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Ulrike Jung

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Promotionsausschuß:
Vorsitzender: Prof. Dr. J. Kurreck
Berichter: Prof. Dr. R. Lauster
Berichter: Prof. Dr. SHE Kaufmann

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The Master in the art of living makes little distinction between her work and her play, her labor and her leisure, her mind and her body, her education and her recreation, her love and her religion. She hardly knows which is which. She simply pursues her vision of excellence in whatever she does, leaving others to decide whether she is working or playing. To her she is always doing both. – Zen Buddhist Text

Table of Contents

1 Introduction **7**
- 1.1 Gene expression in eukaryotes . . . 7
  - 1.1.1 DNA and the composition of a protein-coding gene . . . 7
  - 1.1.2 Transcription . . . 8
  - 1.1.3 Maturation of mRNA . . . 8
  - 1.1.4 Translation . . . 8
- 1.2 Regulation of gene expression by endogenously triggered RNA interference 9
  - 1.2.1 Major components of the RNA interference pathways . . . 10
  - 1.2.2 Gene expression regulation by microRNAs . . . 12
- 1.3 Regulation of gene expression by artificially triggered RNA interference . . 16
  - 1.3.1 siRNAs . . . 16
  - 1.3.2 shRNAs & artificial miRNAs . . . 17
- 1.4 Delivery systems for artificial RNAi platforms and side effects . . . 19
  - 1.4.1 Viral delivery systems for RNAi platforms . . . 19
  - 1.4.2 Possible effects of viral infections on gene expression regulation in eukaryotic cells . . . 20
- 1.5 Tuberculosis . . . 22
  - 1.5.1 Causative agent . . . 22
  - 1.5.2 Diagnosis and pathology . . . 23
  - 1.5.3 Vaccination and therapy . . . 25
  - 1.5.4 Local and global importance . . . 27

2 Aim of this thesis **29**

3 Material and Methods **30**
- 3.1 Material . . . 30
  - 3.1.1 Enzymes . . . 30
  - 3.1.2 Other material . . . 30
  - 3.1.3 Eukaryotic cell lines . . . 31
  - 3.1.4 Bacteria strains . . . 31
  - 3.1.5 Kits . . . 33
  - 3.1.6 siRNAs . . . 33
  - 3.1.7 Primer/oligomers . . . 34
  - 3.1.8 Antibodies and blocking solution . . . 39
  - 3.1.9 Antibiotics and Fungicides . . . 39
  - 3.1.10 Buffer and solutions . . . 39
  - 3.1.11 Software & tools . . . 43
- 3.2 Potential new method to establish gene knockdown and knockout in prokaryotes . . . 43
  - 3.2.1 Design of siRNAs . . . 43
  - 3.2.2 Cellular extract generation . . . 44

3.2.3 Electrocompetent *M. smegmatis* . . . 44
3.2.4 Electrocompetent *L. monocytogenes* . . . 45
3.2.5 Electrocompetent *E. coli* . . . 45
3.2.6 Electrocompetent BCG . . . 45
3.2.7 Electroporation of Prokaryotes . . . 46
3.2.8 Culturing and clone testing (PCR, Fluoroscan) . . . 46
3.2.9 Purification of mRNA . . . 46
3.2.10 siRNA re-purification after electroporation into bacteria . . . 47
3.2.11 Northern Blot for small RNAs after delivery into prokaryotes . . . 48
3.2.12 Isolation of low copy plasmid DNA (*M. smegmatis*) . . . 49
3.2.13 Sequencing and direct methylation analysis . . . 49
3.2.14 Search for potential restriction sites/motifs adjacent to the deleted regions . . . 50
3.2.15 Overexpression of human Ago2-GST in *E. coli* . . . 50
3.2.16 Proof of principle experiments for proposed new method to cause sequence specific mutations by endonucleolytic activity of FEN-1 or Polymerases . . . 50
3.3 Hairpin Protocol . . . 52
3.3.1 Hairpin reverse transcription und TaqMan RT-PCR . . . 52
3.3.2 primer and TaqMan probe design . . . 52
3.4 Establishing a RNAi platform on host side . . . 53
3.4.1 Purification of primary human monocytes from buffy coat and differentiation into dendritic cells . . . 53
3.4.2 Nucleofection of primary human monocytes or THP-1 cells . . . 53
3.4.3 Luciferase assay . . . 54
3.4.4 Reagent based THP-1 cell transfection methods . . . 54
3.4.5 Construction of pHIV8-GFP and pHIV9-Puromycin . . . 56
3.4.6 Design and cloning of amiRNA constructs . . . 57
3.4.7 Northern Blot for snRNA detection . . . 59
3.4.8 Rubidiumchlorid-competent *E. coli* (heat shock competence) . . . 60
3.4.9 Plasmid purification . . . 60
3.4.10 Virus vector packaging . . . 60
3.4.11 Virus vector harvest und purification . . . 61
3.4.12 Target cell transduction . . . 61
3.4.13 FACS . . . 62
3.4.14 Cell sorting . . . 62
3.4.15 Puromycin selection . . . 62
3.4.16 Reverse transcription and SYBR Green RT-PCR . . . 63
3.4.17 Western Blot . . . 63

