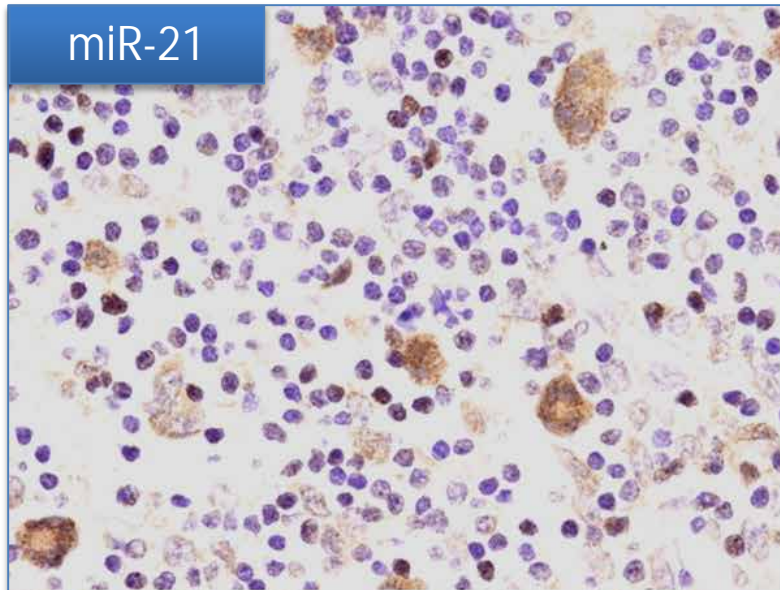
Validation of the 25-microRNA signature in cHL cell lines



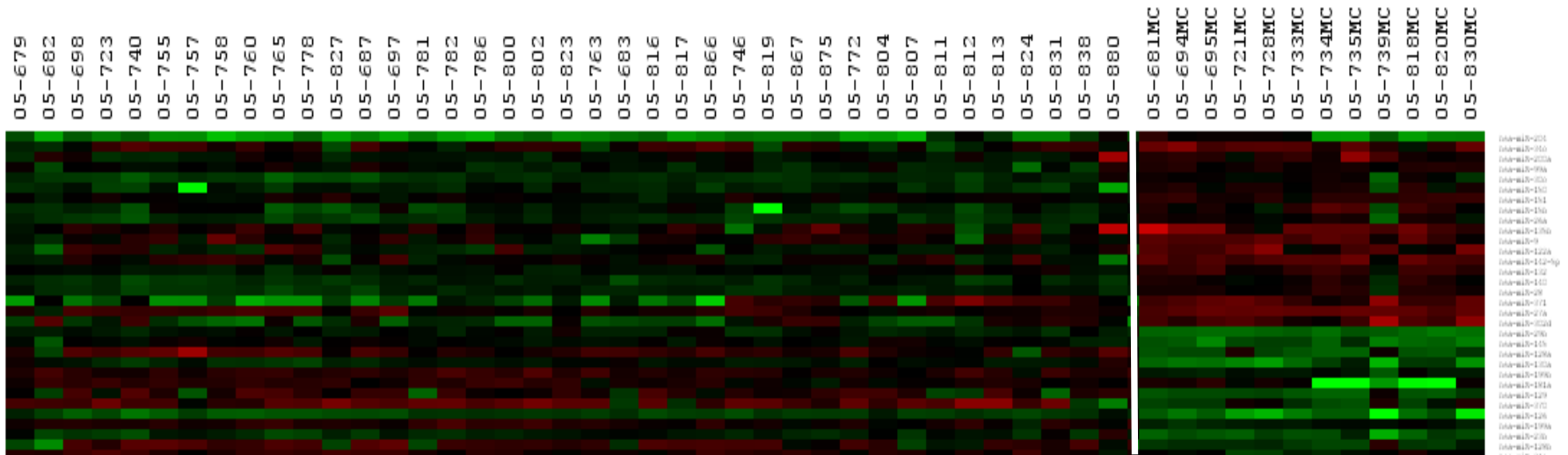
13 H/RS
7 H/RS and microenvironment
5 Microenvironment

miRNA Component	Chromosomal Location	Level Expression	Putative Targets*
miR-21	17q23.2	High	TRAIL-3, PTEN [†]
miR-23b	9q22.32	Low	SUMO1, PLK3, POU4F2
miR-26b	2q35	Low	MMP21, IFNG
miR-27a	19p13.12	High	CD44
miR-30b	8q24.22	Low	CCNE1, IT6B3, IT6A5, TIMP-2, TIMP-3, SERPINE1
miR-31	9p21.3	Low	CD28, CD48, EBF3, TRAF3
miR-124a	8p23.1	High	IT6B1 [†] , ANSPT1 [†]
miR-126	9q34.3	Low	CD97, BAD, IKBKAP, VCAM1, TNFC, TNFS11, PIK3C
miR-134	14q32.31	High	J-CHAIN
miR-135a	3p21.2	Low	MSH2
miR-138	7q32.2	High	PU.1, TCF3, E2A, FAK, HIP-1A
miR-147	9q32.2	High	NOL3, ZAP-70
miR-182*	7q32.2	High	
miR-183	7q32.2	Low	IT6B1
miR-185	22q11.21	High	PBX1, CD79B
miR-198	3q13.33	High	CCND2, BCL7A
miR-204	9q21.13	Low	ATF2, BCL2, CDC25B, BCL9, BCL11A, BCL11B
miR-205	1q32.2	Low	K-RAS, SMAD4, MSH2, PTEN
miR-216	2p16.1	High	BCL11B, BCL9
miR-220	Xq25	High	IRF3
miR-302a	4q25	High	CD45, CD138, RECK, CXCR4
miR-302b	4q25	High	CD45, CD138, RECK, CXCR4
miR-302c	4q25	High	CD45, CD138, RECK, CXCR4
miR-325	Xq21.1	High	NFkB-REPRESSOR FACTOR
miR-335	7q32.2	Low	ANSPT1

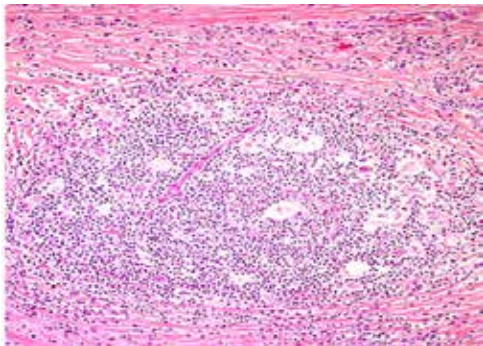
In situ hybridization of miR-21, miR-134, miR-138 and miR-155



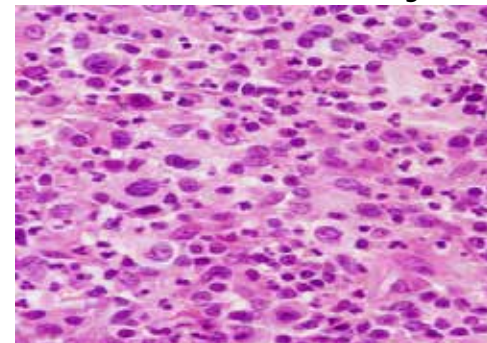
Nodular sclerosis vs mixed cellularity



Nodular sclerosis



Mixed cellularity



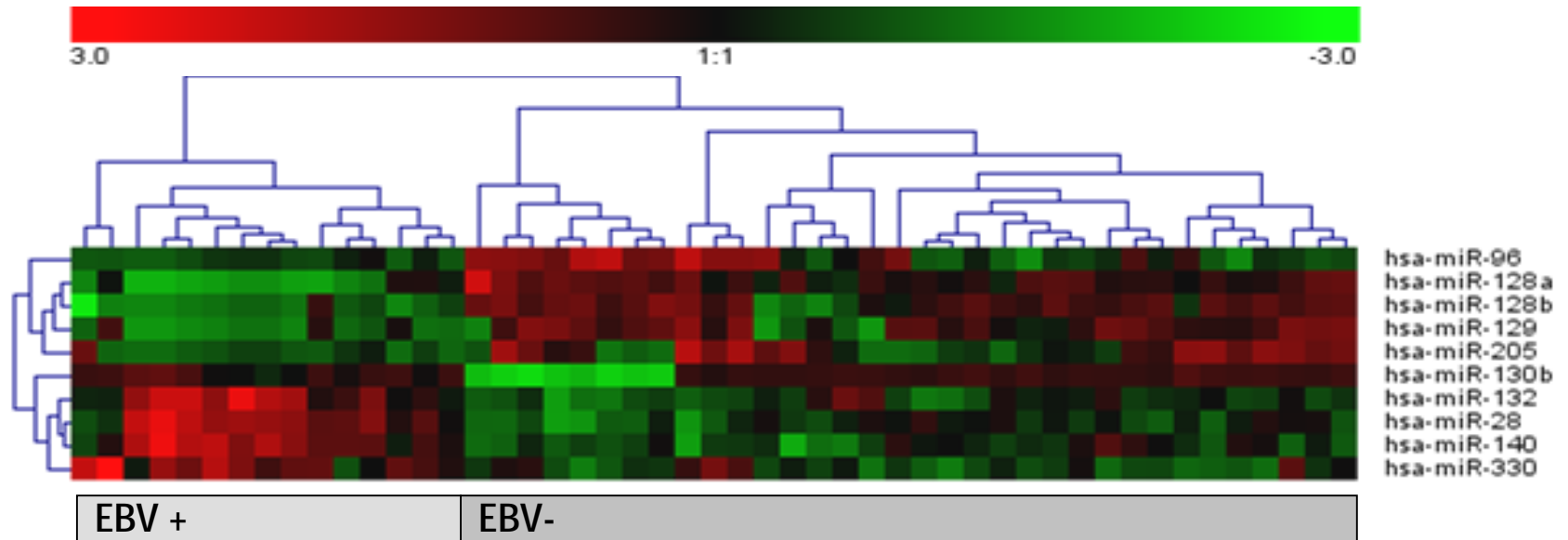
Overexpressed miRNAs

miR-9, miR-15b, miR-26a, miR-27a, miR-28, miR-30c, miR-34c, miR-99a, miR-122a, miR-132, miR-135b, miR-140, miR-142-5p, miR-150, miR-151, miR-200a, miR-204, miR-302d, miR-371

Underexpressed miRNAs

miR-23b, miR-29b, miR-34a, miR-124b, miR-125b, miR-126, miR-128a, miR-128b, miR-129, miR-130a, miR-145, miR-147, miR-154, miR-181a, miR-199a, miR-199b, miR-370

Epstein Bar virus and miRNAs in cHL



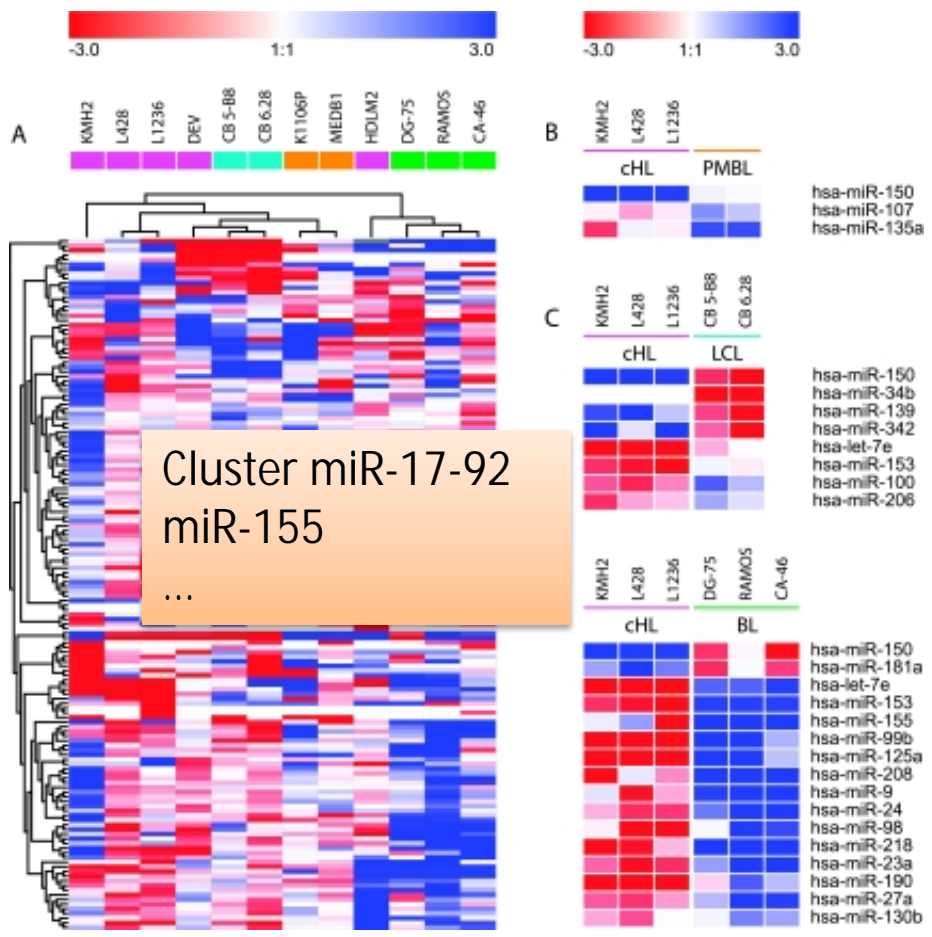
Underexpressed in EBV+ cHL

miR-96
miR-128a
miR-128b
miR-129
miR-205

Overexpressed in EBV+ cHL

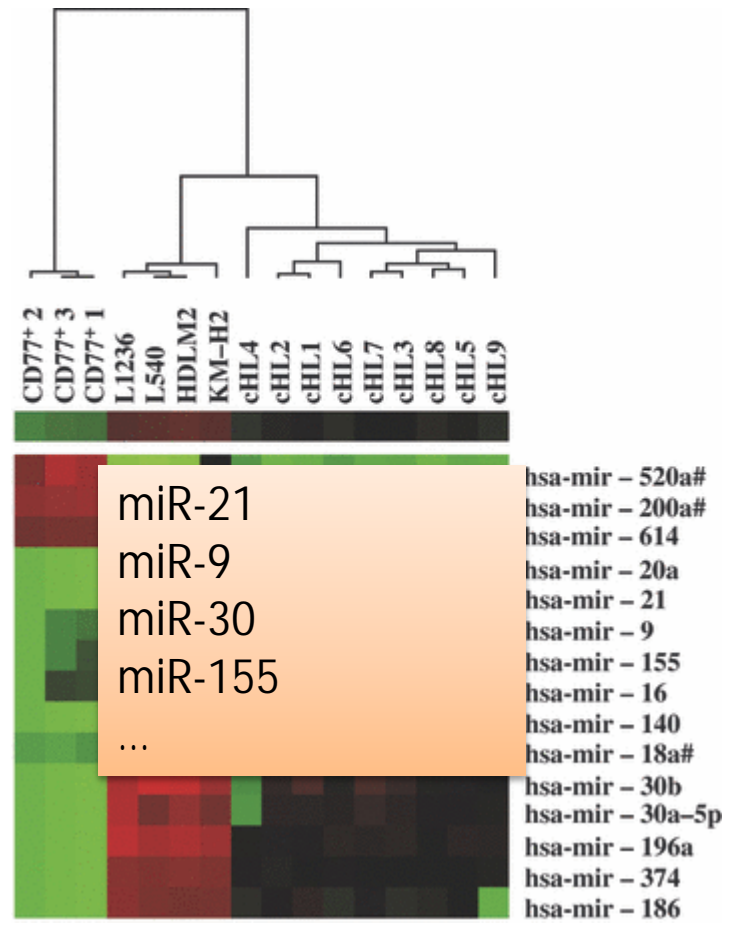
miR-28
miR-130b
miR-132
miR-140
miR-330

MicroRNA profile of Hodgkin lymphoma cell lines



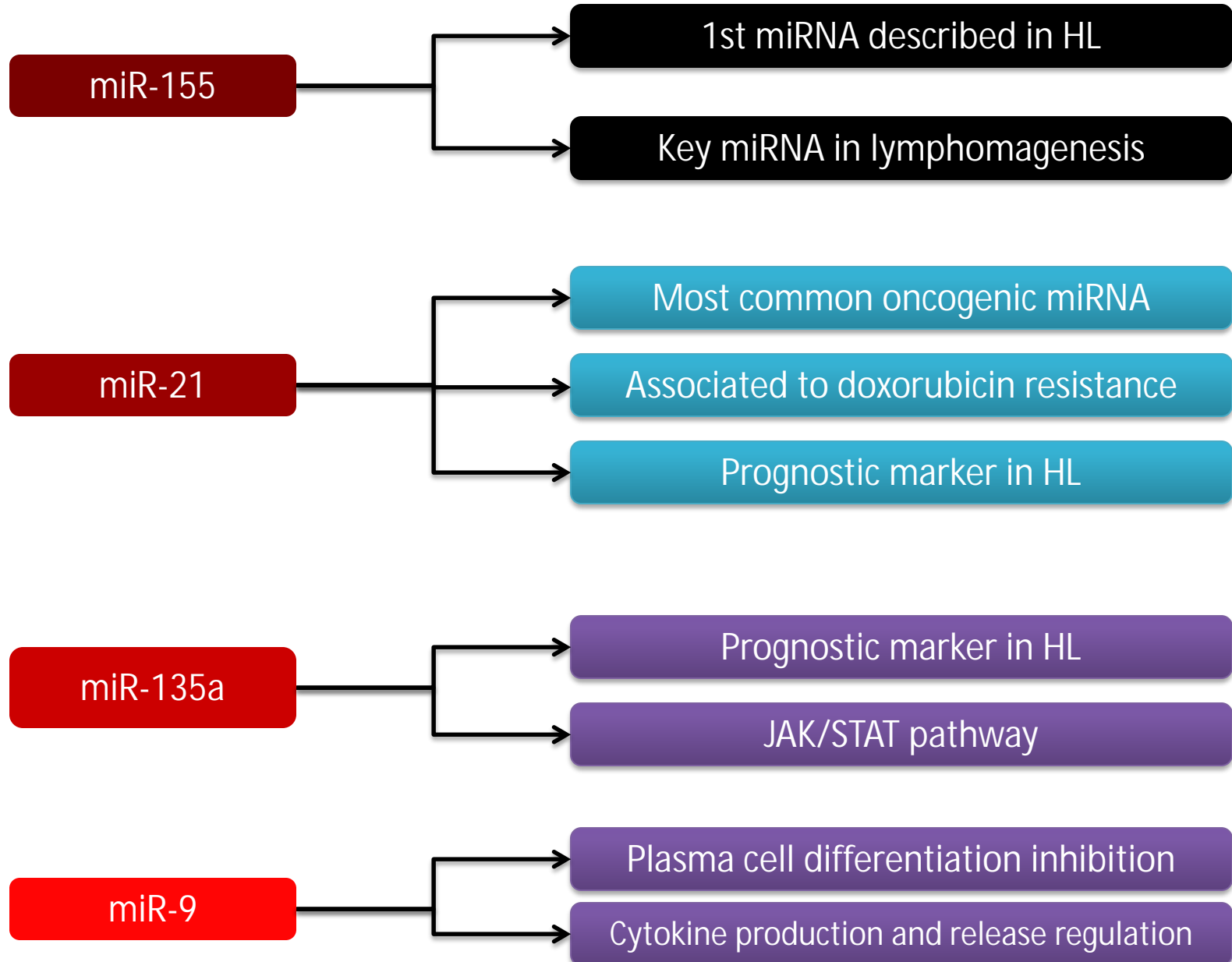
Gibcus JH, et al. *Neoplasia* 2009

MicroRNA profile of microdissected Hodgkin/Reed-Sternberg cells

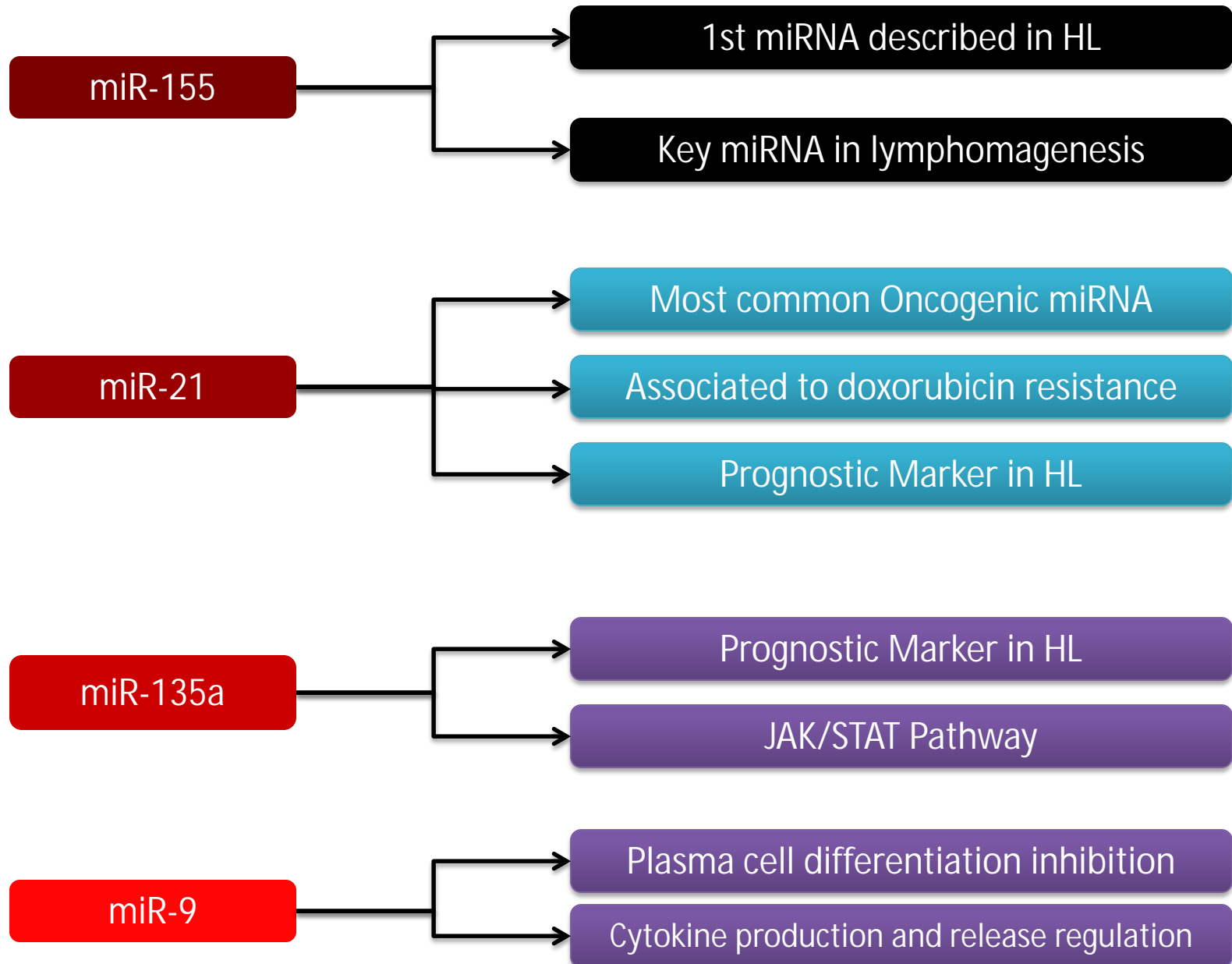


Van Vlierberghe P, et al., *bjh* 2009

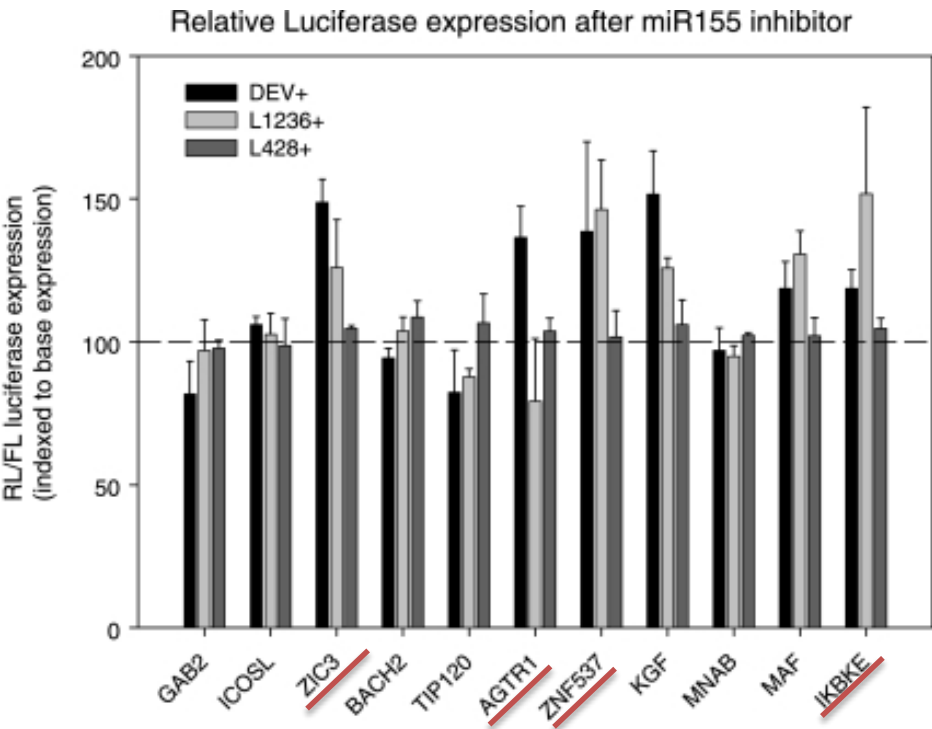
Key miRNAs in HL



Key miRNAs in Hodgkin lymphoma



miR-155 target validation



AGTR1, FGF7, ZNF537, ZIC3, and IKBKE are true miR-155 target genes in HL.