4 Results **64**
4.1 Analysis of a potential new method for gene knockdown and knockout in bacteria . . . 64
4.1.1 Development of a modified TaqMan RT-PCR system . . . 64
4.1.2 Confirmation of delivery of siRNAs into bacteria by TaqMan RT-PCR 65

4.1.3 Search for mRNA slicing and secondary siRNAs . . . 67
4.1.4 Epigenetic and genetic analysis of clones with stable lack of GFP expression . . . 68
4.1.5 RNase III effects and low efficacy of method . . . 70
4.1.6 Hypothesis for a modified method (FEN-1 activation) based on mutations . . . 72
4.2 Summary of results on protocols for gene knockdown or knockout in prokaryotes . . . 76
4.3 Platform for eukaryotic RNAi based gene validation in *M. tuberculosis* infection . . . 77
4.3.1 Temporary siRNA based knockdown and promoter activity in primary human monocytes . . . 77
4.3.2 Comparison of delivery methods in efficiency and toxicity . . . 79
4.3.3 Expression of artificial miRNAs from a combined intron-exon-approach 84
4.3.4 Gene knockdown in eukaryotic cell lines and primary CD34+ derived DC . . . 85
4.3.5 microRNA overexpression in HeLa and THP-1 cell lines . . . 89
4.4 Summary of results on establishment of an RNAi platform in eukaryotic cells 92

5 Discussion 94
5.1 Hairpin primer based TaqMan RT-PCR . . . 94
5.2 Regulation of gene expression in prokaryotes . . . 96
5.2.1 Original method and deletions . . . 96
5.2.2 A hypothesis based on the appearance of mutations . . . 98
5.3 Gene expression regulation on host side . . . 100
5.3.1 Reasons for the use of a lentiviral system and the construction of a new platform . . . 100
5.3.2 Reasons for the selection of an intron derived artificial miRNA and the lentiviral system . . . 102
5.3.3 Influence of promoter-, intron-, artificial miRNA- and artificial miRNA target site sequence as well as infection model on the knockdown effect . . . 104
5.3.4 Gene knockdown with a lentiviral RNAi platform to research the DC-SIGN - Raf1 - PD-L1 signaling cascade . . . 105
5.3.5 Overexpression of endogenous miRNA in different cell lines . . . 107
5.3.6 Outlook on the application of the established lentiviral RNAi platform 108

6 Summary 110

7 References 112

8 Online Resources 127

9 Appendix 128
9.1 Zusammenfassung . . . 128
9.2 Danksagung . . . 130

9.3 Lebenslauf 132
9.4 Plasmidkarten 133
9.5 Supplier 134
9.6 Abbreviations 136

1 Introduction

1.1 Gene expression in eukaryotes

Gene expression is the process leading from the information encoded in the deoxyribose nucleic acid (DNA) within the nucleus to the production of proteins in the cytosol. The transport form of genetic information produced as intermediate step is ribose nucleic acid (RNA) and can code for a protein or can be a non-coding RNA. The expression of a protein-coding gene consists of three steps: transcription, RNA maturation into mRNA (messenger RNA) and translation. The first two of those steps can be involved in generation of non-coding (regulatory) RNAs like small hairpin RNAs (shRNAs) and endogenous or artificial microRNAs (miRNA). Explanation of these steps in detail is necessary to understand where regulatory RNAs can interfere with the expression of a protein coding gene.

1.1.1 DNA and the composition of a protein-coding gene

DNA is the long-term storage form of genetic information and mostly located in the nucleus. It exists usually as a double helical structure of two polymeric complementary strands [2]. Each strand has, due to its asymmetric chemical structure, a "five prime to three prime" (5' - 3') orientation. This orientation determines a reading direction for DNA strand amplification as well as gene expression and allows the definition of terms like "upstream" (5' from another region) and "downstream" (3' from a region) [1]. Complementarity is established between the monomers (nucleotides) of each DNA strand. These nucleotides contain one of four bases linked to the strands deoxyribose phosphate backbone: Adenine (A), Guanine (G), Thymine (T) and Cytosine (C). A can pair with T and G with C (the "Watson-Crick pairing") by non-covalent hydrogen bonds [2]. Genes are encoded at the DNA template strand by the sequence of the nucleotide bases. A protein coding gene includes at least one promoter region [3], a downstream located coding sequence (CDS) and a Polyadenylation signal (PolyA signal) [4]. Near the 3' end of the promoter or between the promoter and the CDS the transcription start site is found [5]. Transcription is the first step of gene expression (see chapter 1.1.2). The region between transcription start site and CDS is called "five prime untranslated region" (5' UTR). Between the CDS and the PolyA signal the region is called three prime untranslated region (3' UTR) [6].