miR-155 transgenic mice (B-cell lineage)

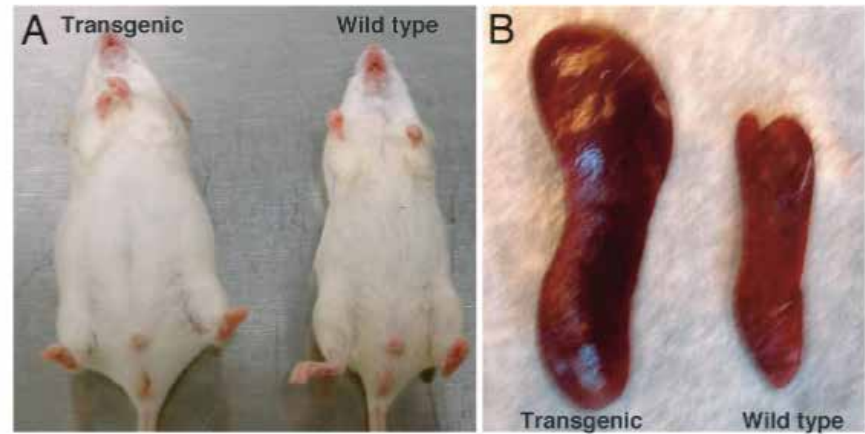
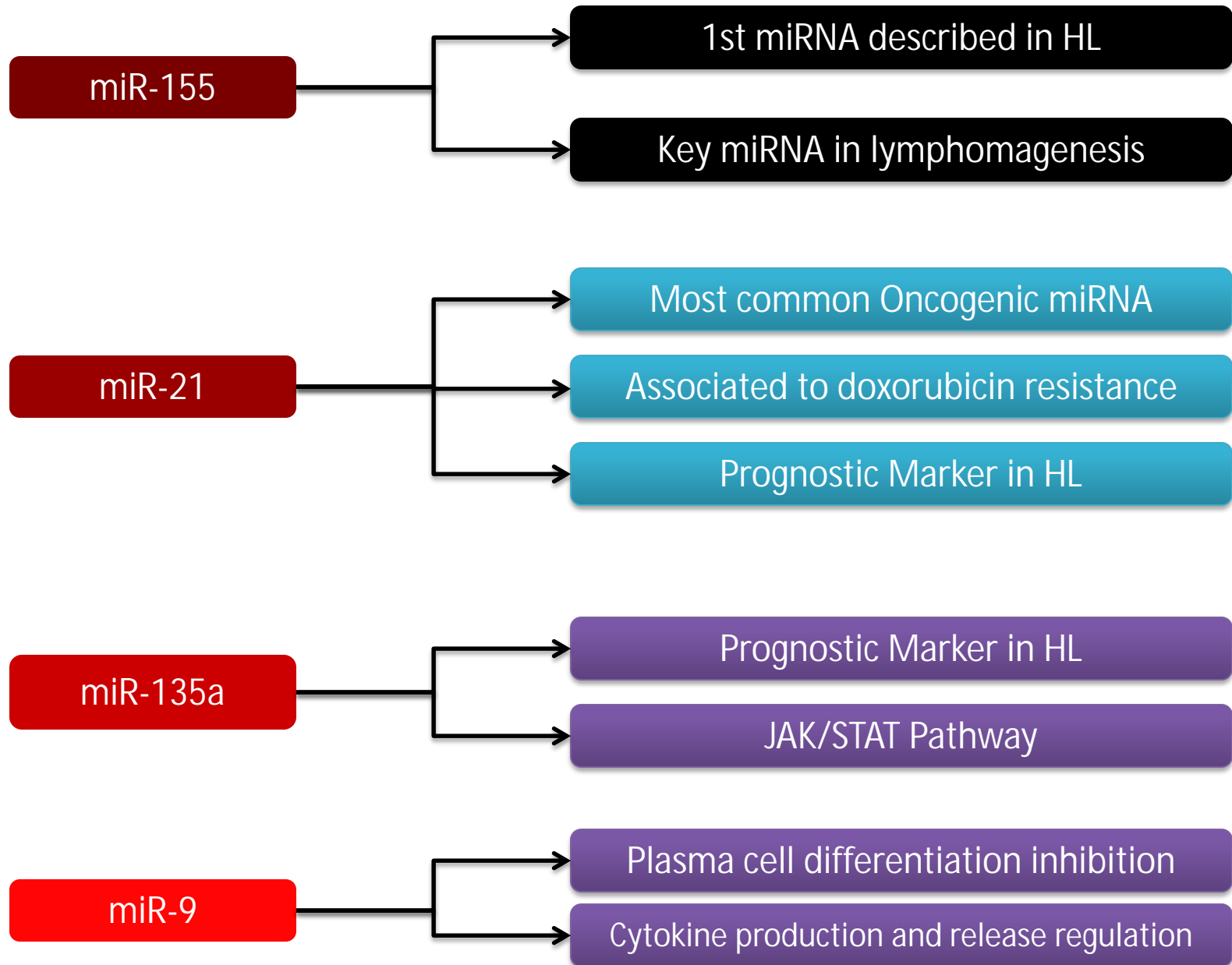


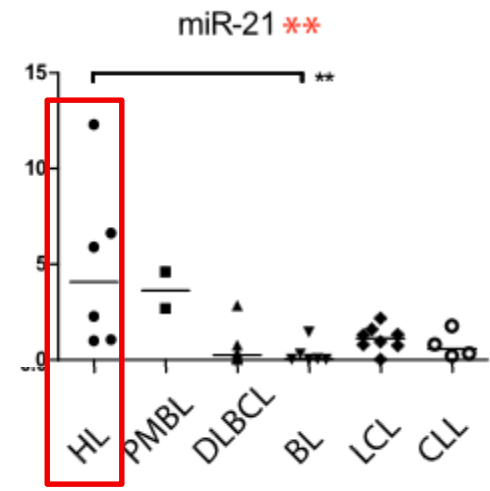
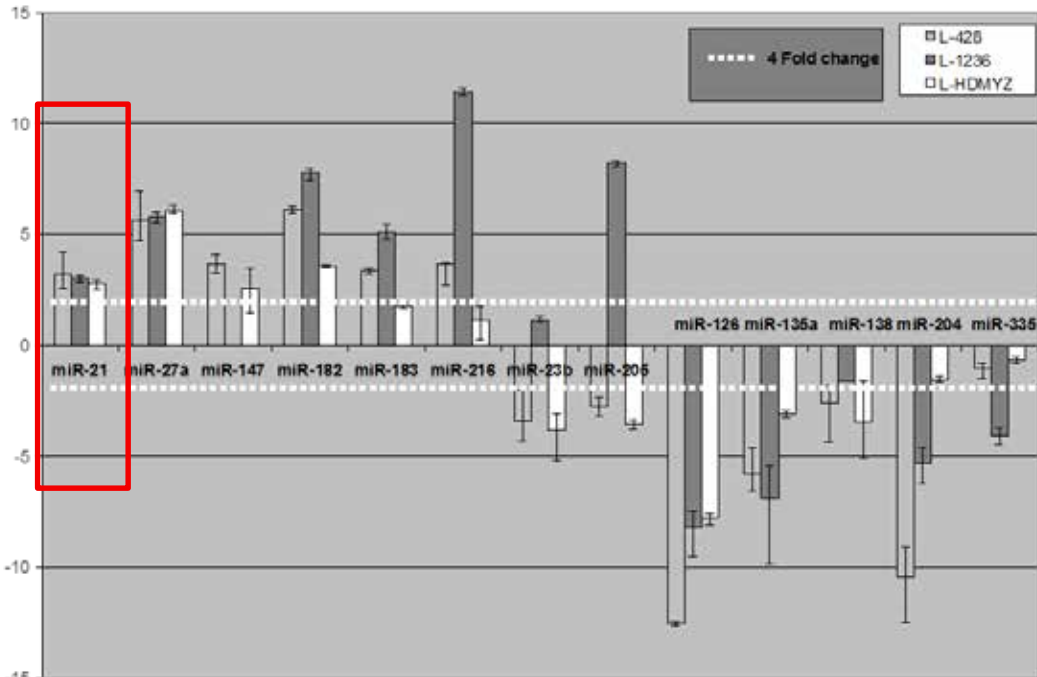
Fig. 2. Transgenic mice, 6 months old, presented an enlarged abdomen and important splenomegaly. (A) Transgenic mice, 6 months old, had a considerably enlarged abdomen compared with wild-type mice, due to the clinically evident splenomegaly. (B) Splens of the mice shown in A. The transgenic spleen is enlarged due to expansion of leukemic/lymphoma cells.

Transgenic mice exhibit initially a preleukemic pre-B-cell proliferation followed by the development of a high-grade B-cell Lymphoma approximately at the age of 6 months.

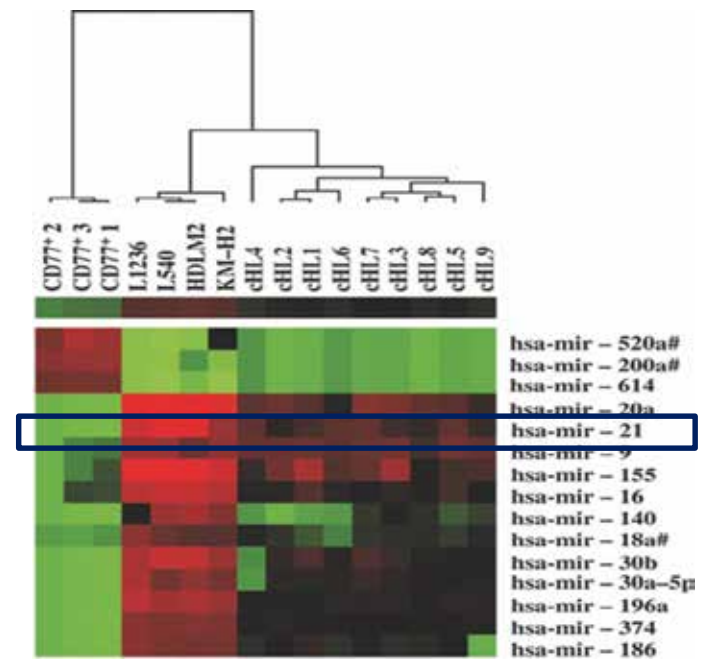
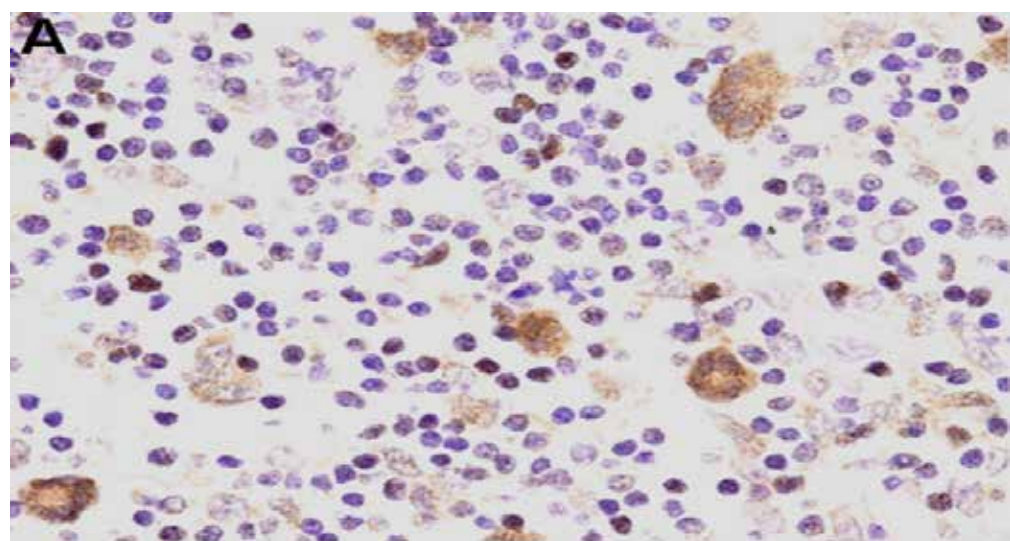
Key miRNAs in Hodgkin lymphoma



miR-21



Gibcus JH, et al. Neoplasia 2009.



Navarro A et al. Blood 2008

Van Vlierberghe P, et al., bjh 2009

miR-21 as prognostic marker

Discovery Set (29 cHL patients) and cHL cell lines

Search of miRNA signatures. Supervised T-test tumour samples vs cHL cell lines

Gene expression profiling microarray is used to identify signatures from either HRS cells or microenvironment

234 miRNAs differentially expressed (FDR < 0.15)
134 miRNAs upregulated in cHL cell lines
100 miRNAs upregulated in tumour samples

Search of potential miRNA candidates Outcome - related

Outcome (F vs U) is used to classify cases (23 Favourable vs 6 Unfavourable)

34 miRNAs are differentially expressed using supervised T-test and univariate regression analysis (P < 0.005)

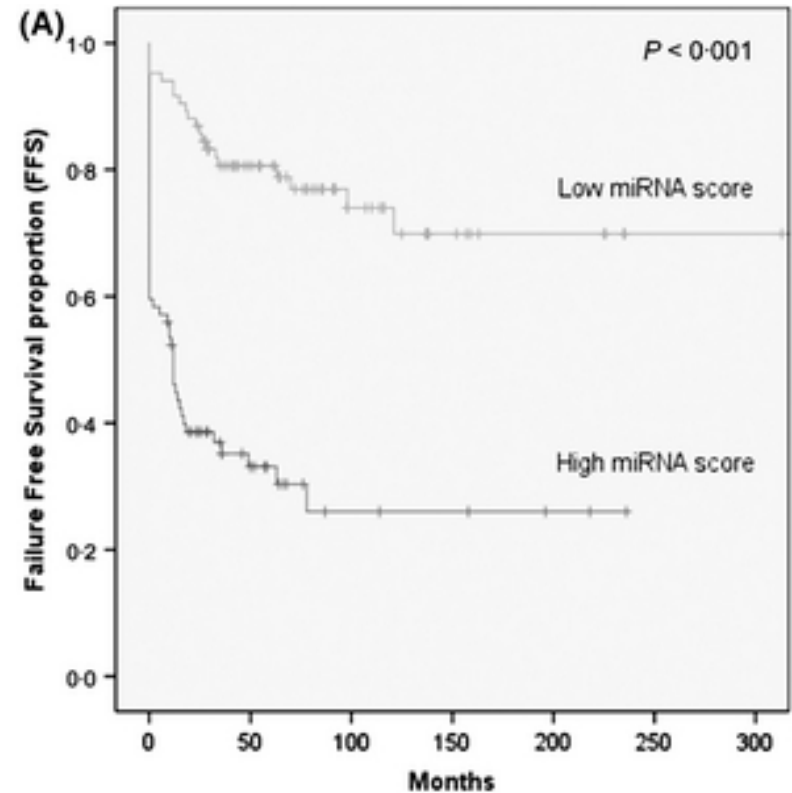
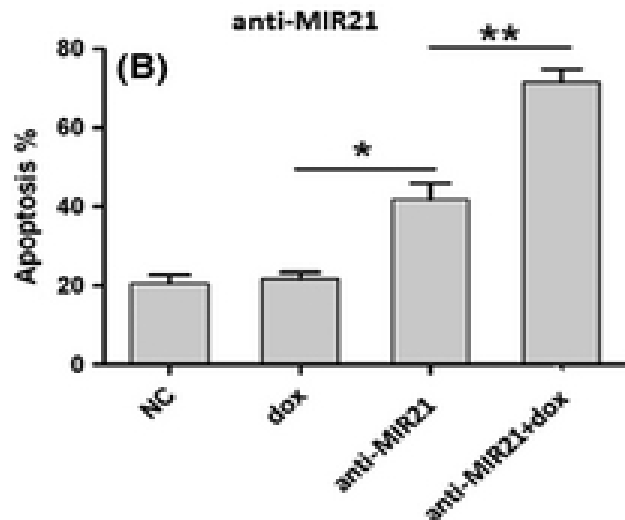
FFPE set (168 cHL patients)

Multiplex Real Time PCR of RNA from FFPE tissue of selected miRNAs (- Δ Ct values for Statistical Analyses)

Derivation of miRNA signature for FFS based on Cox regression analyses of miRNA expression

Kaplan - Meier analysis and log rank test miRNA signature for FFS

miR-21 sensitizes L428 cells to doxorubicin-induced apoptosis

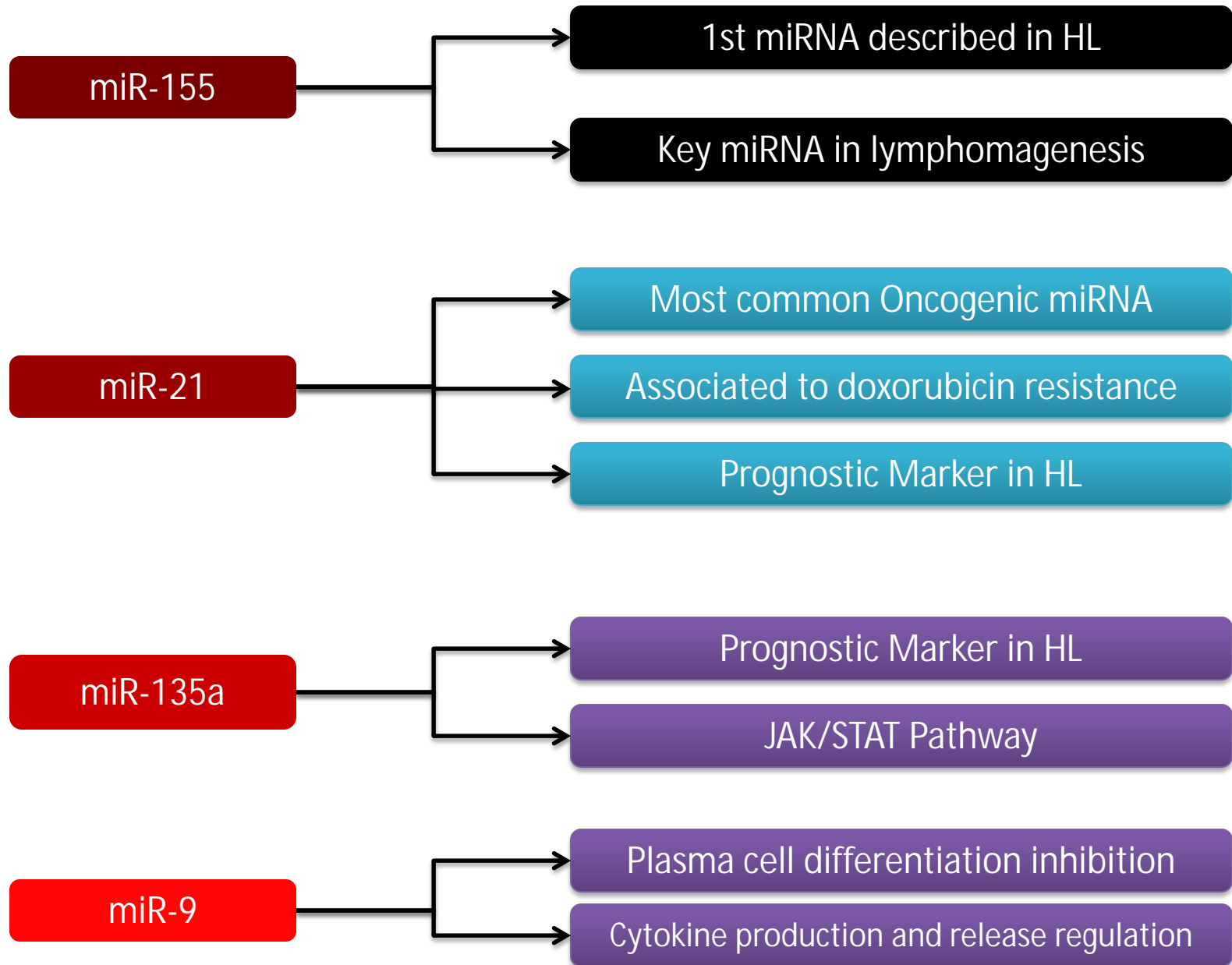


(B)

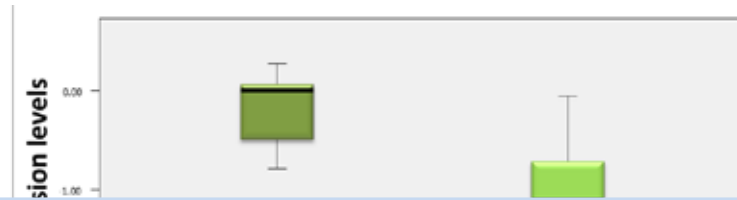
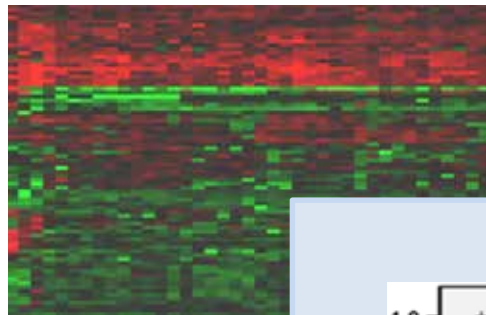
Cox regression backward stepwise model FFPE set (n = 168)

	Regression coefficient (B)	P value	Hazard ratio Exp (B) (95% CI)
MIR21	0.165	0.020	1.179 (1.025-1.357)
MIR92B*	-0.142	0.002	0.868 (0.787- 0.957)
MIR30D	0.118	0.023	1.147 (1.022- 1.287)
MIR30E	-0.138	0.017	0.888 (0.793- 0.993)

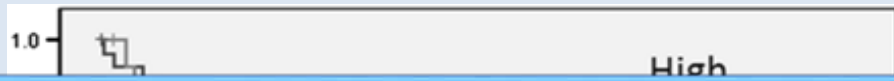
Key miRNAs in Hodgkin lymphoma



miR-135a expression and clinical outcome in 89 cHL patients

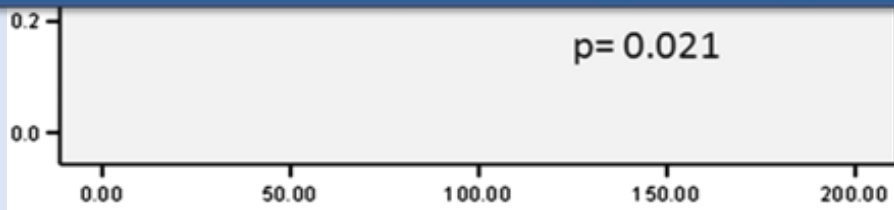


Univariate analysis



Multivariate analysis

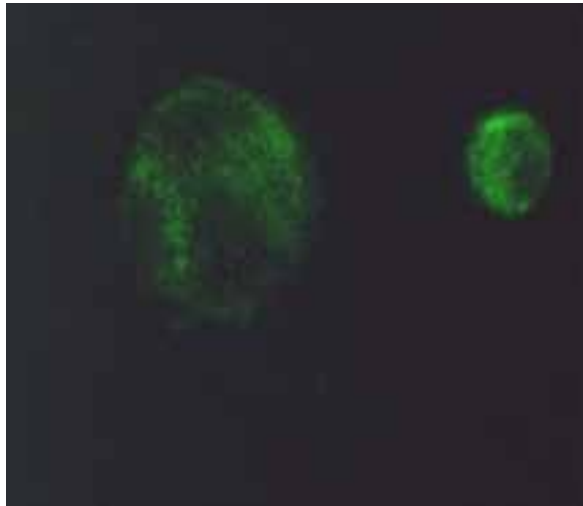
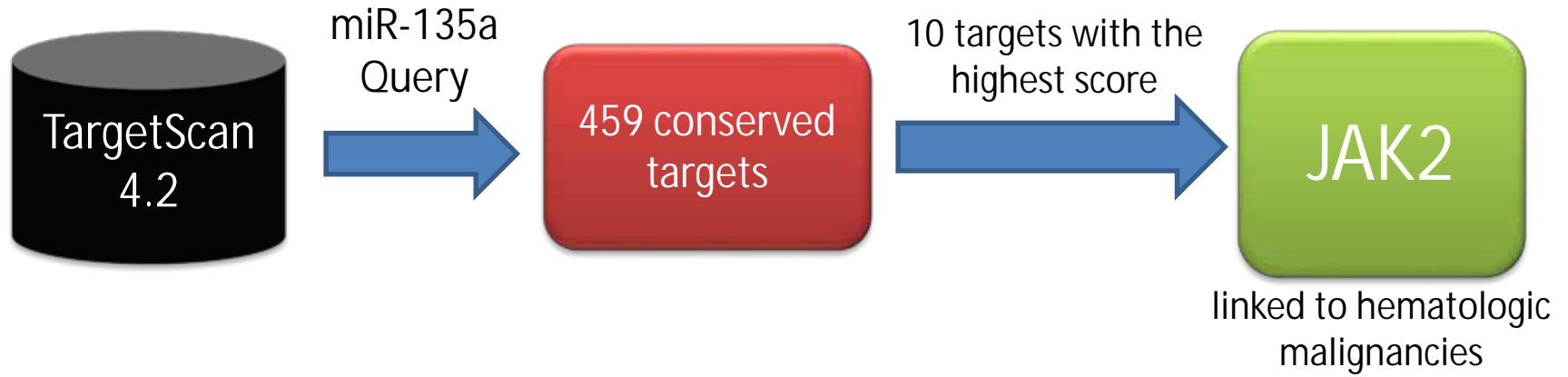
What mechanism is involved in the relation between miR-135a expression and clinical outcome?



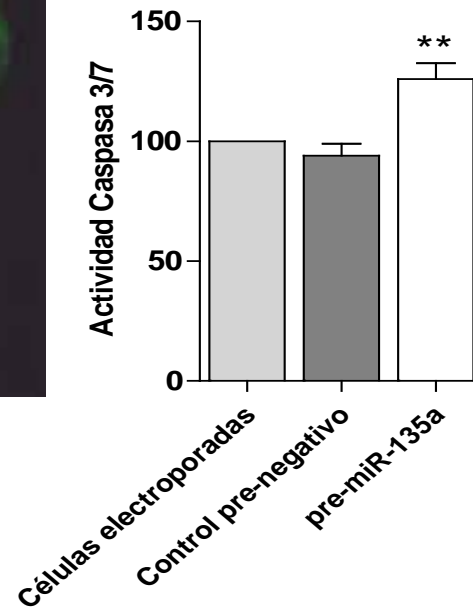
Disease Free survival

Navarro A *et al.* Blood 2009

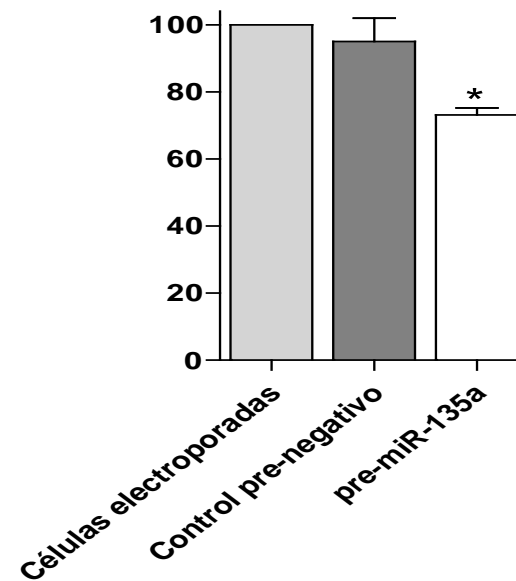
In silico target analysis



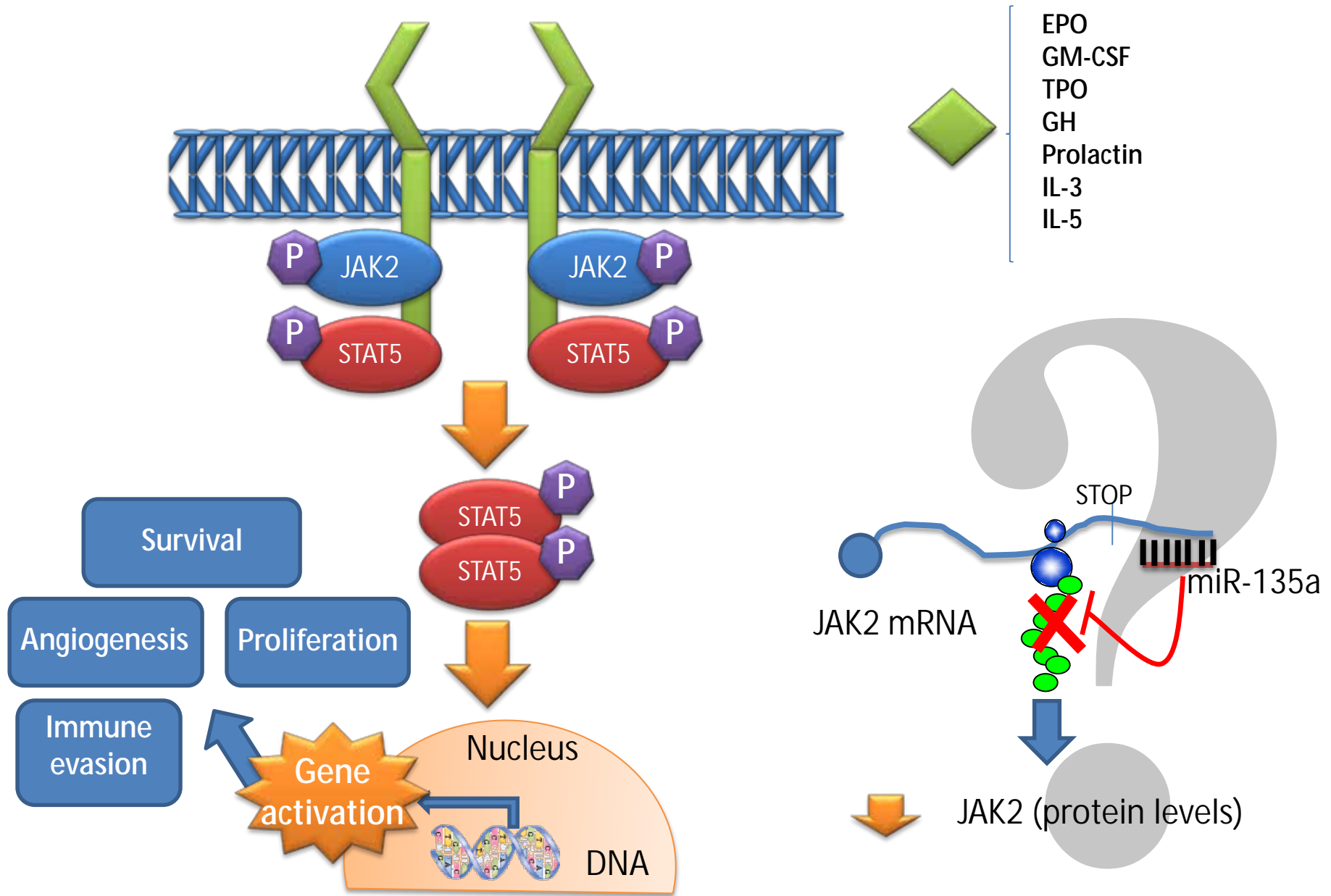
APOPTOSIS



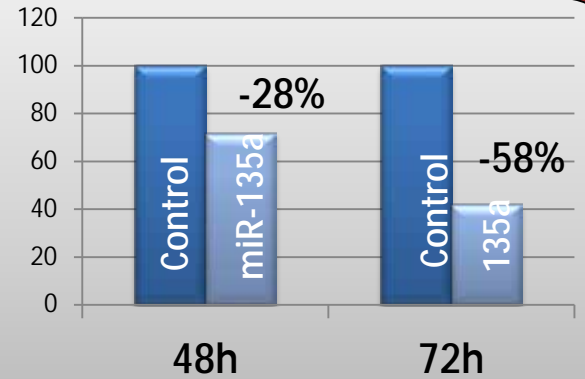
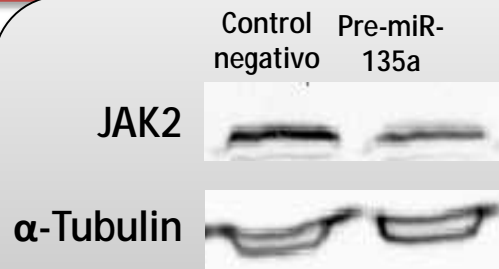
PROLIFERATION



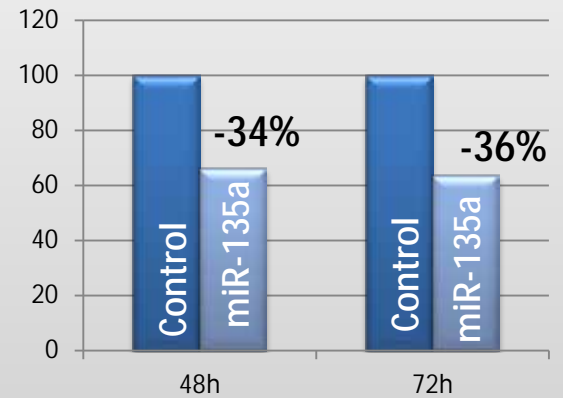
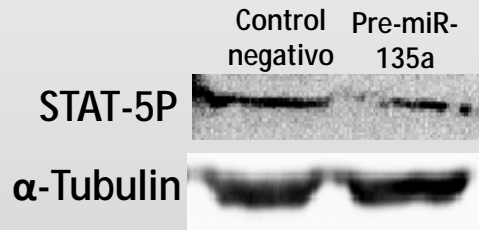
JAK2/STAT signaling pathway



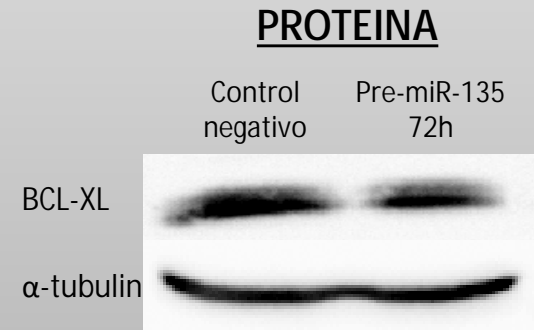
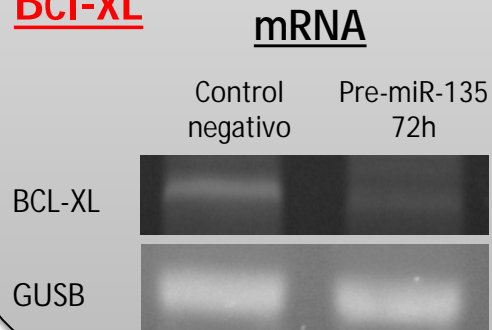
JAK2 protein expression



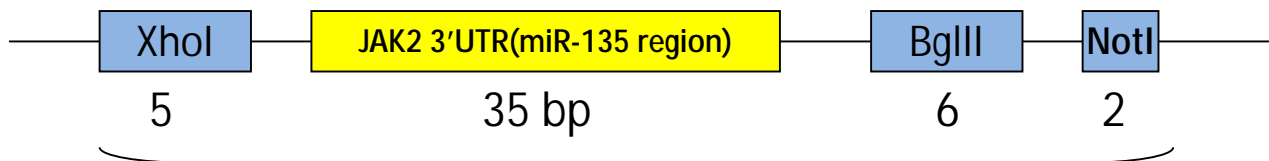
STAT5-P



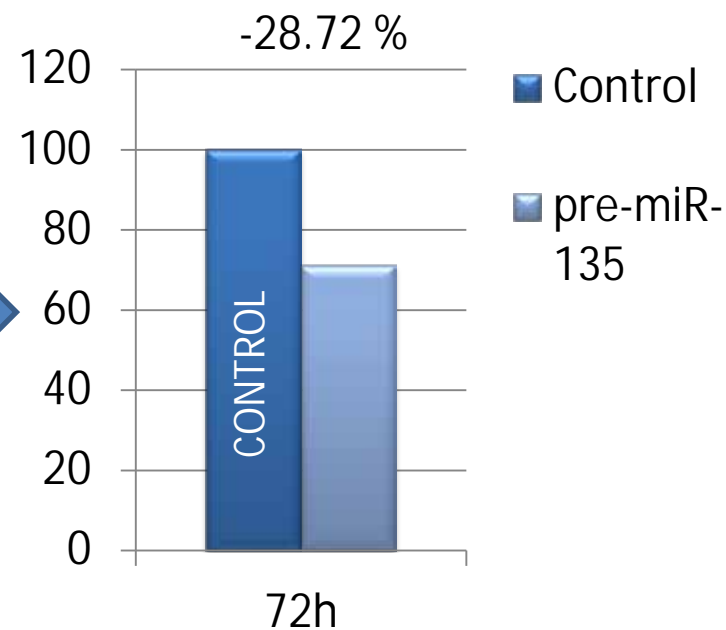
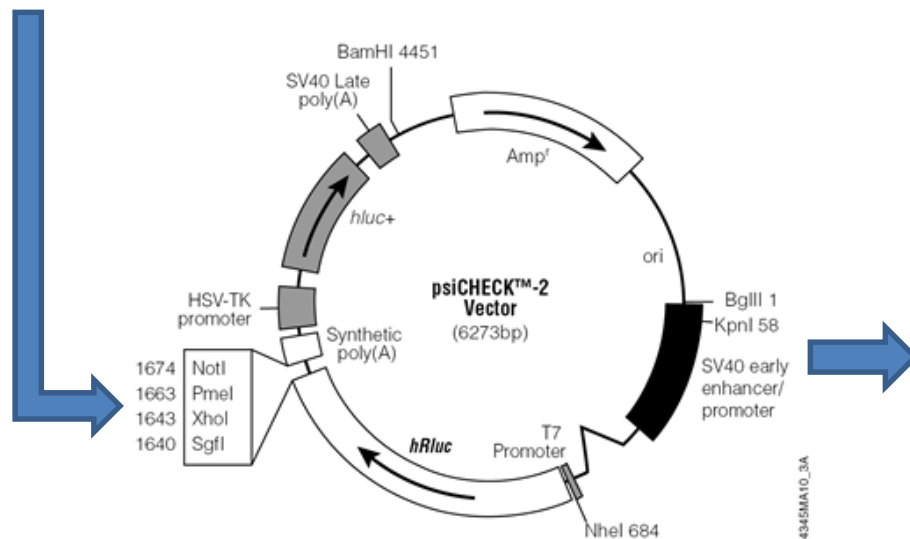
Bcl-xL



Target validation: Renilla/Luciferase assay



48 bp



0.2μM Vector + insert
+
500nM pre-miR-135
or
500nM pre-miR-Negative Control

JAK2

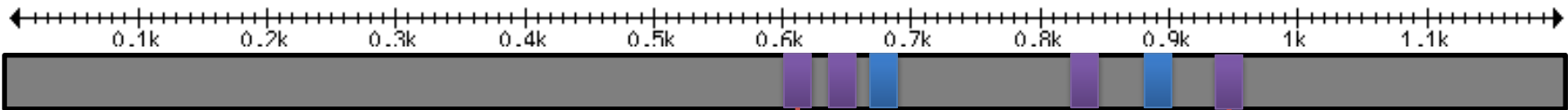
Could other miRNAs regulate the JAK/STAT signalling pathway and impact prognosis in cHL?

223 miRNAs

- ü Expressed in HL
- ü References

375

Human JAK2 3'-UTR region: 1204bp



Type of miRNA binding

■ 8-mer

■ 7-mer-1A

■ miR-101

■ miR-204

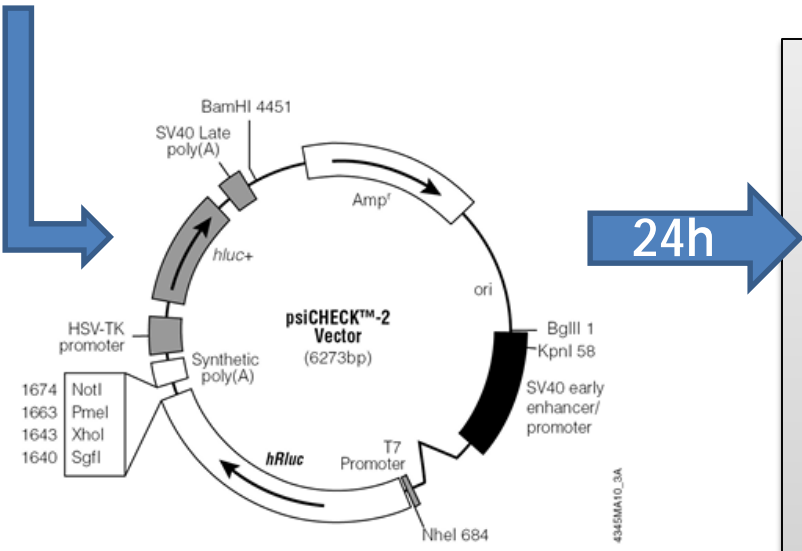
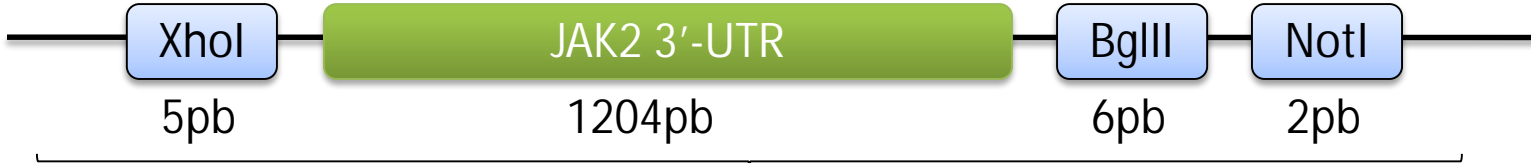
■ miR-375

■ miR-216b

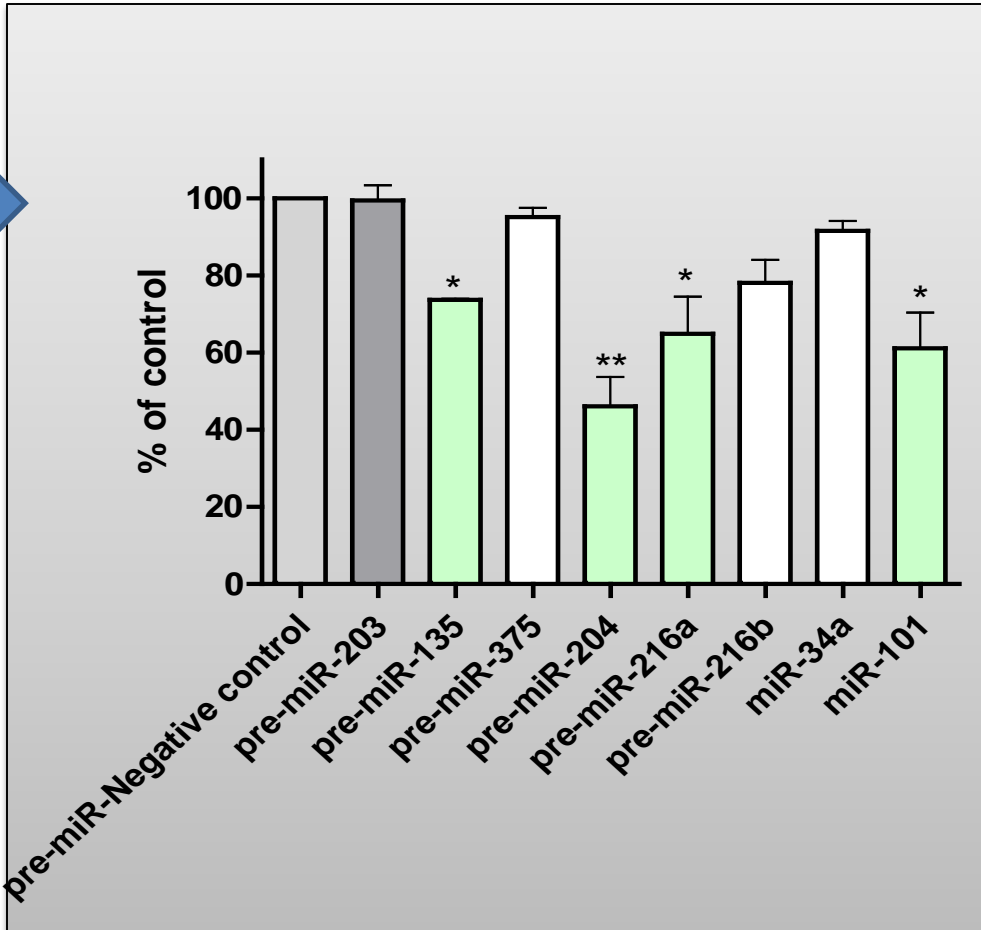
■ miR-34a

■ miR-135a

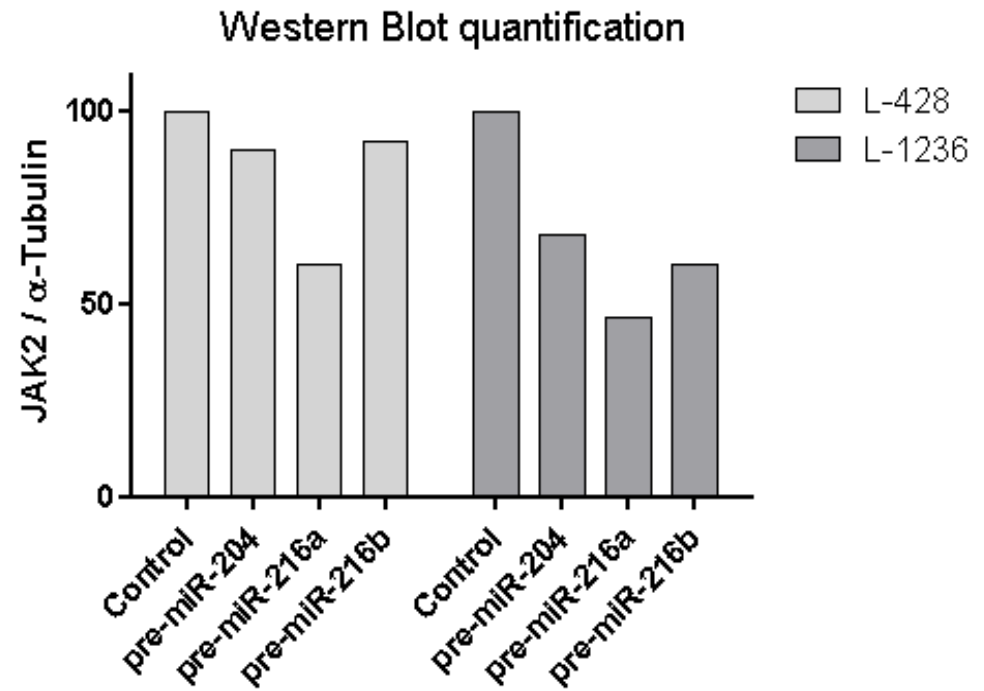
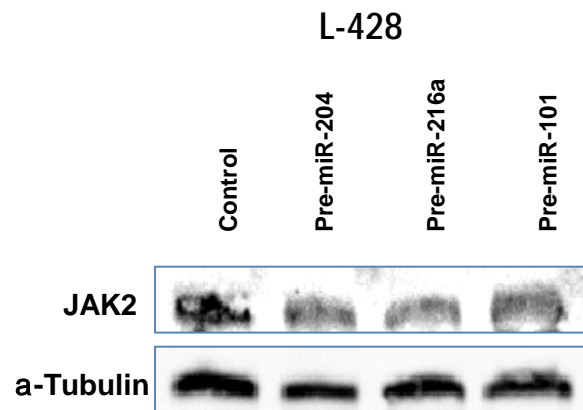
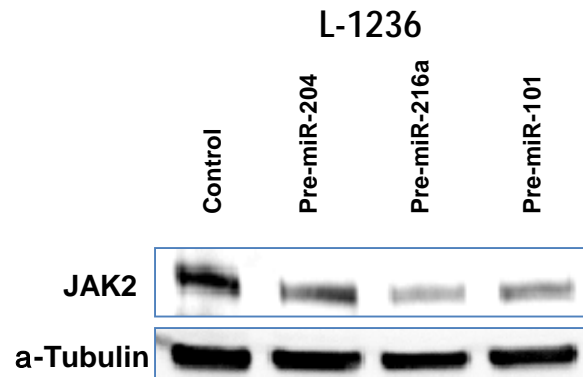
Systematic validation of microRNAs targeting JAK2 by Renilla/Luciferase assay