1.1.2 Transcription

Transcription as the first step of gene expression leads to the generation of a single strand of RNA as copy of the DNA template strand. It is initiated when an enzyme called DNA-dependent RNA polymerase (DdR Pol) binds to the promoter region of a gene. Different promoter types are related to different DdR Pols. Currently four types of DdR Pols are known in eukaryotes: I, II, III and the recently discovered type IV which seems to exist only in plants [7]. Protein coding mRNAs are transcribed from DdR Pol II, non-coding RNAs can be transcribed by all three polymerases [8] [9].

1.1.3 Maturation of mRNA

Maturation of RNA is a co-transcriptional RNA processing step in the nucleus leading to mRNA. It is initiated when DdR Pol II pauses as the nascent immature mRNA is 20-30 basepairs (bp) long. At this step capping occurs [10]. Capping results in a 7-methyl G5′ppp5′N modification of the RNA and protects it from degradation ([11] - [13]). In case the mRNA contains "Intron" sequences "splicing" follows as the next step. Introns are non-coding mRNA sequences that can be found in the 5'UTR and in the CDS. They are defined by conserved motifs referred to as "spliceosom donor" and "spliceosom acceptor" sites and removed from the mRNA by a multimeric complex, the spliceosom [14]. The remaining protein-coding sequences ("Exons") are re-ligated [16]. Introns can contain non-coding RNAs, e.g. immature primary microRNAs (see chapter 1.2.2) which can regulate mRNA stability and translation [15].

Polyadenylation as the last step of mRNA maturation is initiated after the PolyA signal of the RNA is transcribed. The PolyA signal is a highly conserved hexamer of the sequence AAUAAA in the 3' UTR. The RNA is cleaved 10-30 bp downstream of the PolyA-signal and an enzyme called PolyA polymerase adds a tail of several adenosine residues to the 3' end of the now fully mature mRNA [17].

Cap and PolyA tail are necessary for DdR Pol II transcripts to be exported out of the nucleus which is achieved by binding of ribo-nucleo proteins (RNP, referred to as Polypyrimidine-tract-binding protein PTB too). RNPs are multi functional and also play a role as splicing suppressors [18].

1.1.4 Translation

During translation a protein corresponding to the information encoded in the nucleotide sequence of the mRNA is generated in the cytosol.

As first step several eukaryotic initiation factors and the PolyA tail binding protein (PABP) form a complex which connects the cap structure with the PolyA tail. This recruits the 40S subunit of a protein complex referred to as "ribosome". The 40S subunit moves along the mRNA downstream (scanning model) to find the transcription start site which is marked by the codon AUG. Subsequently, the other subunit (60S) is recruited and the holo-ribosome formed. The holo-ribosome complex moves along the RNA sequence in three-nucleotide steps (codons). For every codon the process is stalled until a complementary transferRNA (tRNA) is found. The tRNA is a non-coding RNA which has a codon specific amino acid attached. The amino acid is bound covalently to the nascent poly-peptide chain [19]. Translation is terminated once the stop codon is reached [20].

Cap independent translation initiation is possible as well if the mRNA contains a sequence referred to as internal ribosomal binding sites (IRES) which are usually found in viral mRNAs [19]. Small non-coding regulatory RNAs can influence mRNA stability as well as translation initiation and the ongoing translational process (see chapter 1.2.2).

1.2 Regulation of gene expression by endogenously triggered RNA interference

There have been many different pathways discovered, how non-coding RNA molecules in pro- and eukaryotes can regulate gene expression. RNaseIII proteins are defined as endoribonucleases specifically cleaving double stranded RNAs (dsRNAs) and play a role in processing and decay of most eukaryotic and prokaryotic RNA [21]. RNaseIII class I proteins and their functions in gene expression regulation are long known in eukaryotes and prokaryotes [21] [22]. However in 1998 Craig Mello and Andrew Fire discovered a new regulatory mechanism in eukaryotes which was named RNA interference (RNAi) and awarded with the Nobel Price for Medicine in 2006 [23]. RNAi is defined as a mechanism for gene expression regulation which is triggered by short non-coding double stranded RNAs (dsRNAs) and independent of RNaseIII class I proteins [24]. Several different RNAi pathways are currently known which are active on epigenetic and post-transcriptional level. As epigenetic pathways are mostly described for non-mammalian organisms [25] this introduction will go into detail only on post-transcriptional RNAi.