0.2μM modified Vector
 +
 500nM pre-miRNA
 0
 500nM pre-miRNA Negative Control

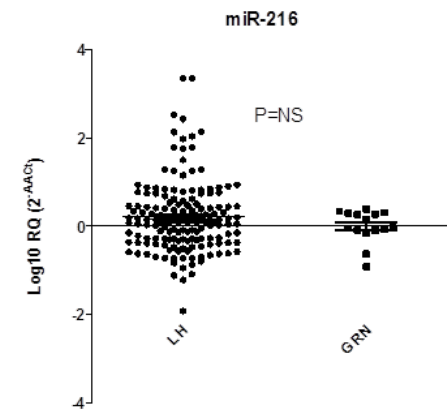
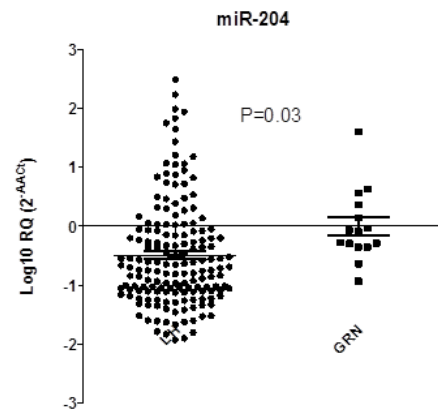
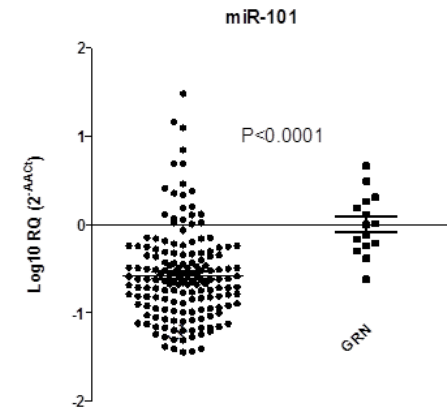
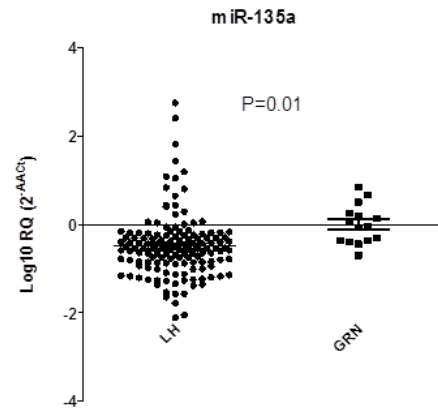


Western Blot Analysis of microRNA modulation of JAK2 protein levels

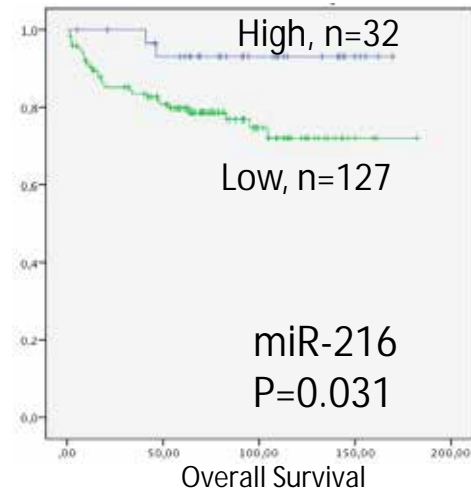
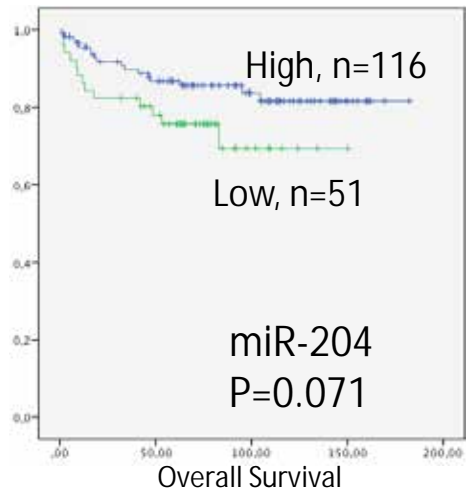
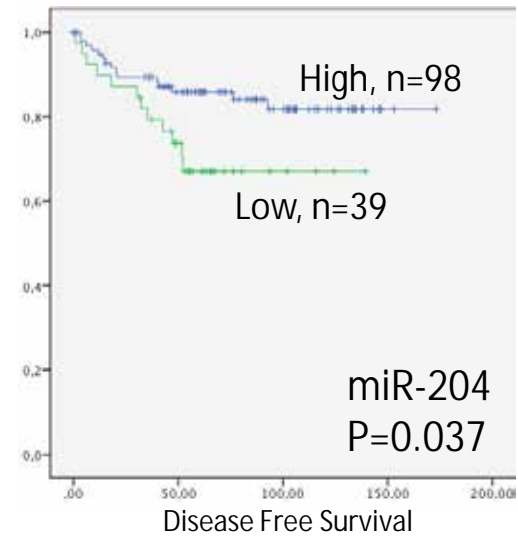
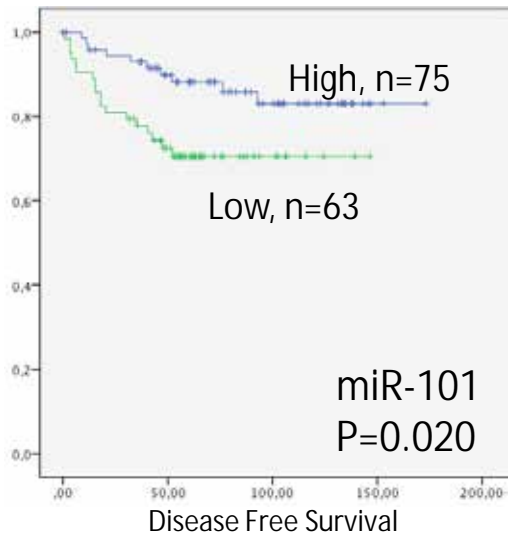


Patients

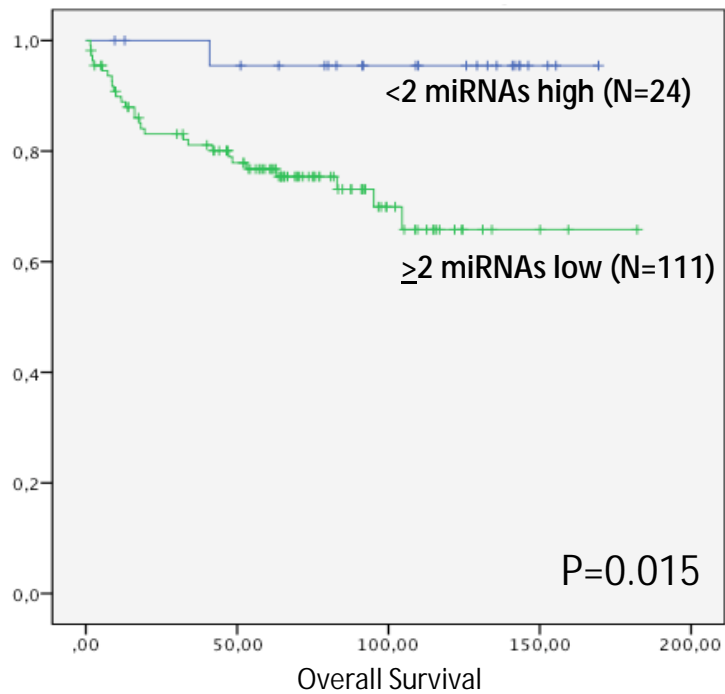
Characteristics	N=168
Age(median, range)	33 (15-89)
Sex(M/F)	86/82 (51.2%-48.8%)
Histology	
NE	95 (56.5%)
MC	27 (16%)
EBV+	56 (43.8%)
HIV+	21 (12.5%)
B Symptoms	72 (43.6%)
Bulky mass	32 (19%)
Anemia(Hb<10 ⁵ g/L)	36 (21.4%)
Leucocytosis (15 x10 ⁹ /L)	20 (11.9%)
Lymphocytopenia	17 (10.2%)
Hypoalbuminemia	60(38.5)
High LDH	55 (33.1%)
High B-2-microglobulin	37(28.5%)
Ann Arbor Stage I-II	102 (60.71%)



Survival analysis according to JAK2-miRNA expression



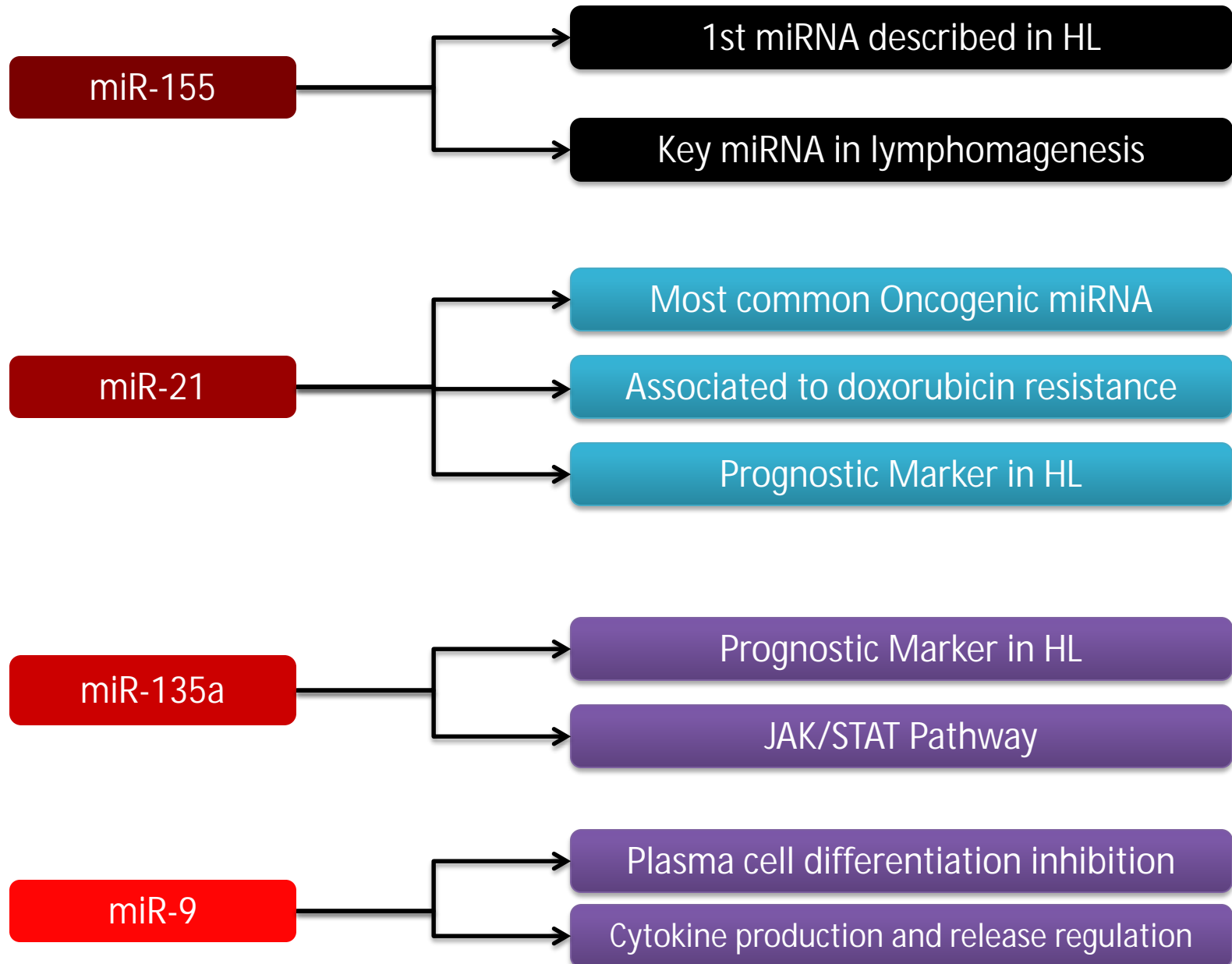
4-microRNA Signature Targeting JAK2



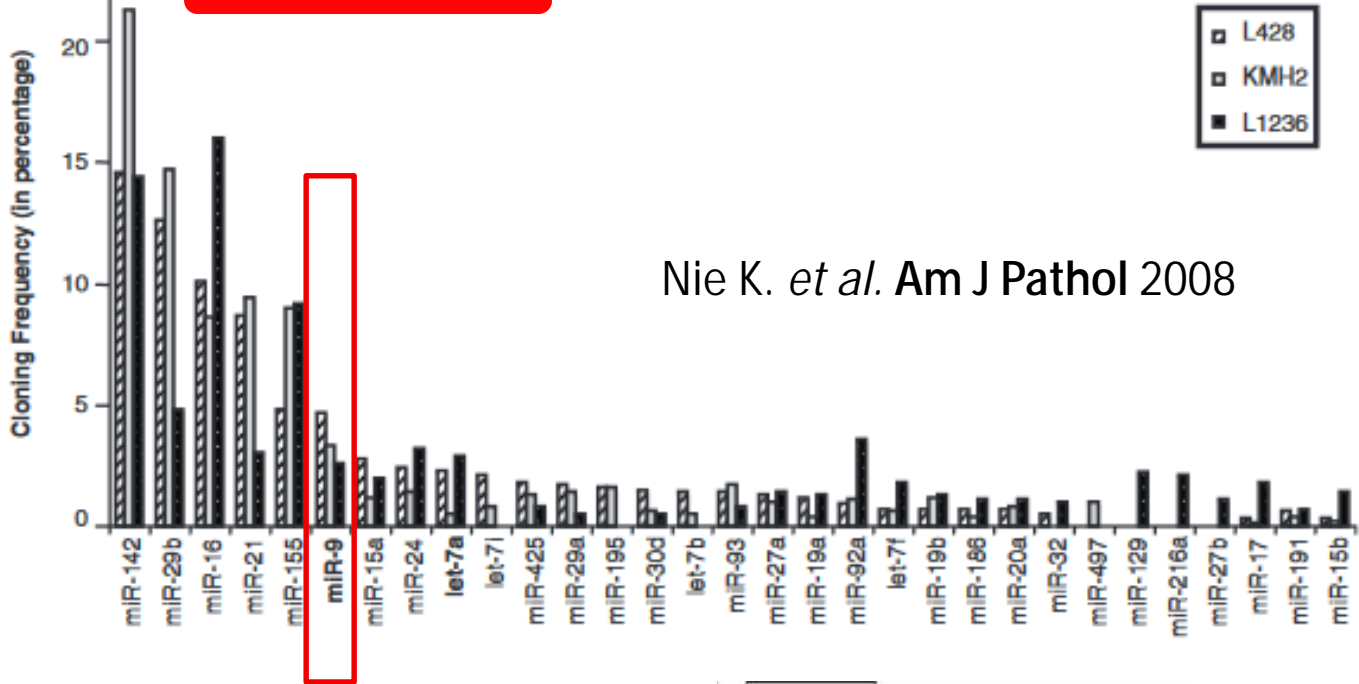
Multivariate analysis

Factor	OR (95%CI)	P-value
4-miRNA signature	7.6 (1.1-57.2)	0.048
Age ≥ 45	5.9 (2.4-14.4)	P<0.001
B Symptoms	2.6 (1.09-6.3)	P=0.03

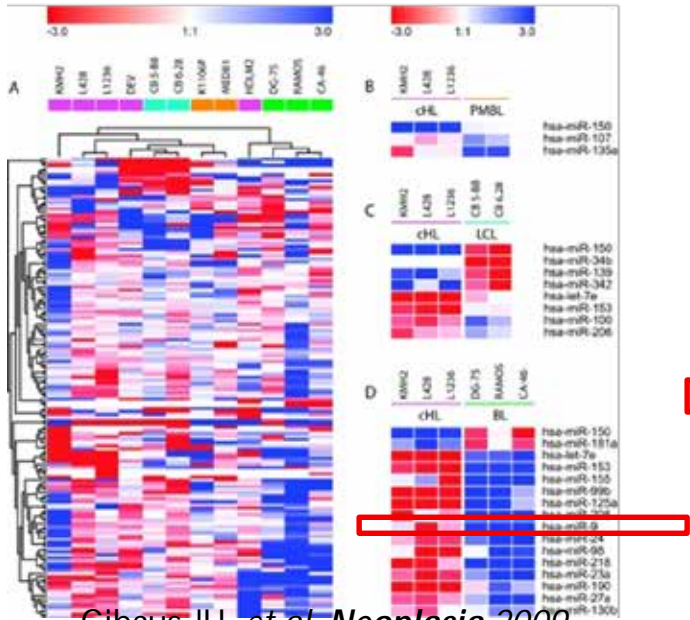
Key miRNAs in Hodgkin lymphoma



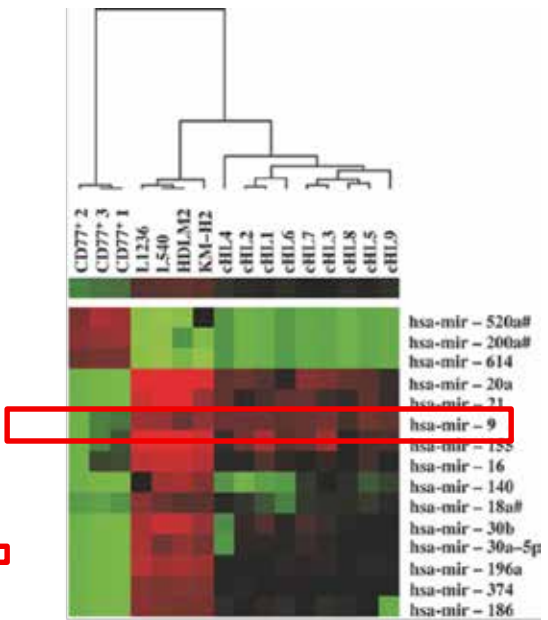
miR-9



Nie K. *et al.* Am J Pathol 2008



Gibcus JH, *et al.* Neoplasia 2009

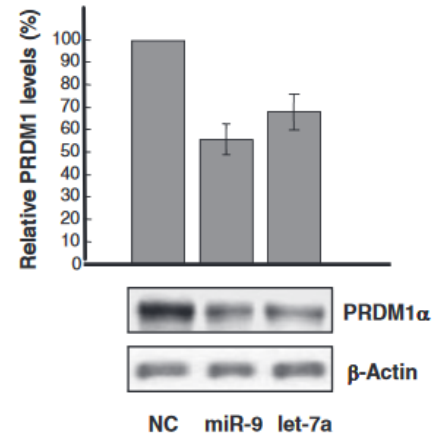


Van Vlierberghe P, *et al.*, *bjh* 2009

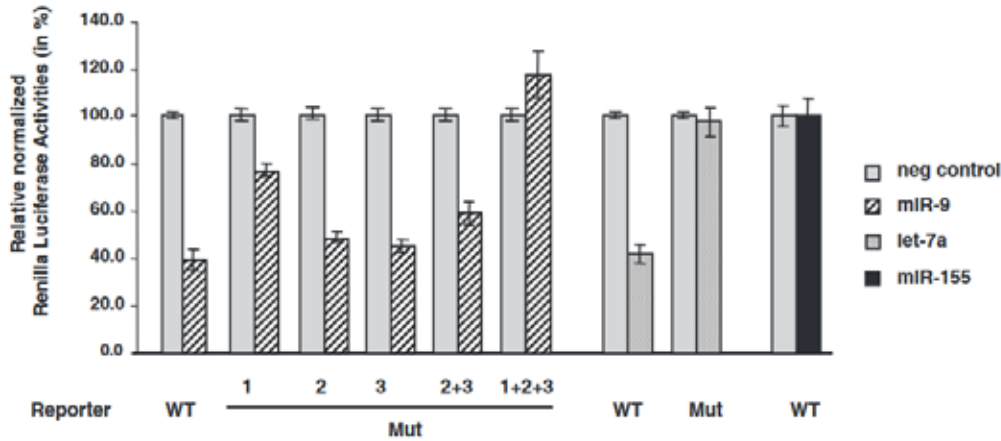
PRDM1 3'UTR



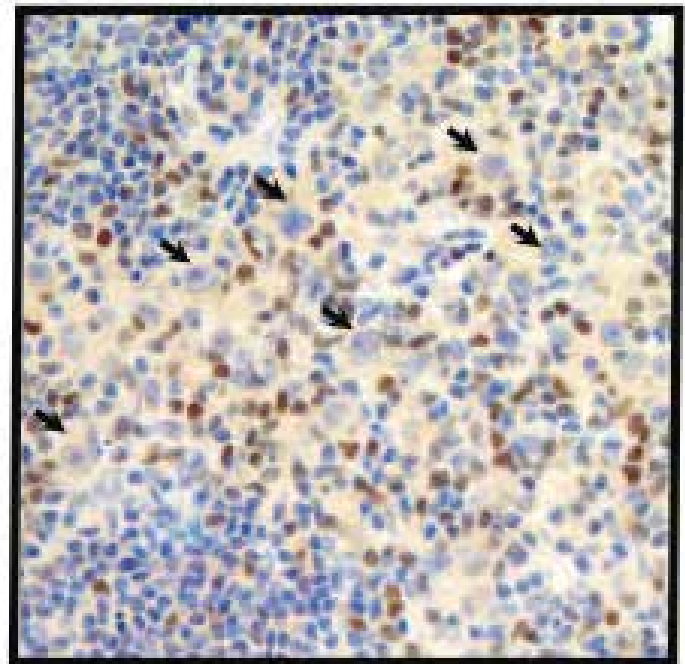
A



A



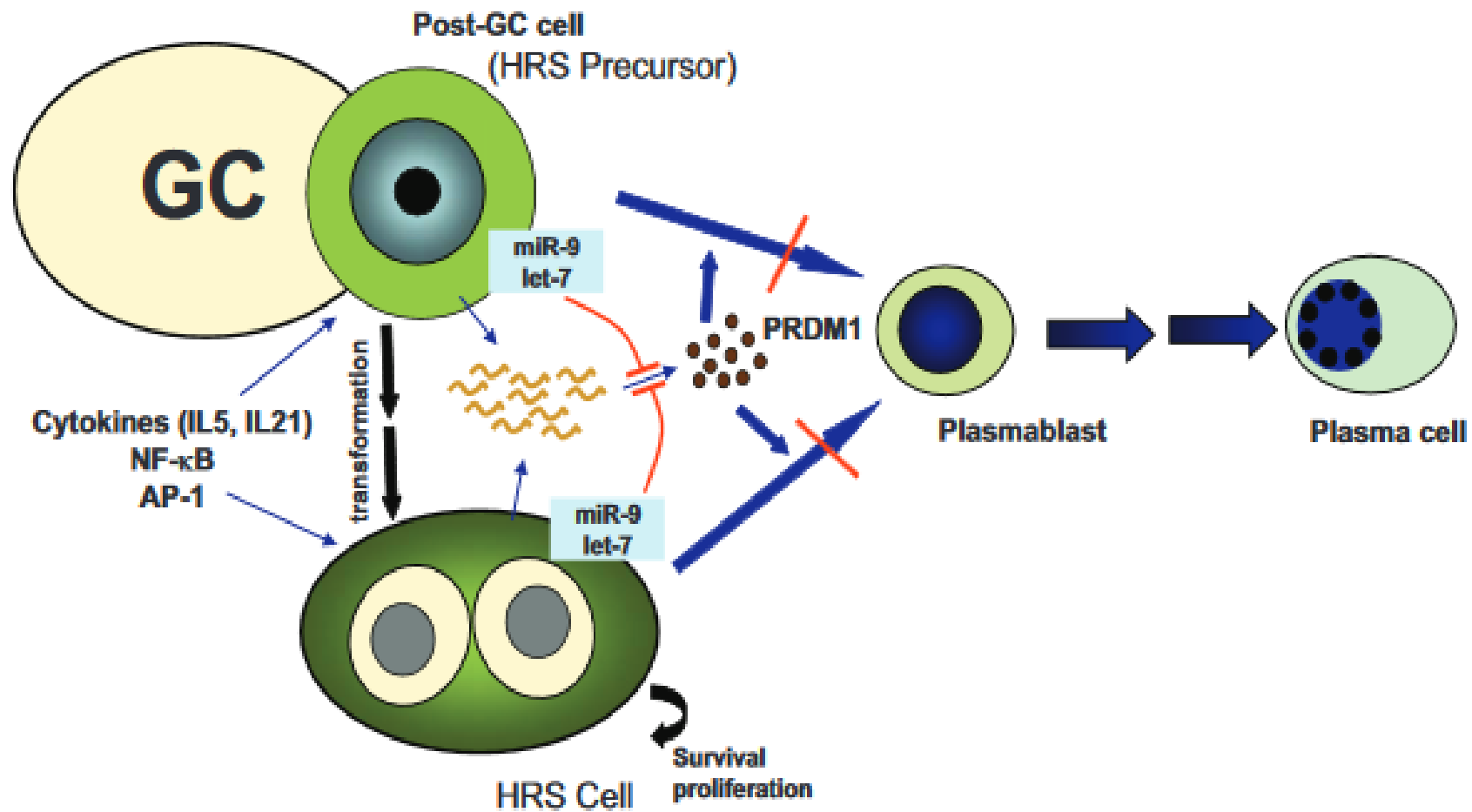
ii



PRDM1-negative HRS cells

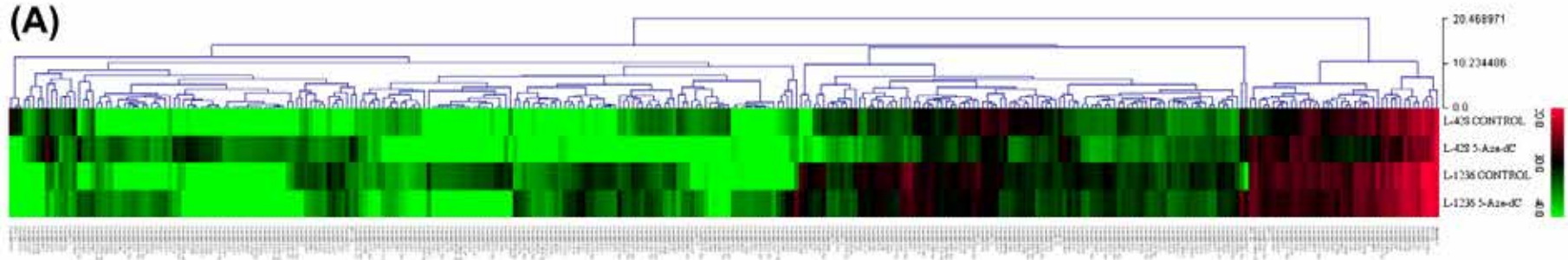
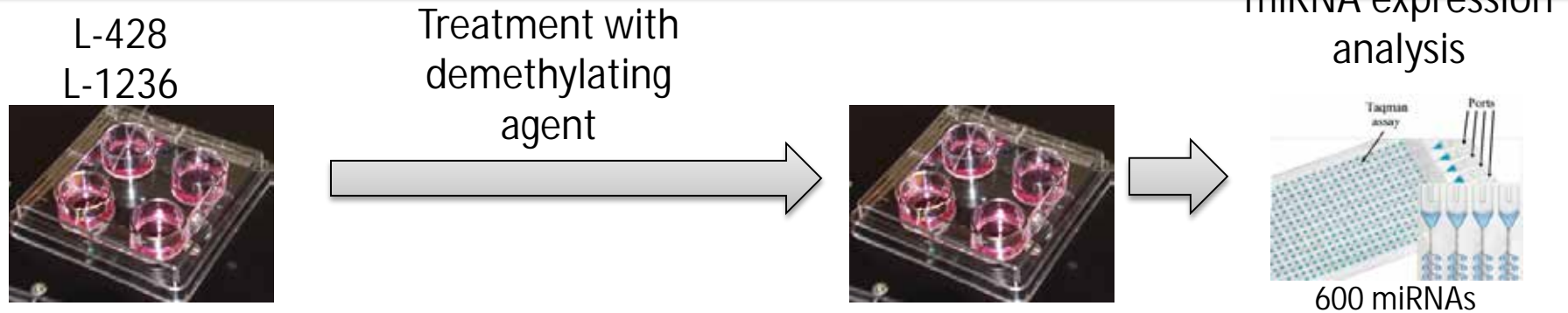
Nie K. *et al.* Am J Pathol 2008

A hypothetical model of microRNA-mediated *PRDM1* inactivation in pathogenesis of HRS cells



1. General epigenetics features of HL
2. Generalities of Non-coding RNAs
3. microRNAs regulating HRS cells crucial pathways
4. **microRNAs regulated by methylation in HL**
5. Conclusions

Identification of miRNAs regulated by methylation in cHL



1p36.22



14q32.33

Isla CpG

MIRNA203 gene

§miR-34a

§miR-99b*

§miR-105

§miR-135b*

§miR-203

§miR-337-5p

§miR-342-5p

§miR-490-3p

§miR-512-3p

§miR-517a

§miR-517c

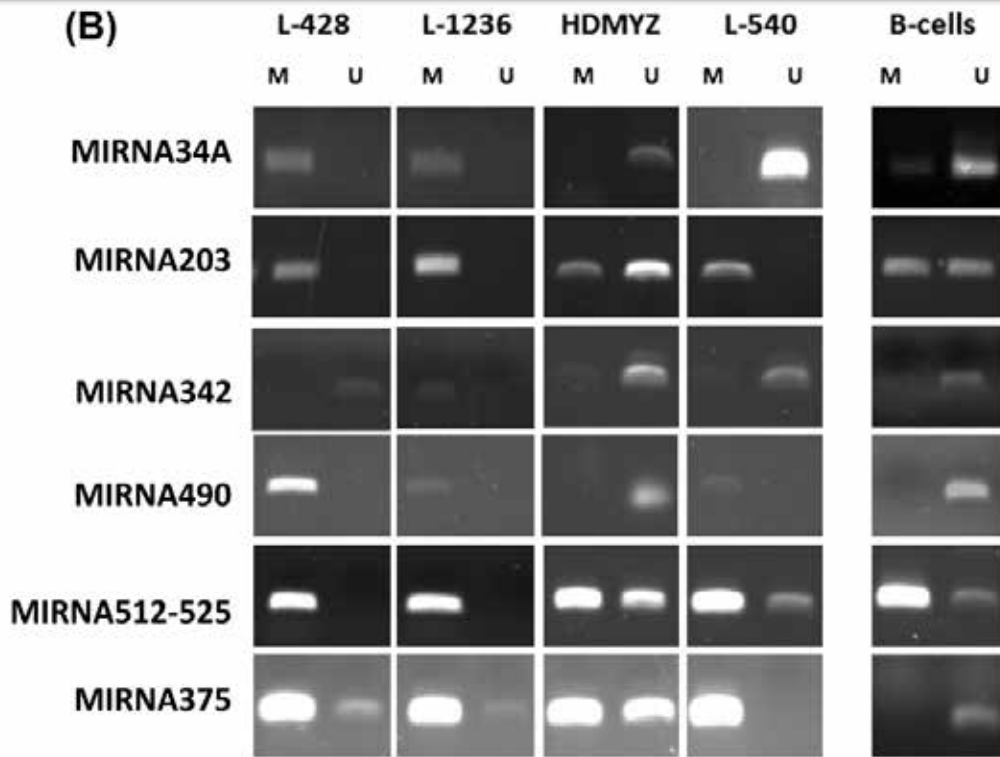
§miR-518f

§miR-519a

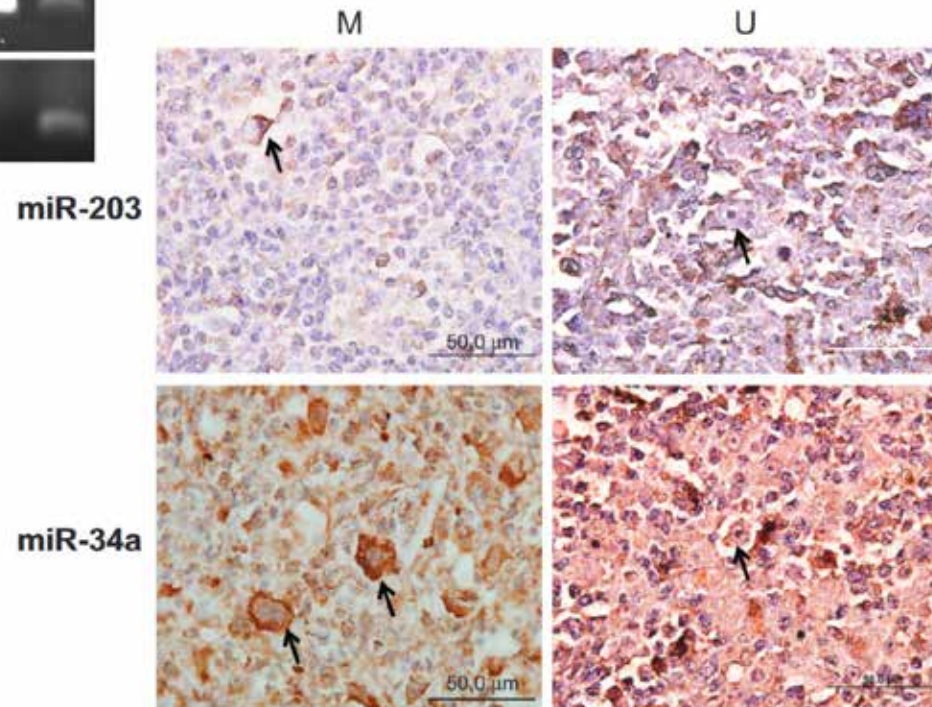
§miR-520g

§miR-525-3p

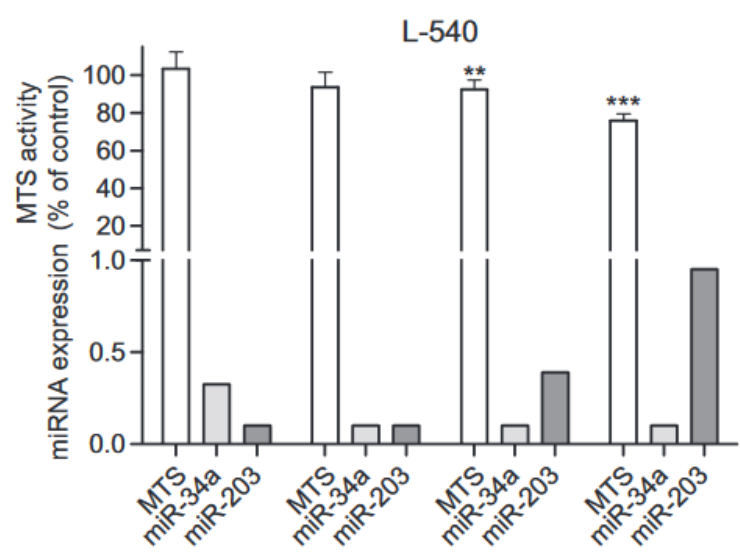
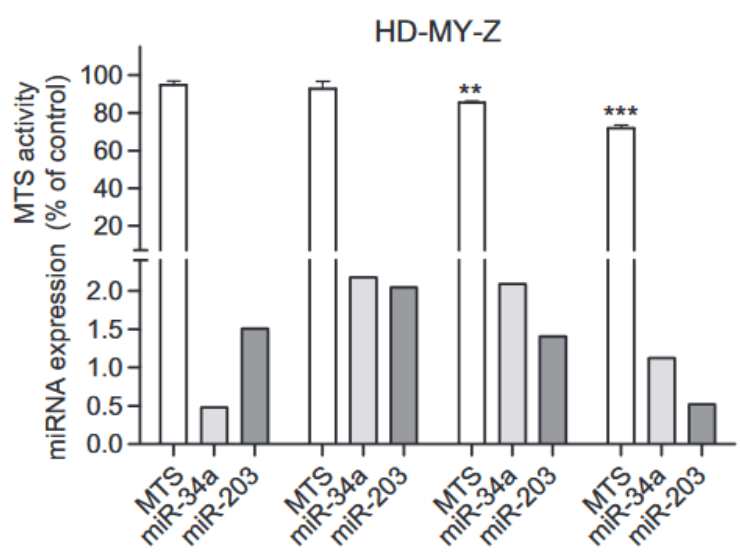
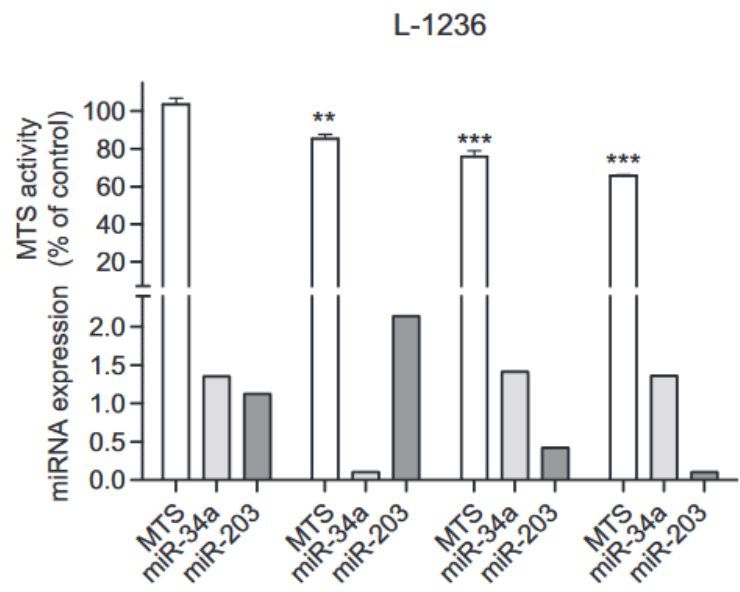
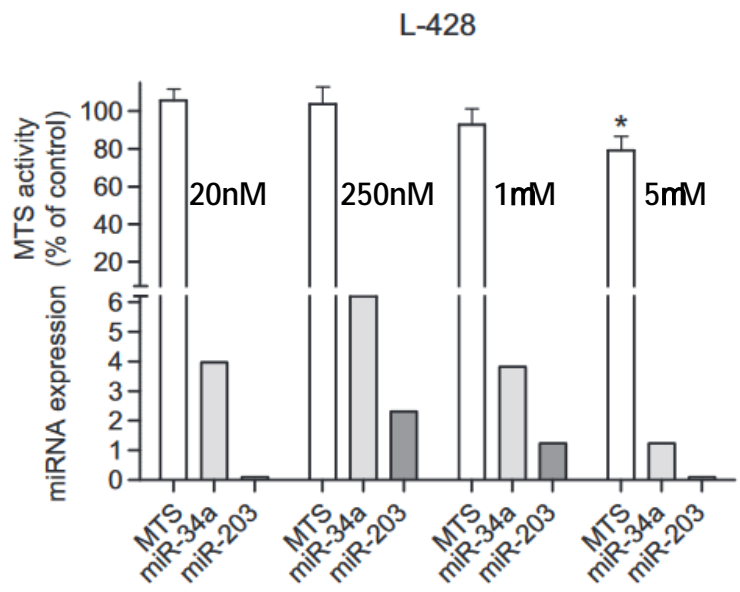
Validation by MS-PCR and by in situ MSPC



In situ MS-PCR



5-Aza-dC treatment inhibits proliferation at high doses and produces re-expression of miR-34a and miR-203 at low-intermediate doses.

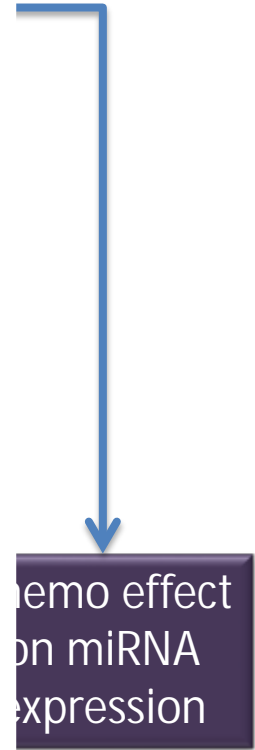


1. General epigenetics features of HL
2. Generalities of Non-coding RNAs
3. microRNAs regulating HRS cells crucial pathways
4. microRNAs regulated by methylation in HL
- 5. Conclusions**

Therapeutic applications?



miR-135a and JAK2



Therapeutic effect
on miRNA
expression

AZA
PBA
R-127 and BCL6

Ongoing miRNA-based clinical trials on lymphoma

A Multicenter Phase I Study of MRX34,
MicroRNA miR-RX34 Liposomal Injection



Recruiting
participants

Primary Liver Cancer
SCLC
Lymphoma
Melanoma
Multiple Myeloma
Renal Cell Carcinoma
NSCLC

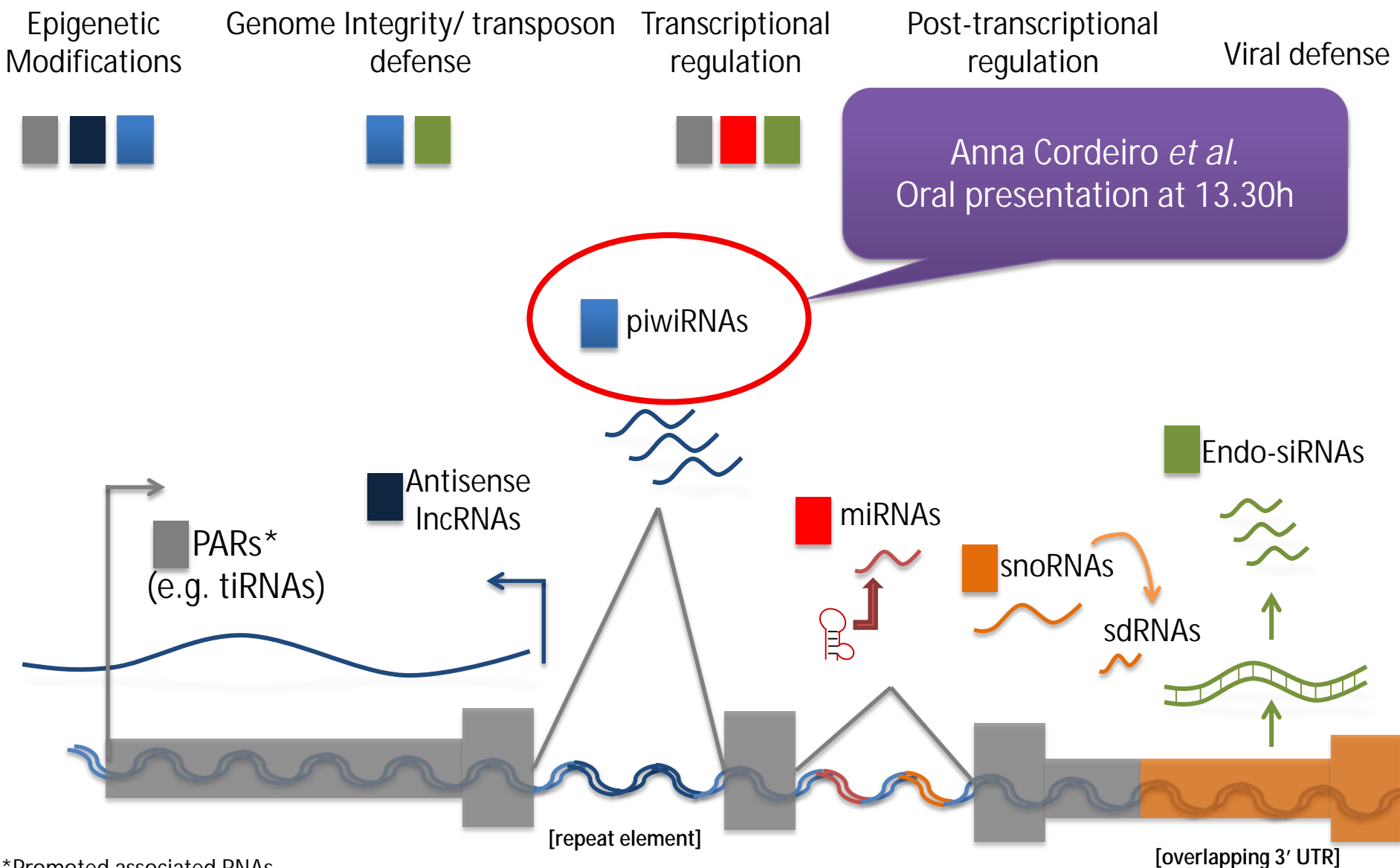
miRNA
THERAPEUTICS



MRX34 is a first-in-class
cancer therapy and the
first microRNA mimic to
enter clinical trials.

There is still a long way to go on miRNA based treatments...

There are a lot of non-coding RNAs waiting to be studied in HL



*Promoted associated RNAs

Acknowledgments

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Dolors Fuster
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Dr. Antonio Martínez
Dr. Blanca Gonzalez

Hematology - Hospital Clínic

Dr. Anna Gaya
Dr. Carmen Martínez
Dr. Marina Díaz-Beyá



Molecular Oncology and Embryology Laboratory

Human Anatomy Unit

School of Medicine – UB

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UNIVERSITAT DE
BARCELONA

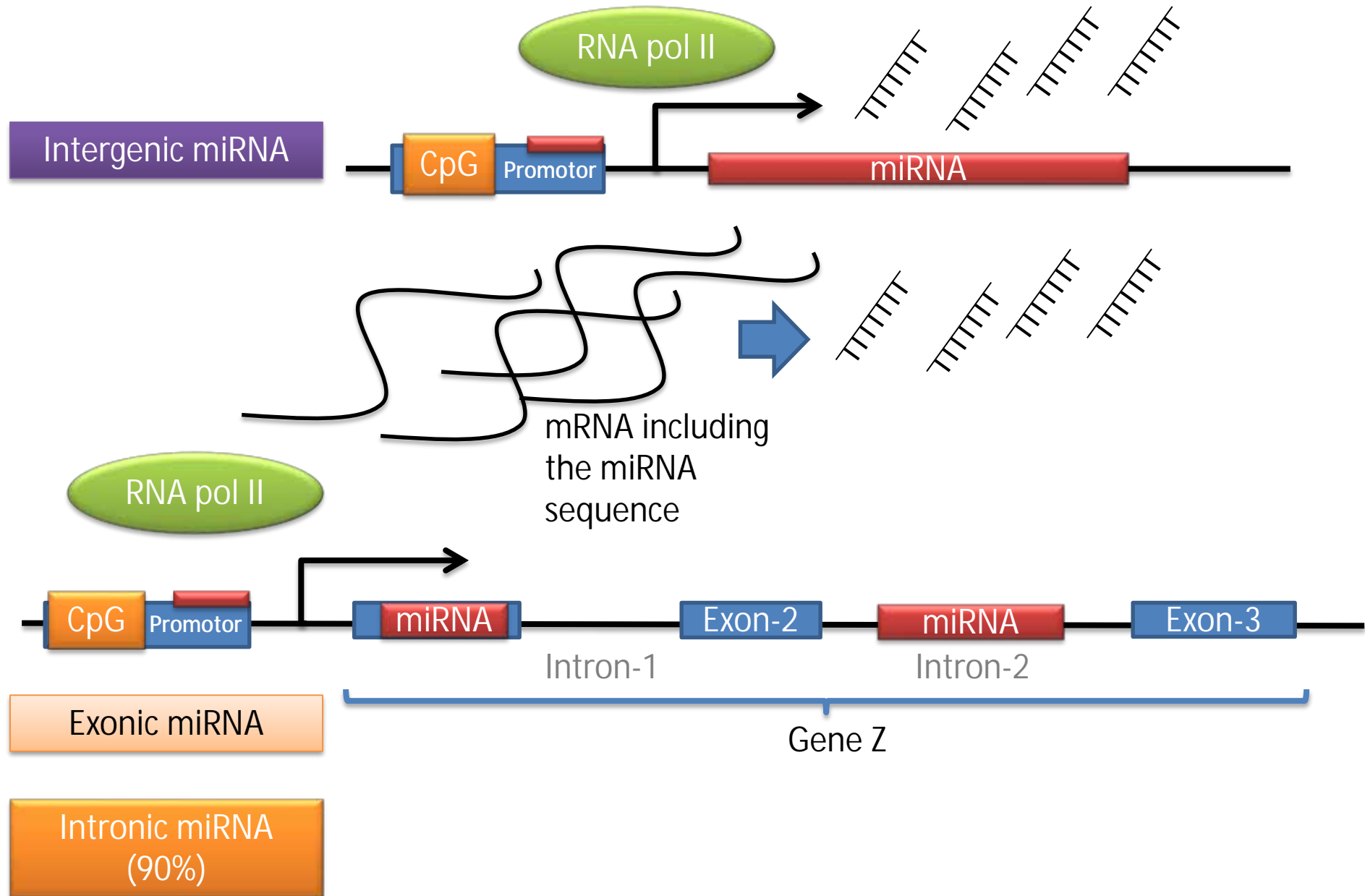
CLÍNIC
BARCELONA
Hospital Universitari

Funding

AECC-Cataluña



Transcriptional regulation of microRNAs



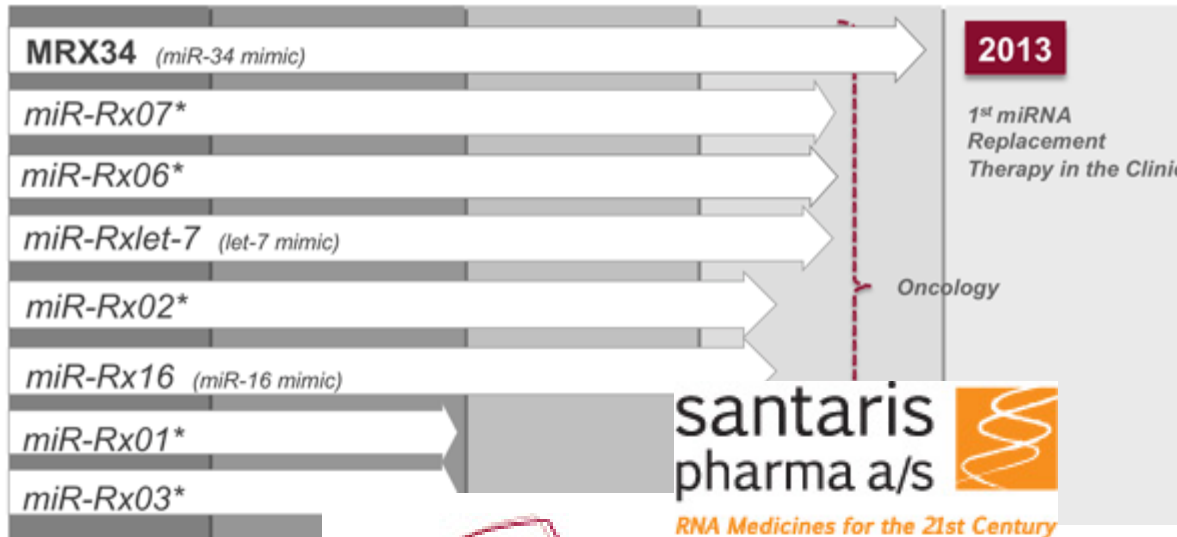
Ongoing miRNA-based clinical trials

miRNA	Indication	Phase	Status
miRNA antagonist			
miR-122 <i>Drug: Miravirsen</i>	Hepatitis C virus	Phase 2	Completed Janssen, et al. N Engl J Med 2013
miRNA replacement			
miR-34 <i>Drug: MRX34</i>	Primary unresectable liver cancer or other primary tumors with liver metastases	Phase 1	Recruiting participants

santaris
pharma a/s 
RNA Medicines for the 21st Century

miRNA
THERAPEUTICS

miRNA-based molecules in preclinical development

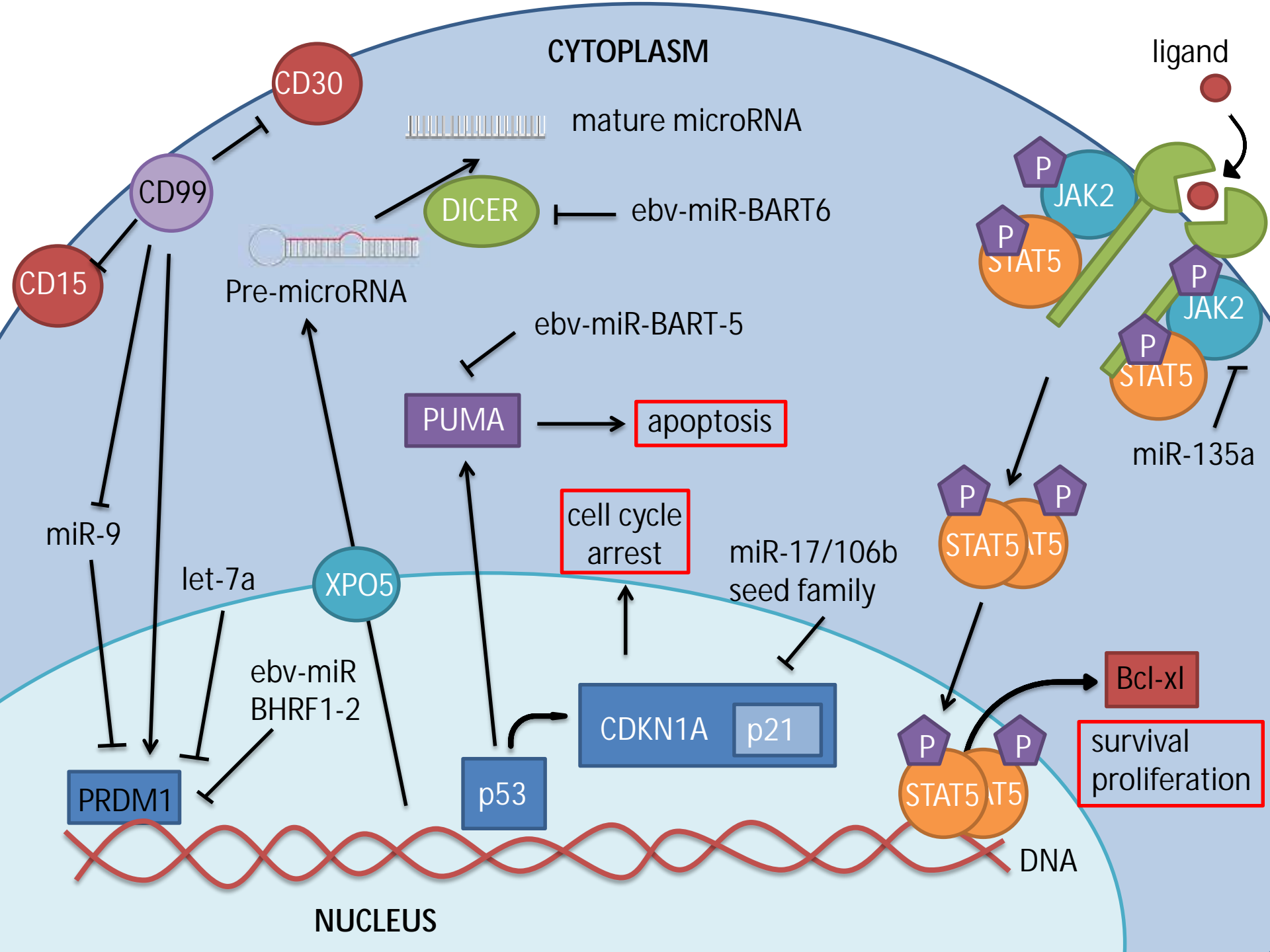


DISCOVERY **EFFICACY**

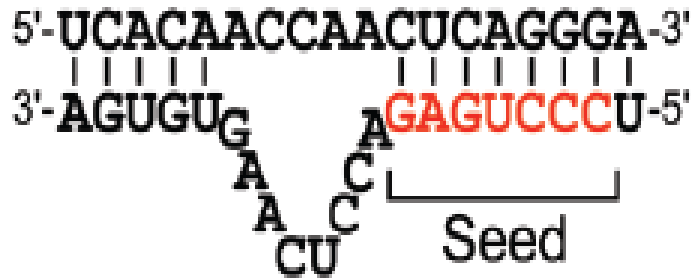
* miRNA mimics undisclosed



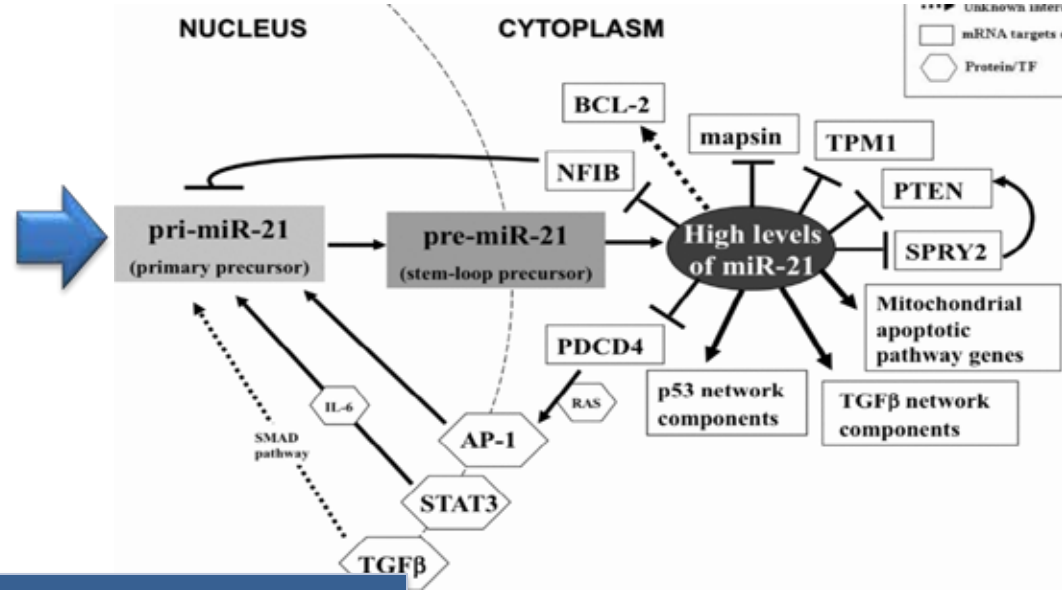
INDICATION	miR TARGET	TARGET VALIDATION	LEAD OPTIMIZATION	PRE-CLINICAL	PHASE I
AntimiR Programs					
MGN-9103	Chronic Heart Failure	208/499			
MGN-1374	Post-MI Remodeling	15/195			
MGN-4893	Polycythemia Vera	451			
PromiR Programs					
MGN-4220	Cardiac Fibrosis	29			
Target Validation Programs					
MGN-6114	Peripheral Arterial Disease	92			
MGN-5804	Cardiometabolic Disease	378			
MGN-2677	Vascular Disease	143/145			
MGN-8107	Amyotrophic Lateral Sclerosis	206			



Targets of miRNAs



“Seed sequence”



Database	Web address
MIRBASE	http://microrna.sanger.ac.uk/
TARGETSCAN	http://www.targetscan.org/
TARBASE	http://www.diana.pcbi.upenn.edu/tarbase.html
MIRANDA	http://www.microrna.org/
miRNAMap	http://mirnamap.mbc.nctu.edu.tw/
miRGen	http://www.diana.pcbi.upenn.edu/miRGen.html
DIANA-MicroT Analyzer	http://diana.pcbi.upenn.edu/

- They have more than one target
- Their expression is tissue and cell type dependent

Main functions of miRNAs in hematology

- Hematopoietic stem cell regulation
 - Ø Hematopoietic differentiation
- Active role in carcinogenesis
 - Ø miRNAs as oncogenes
 - Ø miRNAs as tumor suppressor genes

Normal hematopoiesis and miRNAs



MicroRNAs Modulate Hematopoietic Lineage Differentiation

Chang-Zheng Chen, *et al.*

Science **303**, 83 (2004);

DOI: 10.1126/science.1091903



miR-181a, miR-223 y miR-142

They are specifically expressed in hematopoietic cells and their expression is dynamically regulated during early hematopoiesis and lineage commitment.

miRNAs in normal hematopoiesis

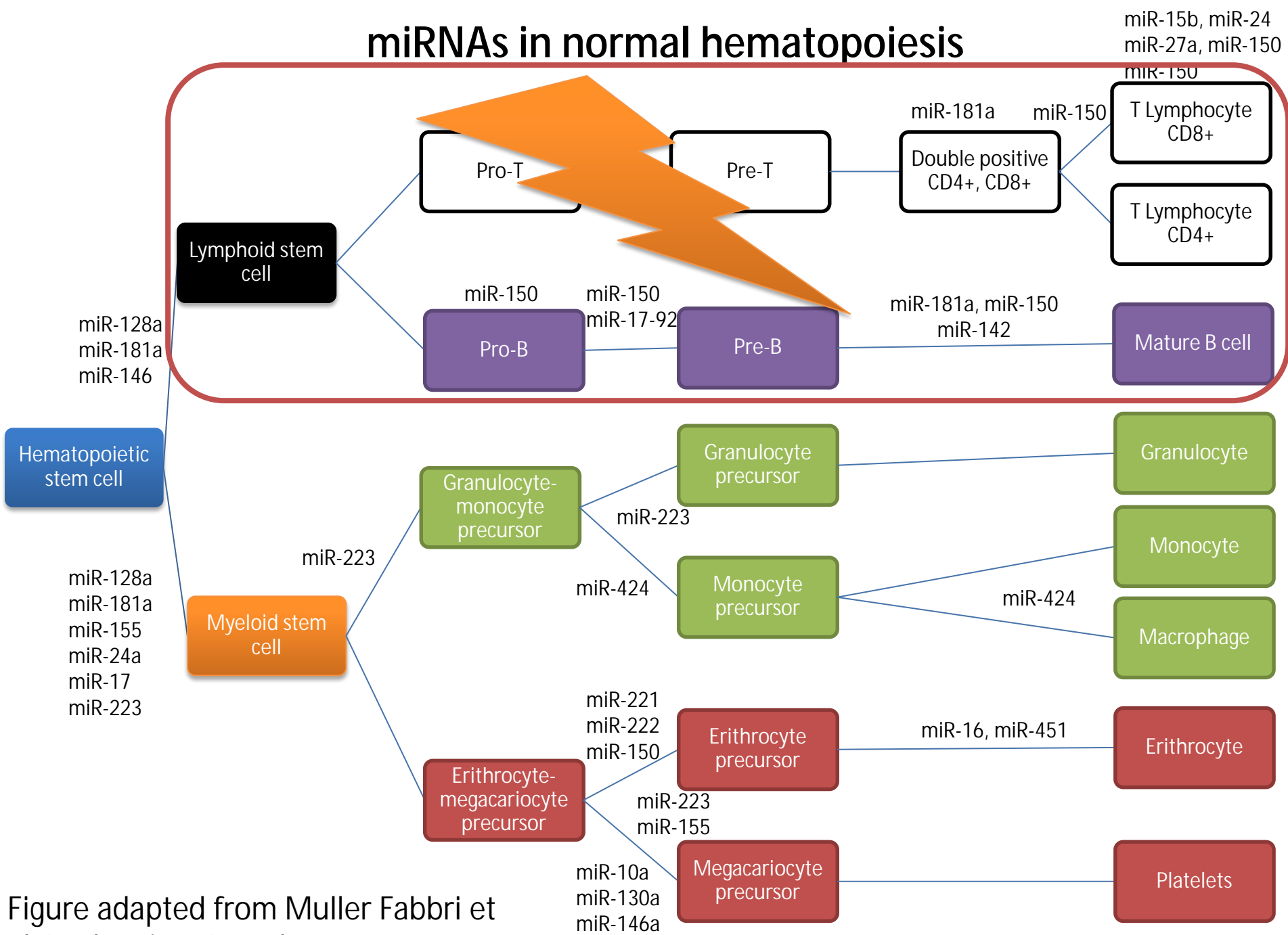
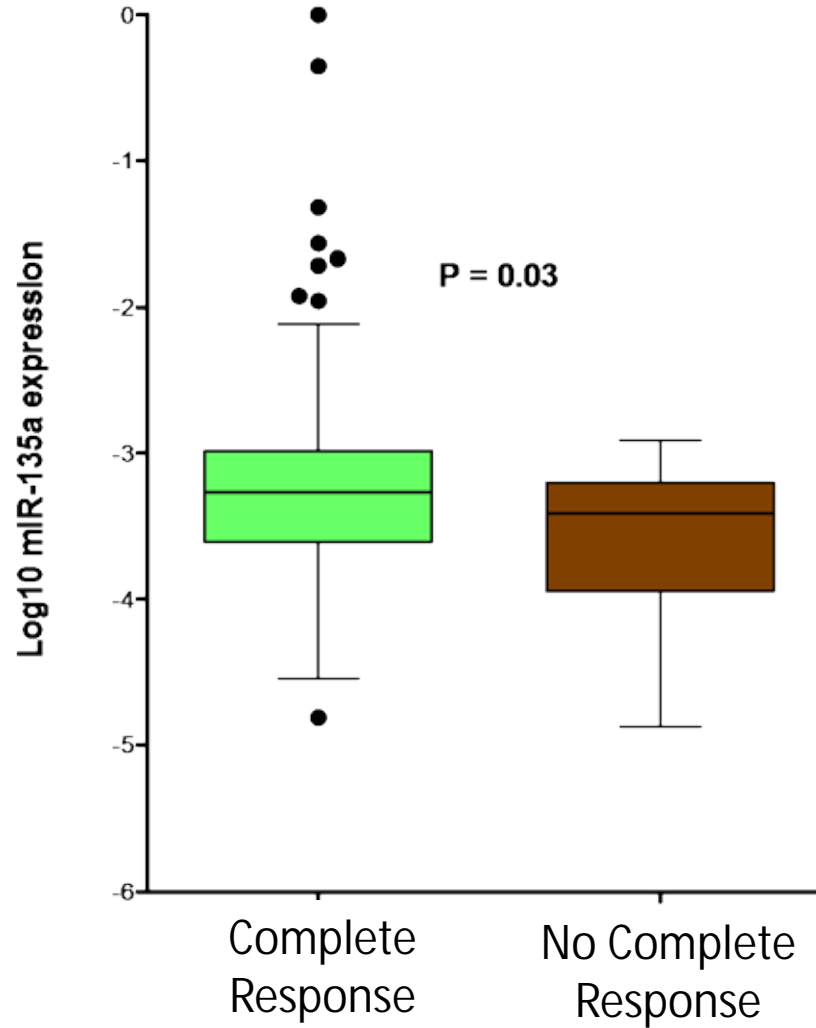


Figure adapted from Muller Fabbri et al. Leukemia & Lymphoma 2009

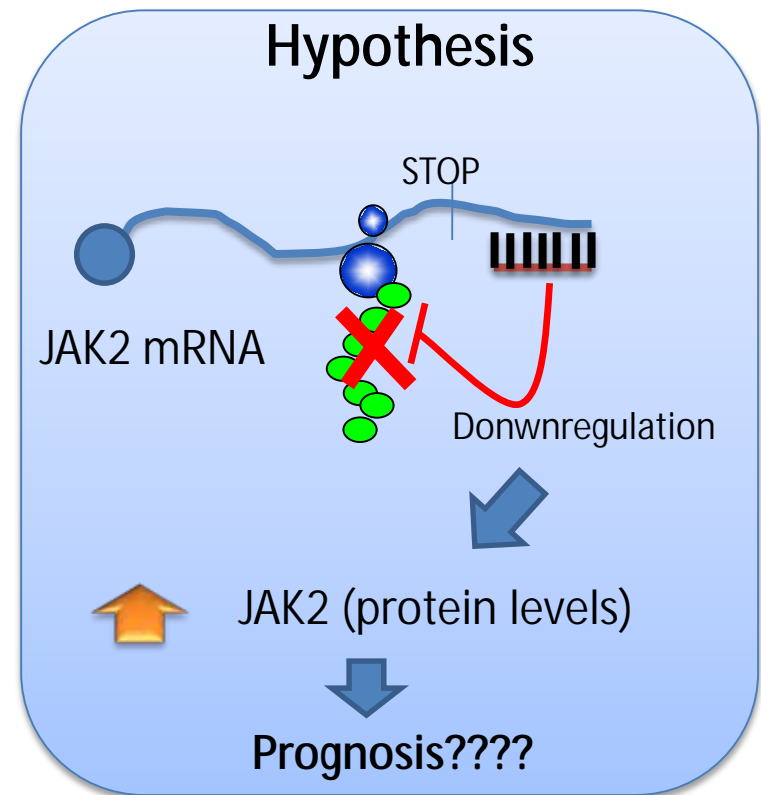
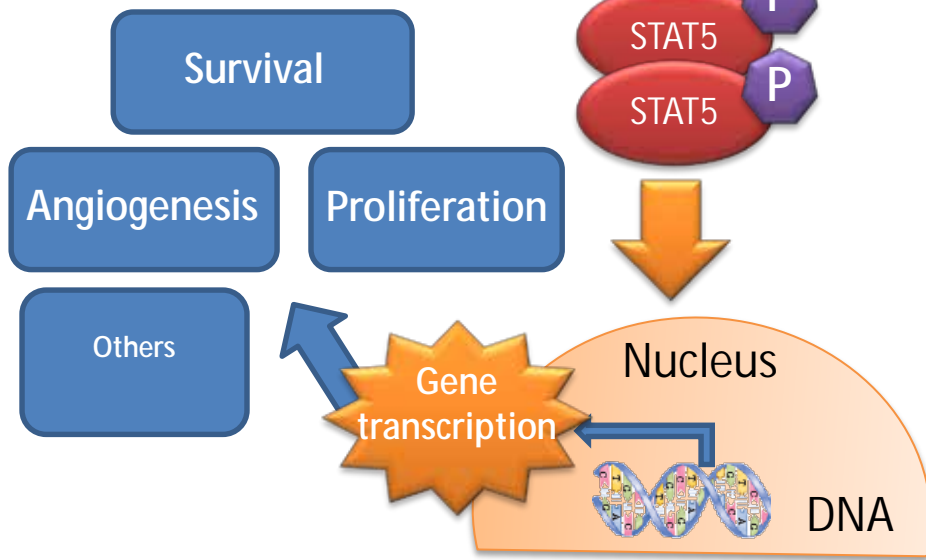
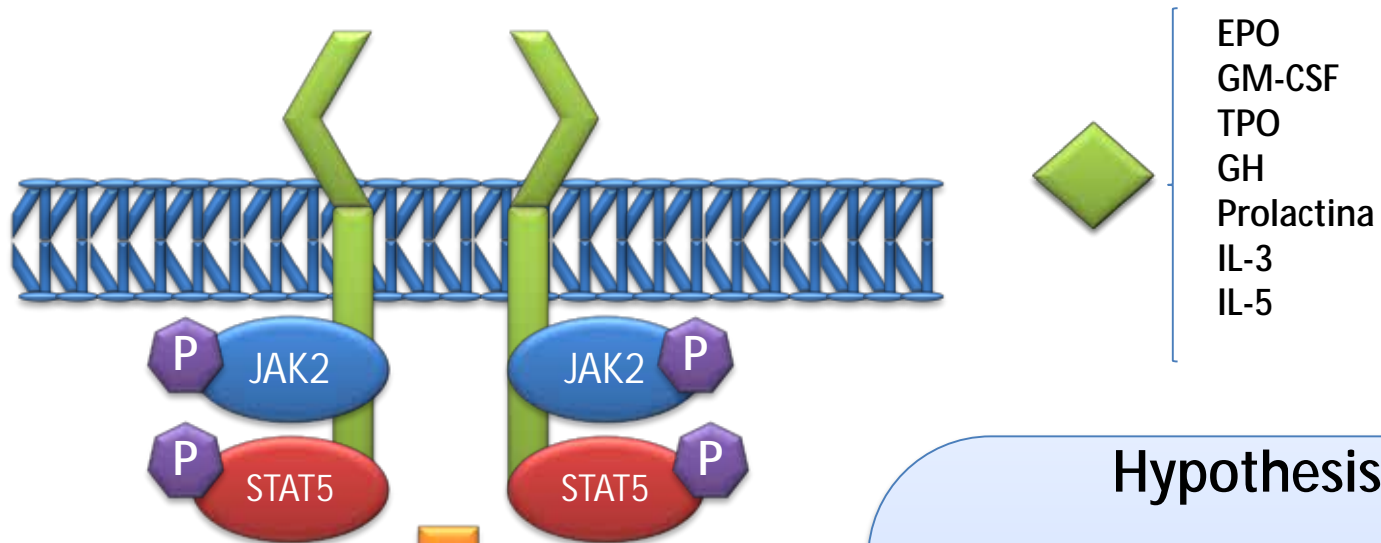
miR-135a levels and treatment response

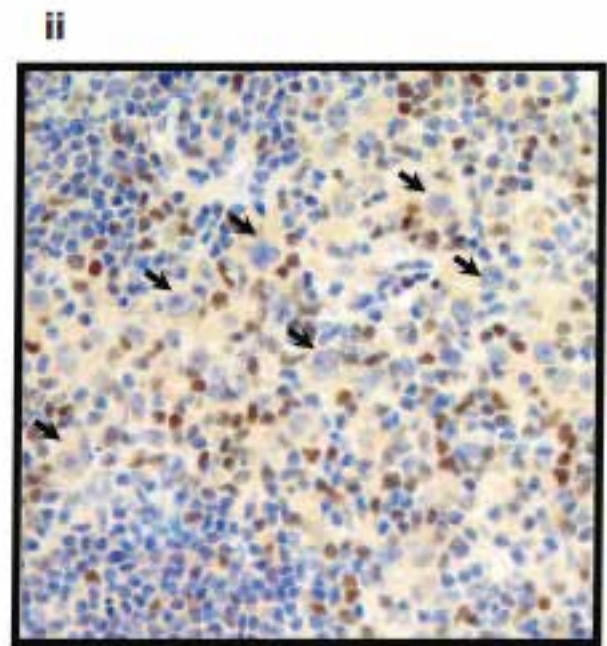
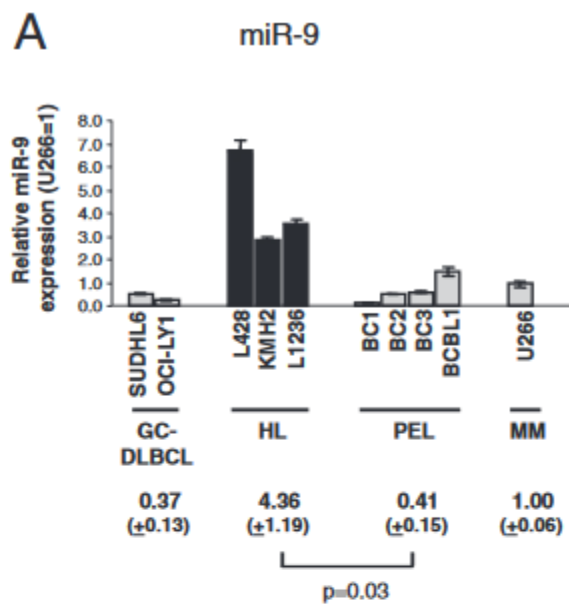


Epstein Barr virus in HL

- Infects B cells
- Type II latency with expression of EBNA-1, LMP-1, LMP-2A/B & EBERs
- 40% of cHL cases are EBV+
- LMP1 mimics CD40 and activates survival mechanisms in B cells
- More frequently found in mixed cellularity subtype

Could other miRNAs regulate the JAK/STAT signalling pathway and impact prognosis in cHL?





PRDM1-negative HRS cells

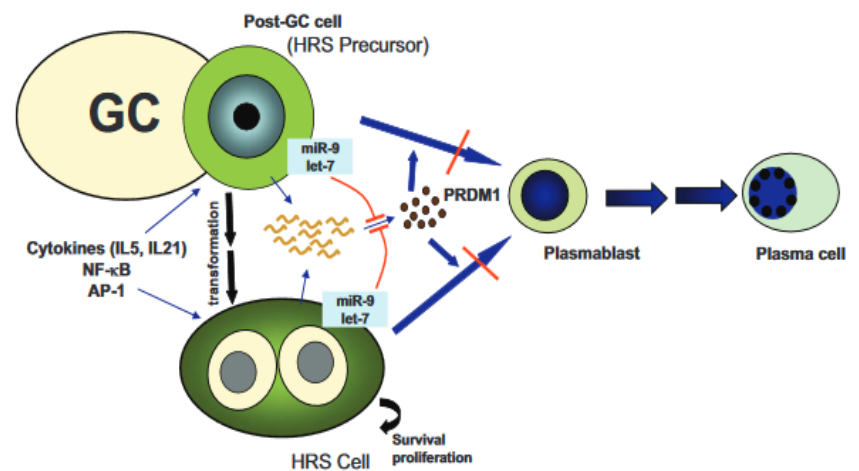
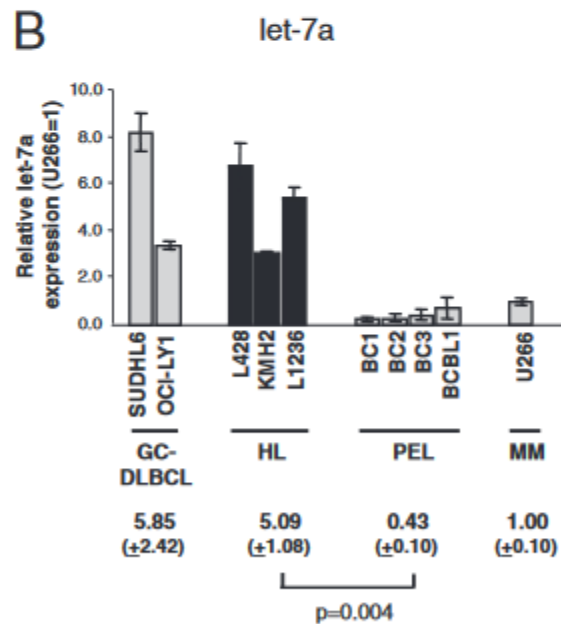
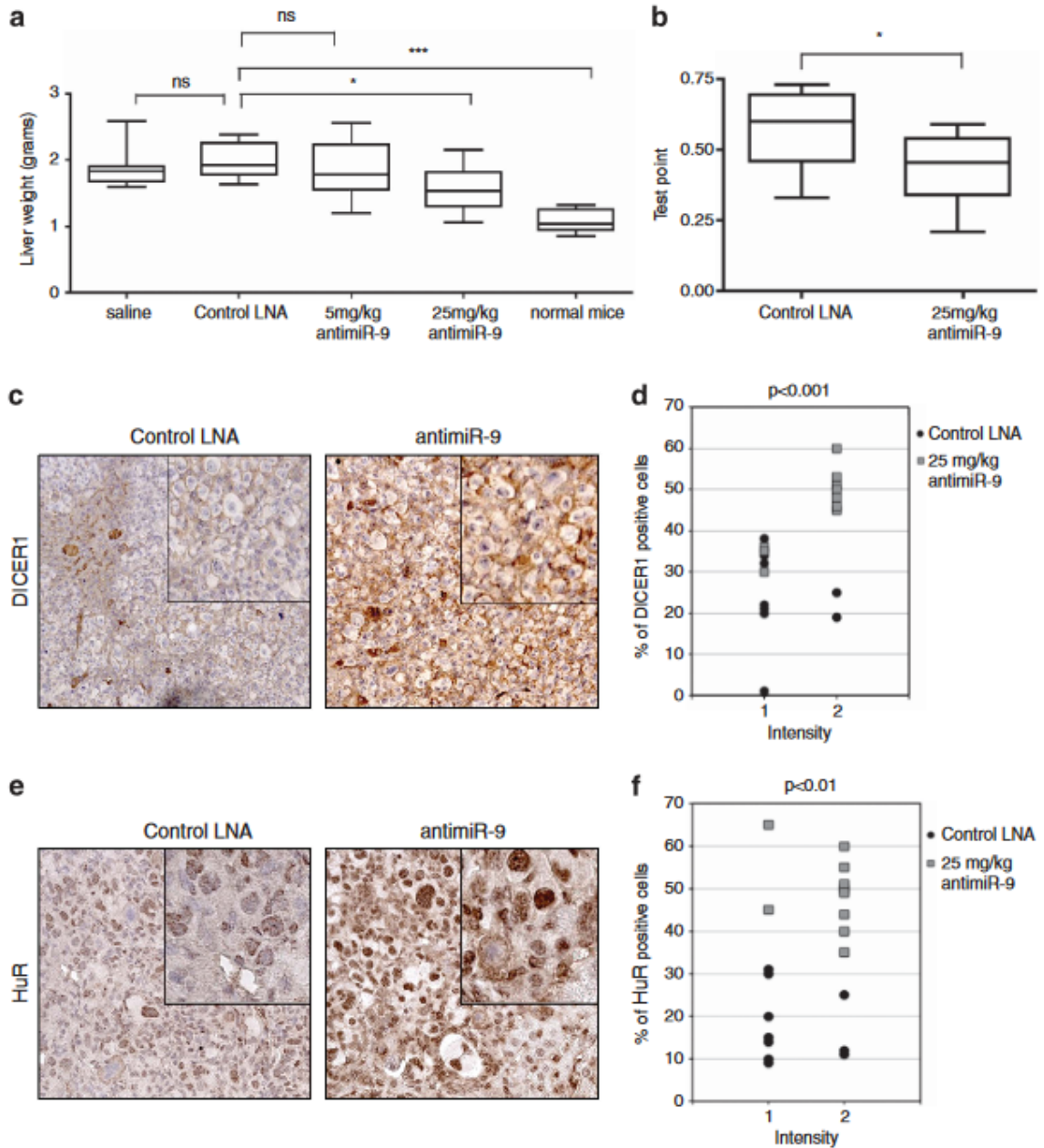
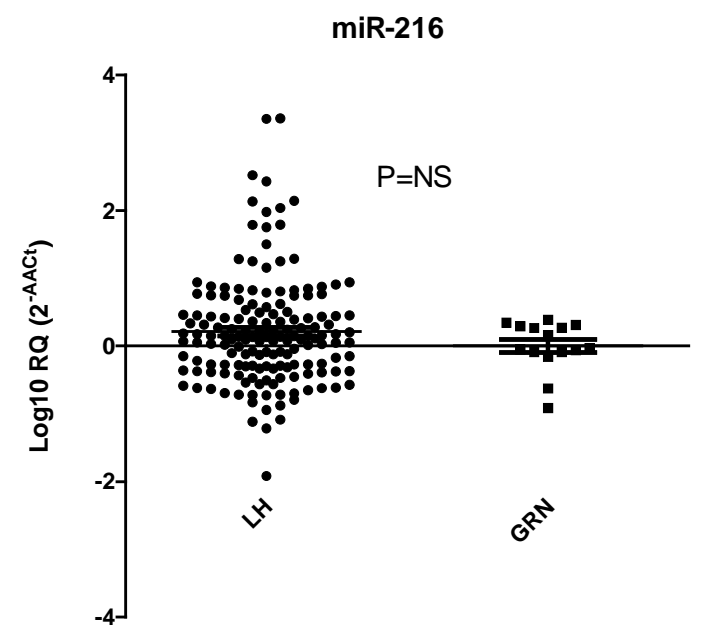
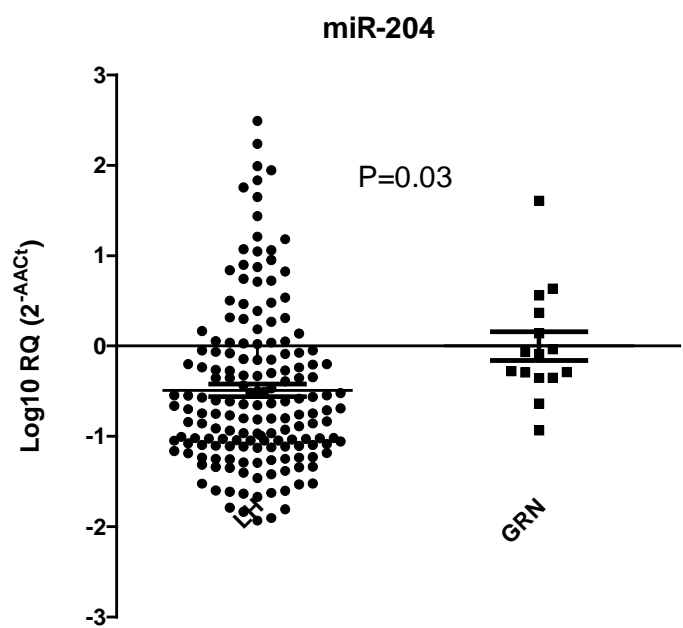
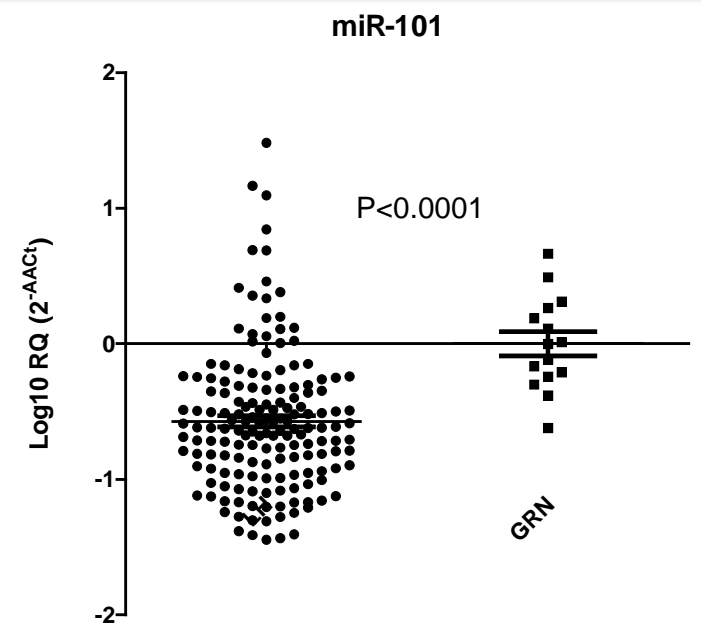
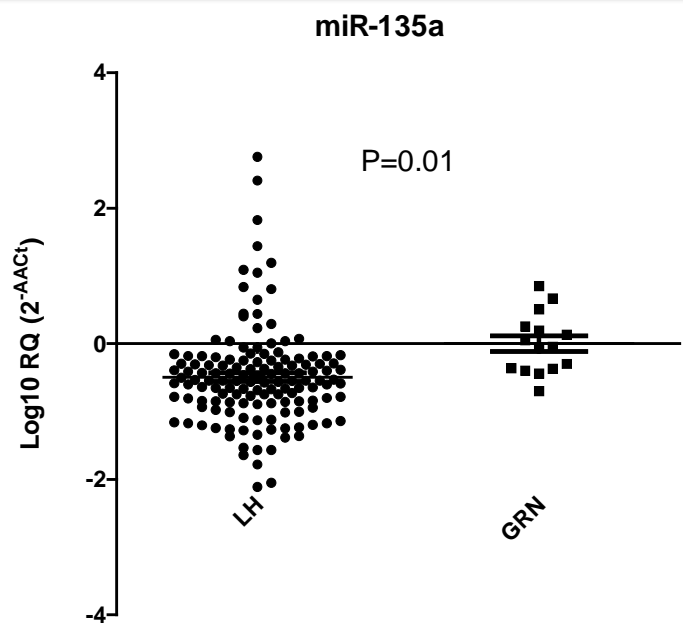


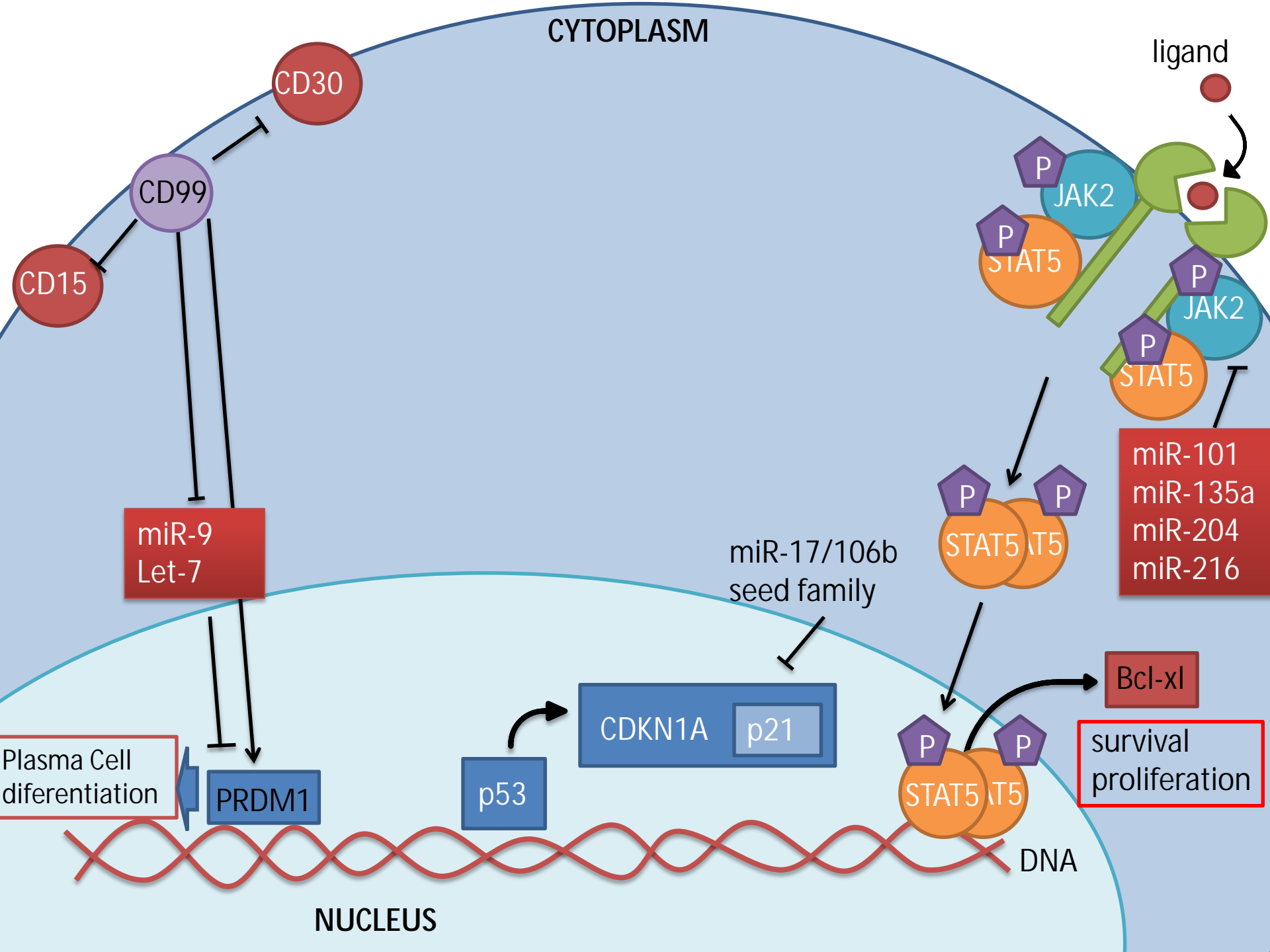
Figure 4. HL cell lines express relatively high levels of miR-9 and let-7a. **A** and **B:** The relative miR-9 and let-7a levels in GC-DLBCL, HL, PEL, and myeloma cell lines determined by quantitative real-time PCR were shown. The level in U266 was arbitrarily set as 1.

Silencing of miR-9 by anti-miR-9 reduces HL tumour growth in NOG mice.



Expression levels of JAK2-miRNAs in HL lymph nodes and reactive lymph nodes





CYTOPLASM

ligand

CD30

CD99

CD15

JAK2

STAT5

JAK2

STAT5

miR-101
miR-135a
miR-204
miR-216

miR-17/106b
seed family

STAT5

STAT5

miR-9
Let-7

PRDM1

Plasma Cell
differentiation

p53

CDKN1A p21

STAT5

STAT5

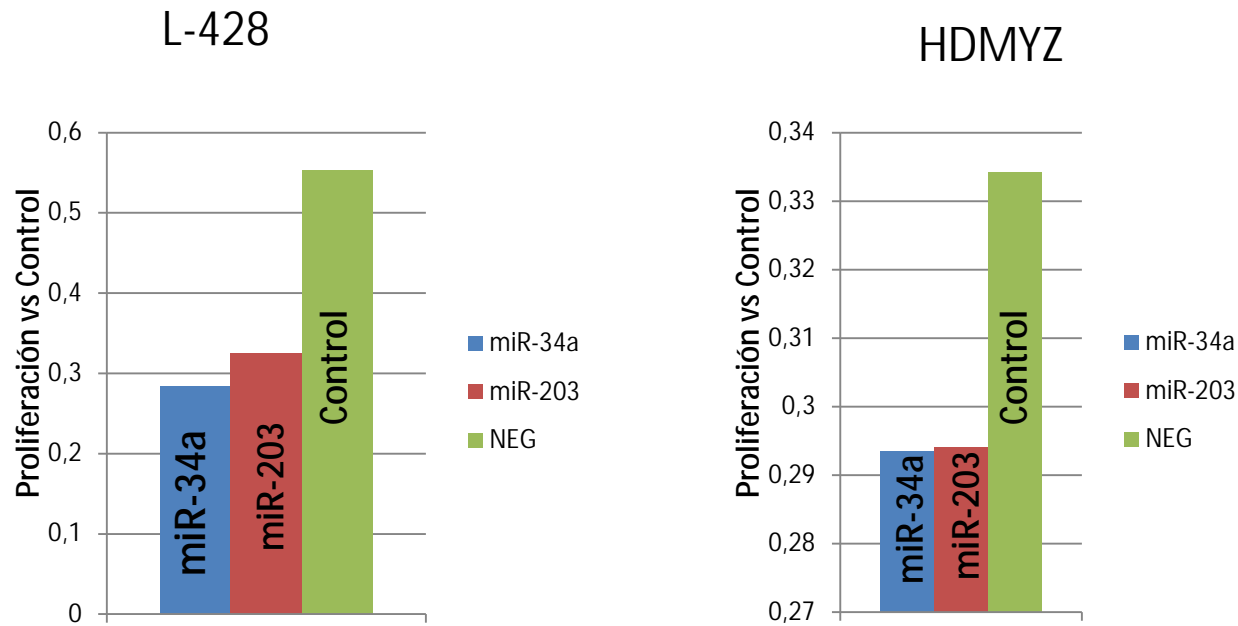
Bcl-xl

survival
proliferation

NUCLEUS

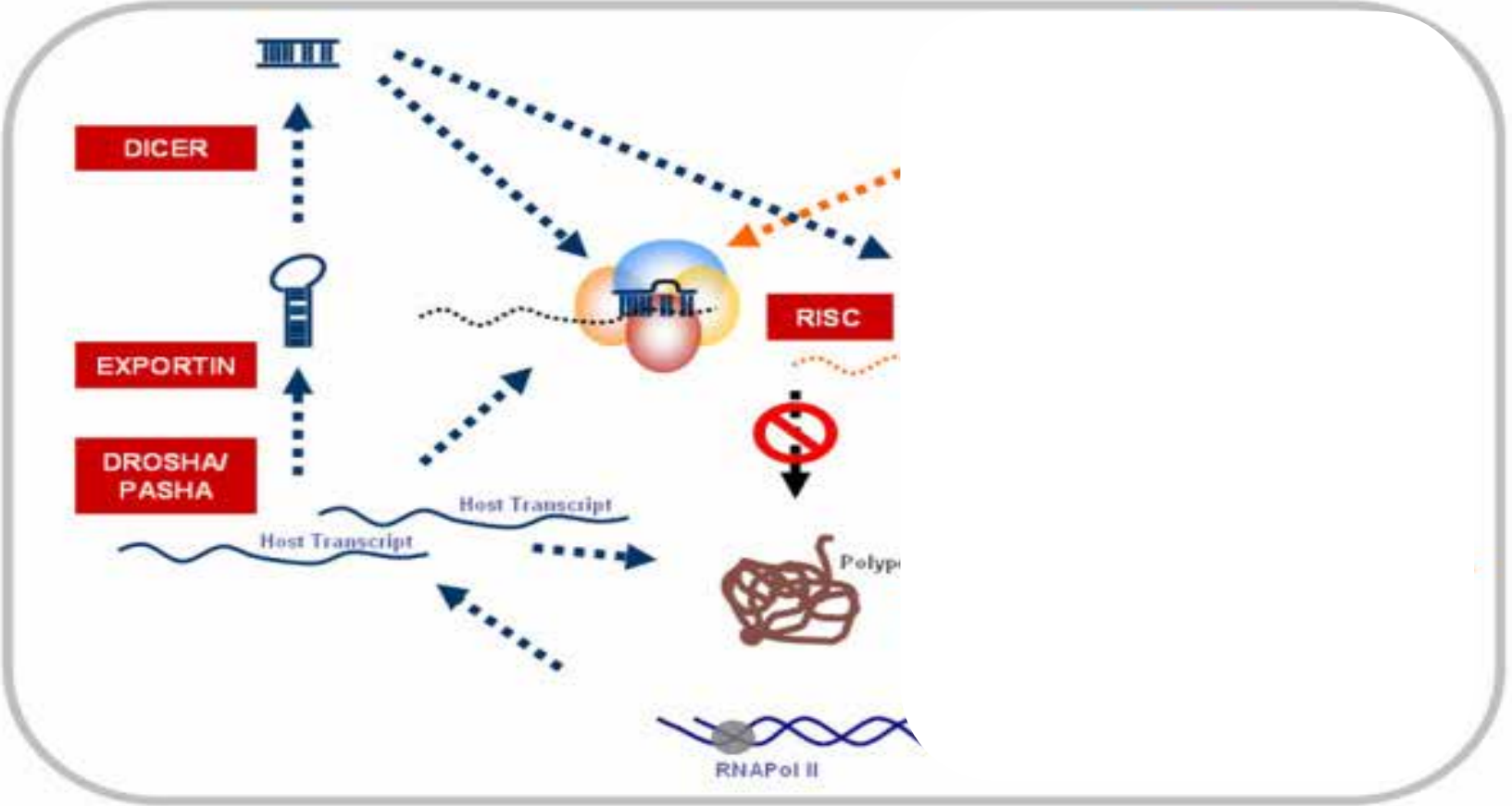
DNA

ANÁLISIS DE PROLIFERACIÓN CELULAR DESPUÉS DE TRANSFECTAR LAS LÍNEAS CELULARES CON pre-miR-34a y pre-miR-203

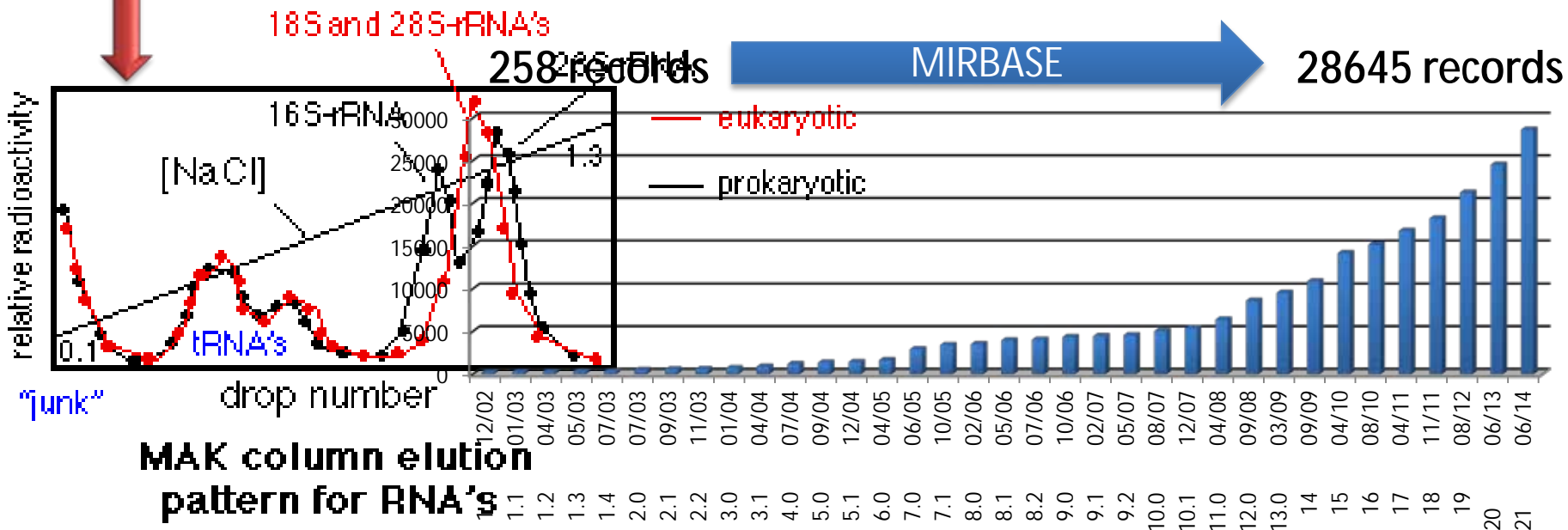
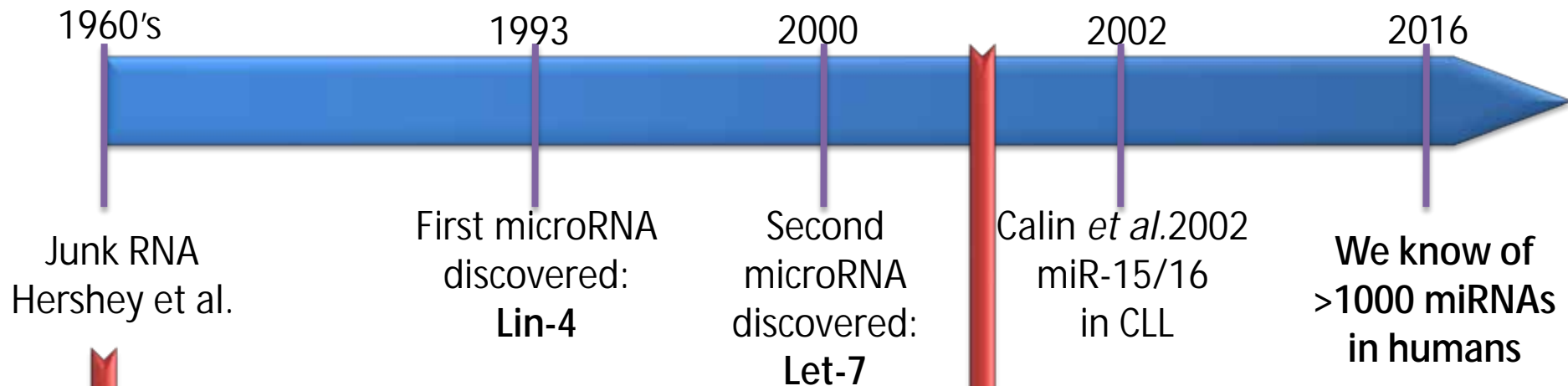


**miR-34a y miR-203 ACTUAN COMO
GENES SUPRESORES DE TUMORES EN LH**

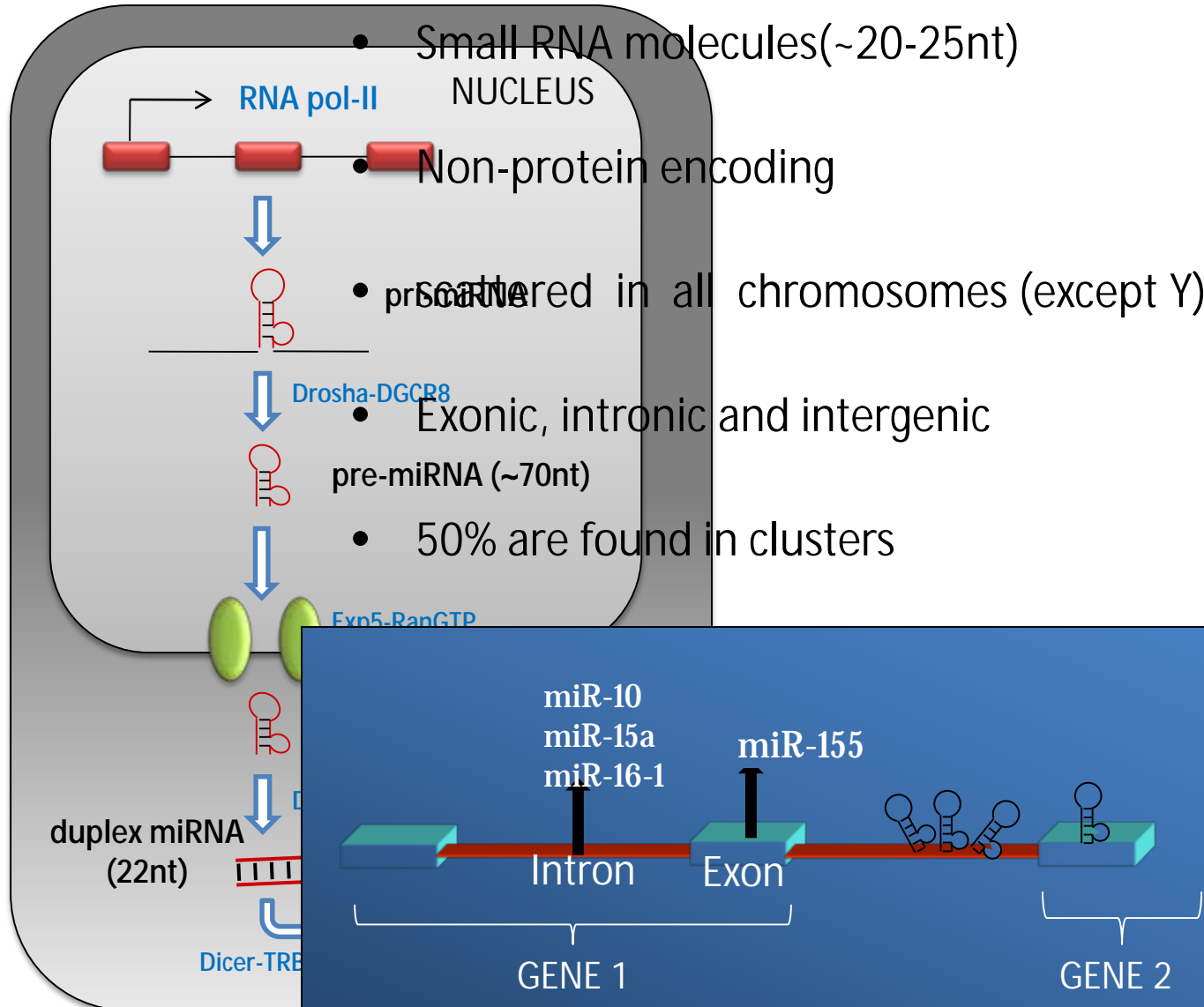
Virus and miRNAs



HISTORY

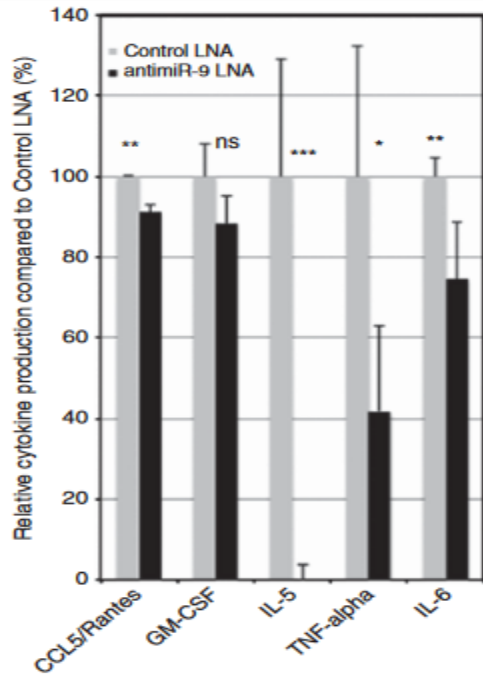


microRNAs

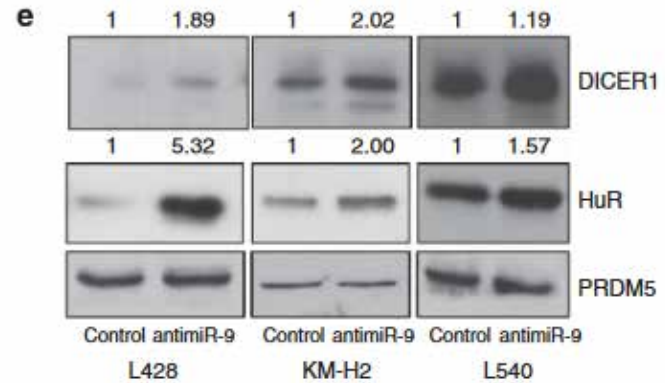


- General epigenetics features of HL
- **Generalities of Non-coding RNAs**
- microRNA profiling phase in HL
- microRNAs regulating HRS cells crucial pathways
 - miR-21
 - miR-155
 - miR-135, miR-204
 - miR-9
 - miR-30 family
- microRNAs regulated by methylation in HL

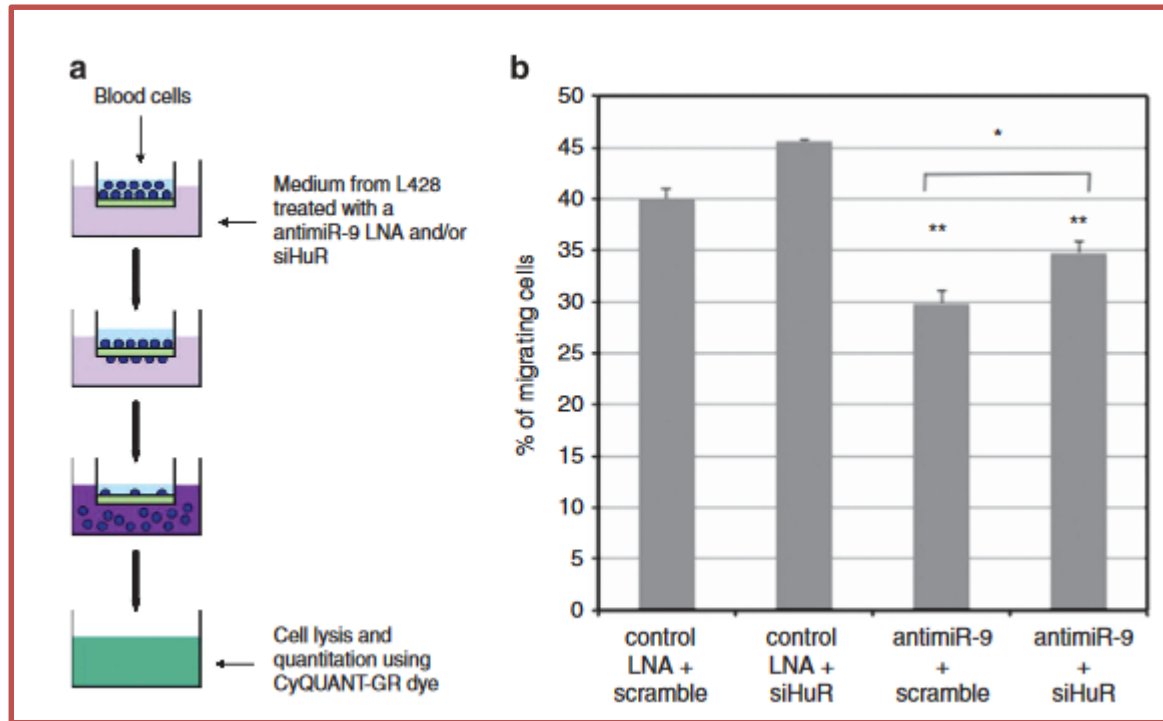
miR-9 inhibition reduces cytokine secretion



miR-9 targets DICER1 and HuR



AntimiR-9-mediated upregulation of HuR impairs the ability of HL cells to attract normal blood cells.



Int. J. Cancer: **122**, 969–977 (2008)

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SPECIAL SECTION PAPER

MicroRNAs and cancer: Profile, profile, profile

Elisa Barbarotto^{1,2}, Thomas D. Schmittgen^{3*} and George A. Calin^{1*}

¹*Department of Experimental Therapeutics, University of Texas, MD Anderson Cancer Center, Houston, TX*

²*Department of Morphology and Embryology, Human Anatomy Section, University of Ferrara, Ferrara, Italy*

³*College of Pharmacy, Ohio State University, Columbus, OH*

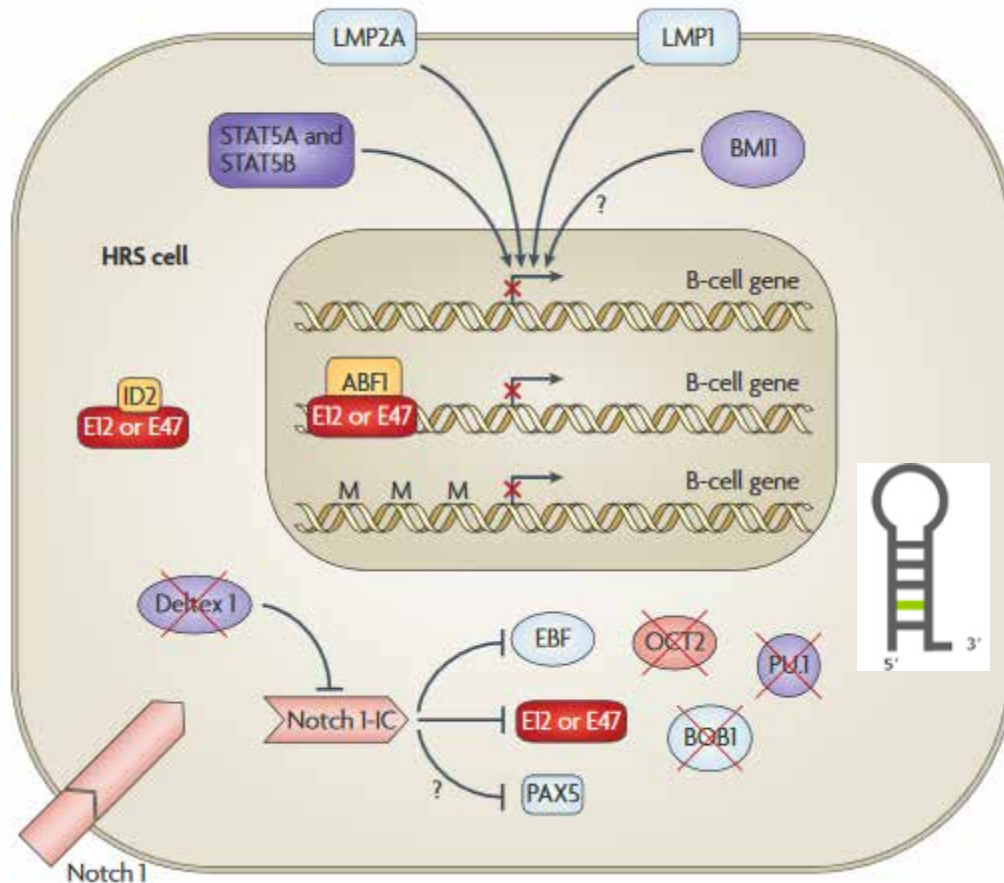


MicroRNA expression profiling in classic Hodgkin lymphoma

Alfons Navarro,^{1,2} Anna Gaya,^{2,3} Antonio Martinez,^{2,4} Alvaro Urbano-Ispizua,^{2,3} Aina Pons,^{1,2} Olga Balagué,^{2,4} Bernat Gel,⁵ Pau Abrisqueta,^{2,3} Armando Lopez-Guillermo,^{2,3} Rosa Artells,^{1,2} Emili Montserrat,^{2,3} and Mariano Monzo^{1,2}

BLOOD, 1 MARCH 2008 • VOLUME 111, NUMBER 5

Mechanisms of reprogramming a Hodgkin and Reed-Sternberg cell: loss of the B-cell phenotype



§ Deregulated expression of inhibitors of B-cell molecules

ü ID2, ABF1, Notch1

§ Downregulation of B-cell transcription factors

ü OCT2, BOB1, PU.1

§ Epigenetic silencing of B-cell genes

ü CD19, IgH

§ Non-coding RNAs (microRNAs)

§ EBV