1.2.1 Major components of the RNA interference pathways

The RNAi pathway was discovered in *C. elegans* but meanwhile it has been shown that its components are highly conserved in eukaryotes like plants, mammals and insects [26]. Two protein classes play major roles in eukaryotic RNAi processes: RNaseIII proteins of class II and III and the member of the Argonaute superclass family.

1.2.1.1 Drosha

Drosha is a member of the RNase III class II family which processes the endonucleolytic cleavage of primary microRNAs (pri-miRNA) into preliminary microRNAs (pre-miRNA). This step takes place in the nucleus. The resulting 2bp overhang at one 3' end of the pre-miRNA is necessary for its export into the cytosol by Exportin5 [29]. At least one additional co-factor is involved in processing by Drosha: Pasha in *D. melanogaste*r or DGCR8 (DiGeorge syndrome critical region gene 8) in *H. sapiens* [27] [28].

1.2.1.2 Dicer

Dicer is a cytosolic enzyme which contains two RNaseIII and one PAZ (Piwi Argonaut Zwille) domain. The RNaseIII domain provides endonucleolytic cleavage activity. The PAZ domain binds the 2 bp overhang of a dsRNA. The double strand of the dsRNA is loaded into the Dicer cleft and cleaved with the RNaseIII domains leaving a second 2 bp overhang at the other 3' end (see fig. 1.1) [30]. This kind of overhang is crucial for the efficacy of the subsequent process [31]. The length of the resulting small dsRNA is determined by the spatial distance caused by a helical motif between RNaseIII and PAZ domain [30].

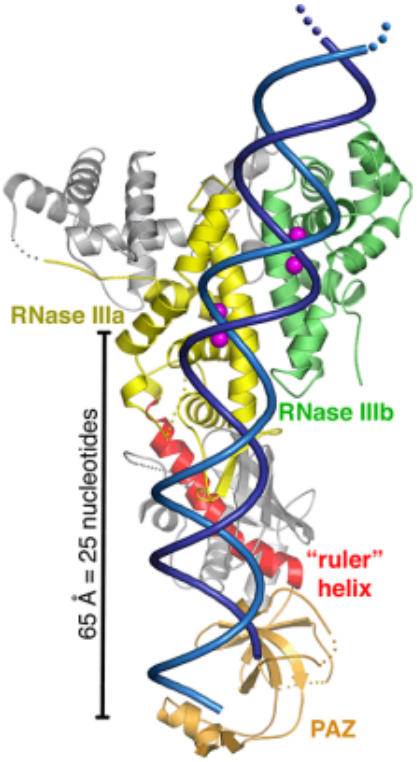

Figure 1.1: Structure of *Giardia* Dicer with a long dsRNA incorporated (fig. from [34])

The number of existing Dicer variants depends on the organism. Mammals seem to have only one type. *D. melanogaster* has two Dicer variants with different tasks: dcr1 processes imperfectly matched dsRNAs with a hairpin loop at one end (see chapter 1.2.2) and dcr2 processes perfectly matched dsRNAs which leads to mRNA degradation [32]. In plants the number of Dicer variants differs among different species. For example *A. thaliana* has four different variants which are part of different regulatory pathways [33].

1.2.1.3 Argonaute-like and Piwi-like Proteins

The Argonaute protein family is a very diverse protein family with all members containing at least one PAZ domain and one PIWI (P-element induced wimpy testis) domain [30] [35]. The PAZ domain binds the 2 bp overhang of the 3' end of a small non-coding dsRNA. The PIWI domain belongs to the RNaseH domains and provides the Argonaute with endonucleolytic activity [30] [35].

Argonautes (Ago) are incorporated into two complexes: RISC (RNA-induced silencing complex) and RLC (RISC loading complex [36]). In the Argonaute containing RLC the dsRNA is unwound in an ATP-depending fashion by a helicase domain. One of the

resulting RNA strands (the "guide strand") remains bound to the Argonaute protein. The other strands ("passenger strand") phosphodiester ribonucleic backbone is cleaved at the position opposing nucleotide ten and eleven of the guide strand. The cleavage of a strand leads to its removal from the RLC and the RLC turns into the RISC [37]. RISC binds a complementary mRNA which undergoes the same fate as the passenger strand at the same position [38] [39].

Several Argonaute paralogs have been identified (one *in S. pombe,* eight in *H. sapiens* and twenty in *C. elegans)* [40] - [42]. Argonaut protein functions seem to be non-redundant. In humans small non-coding dsRNAs seem to bind to Argonaut 1 to 4. [43] Incorporation into human Ago 2 RISC leads to cleave of the target mRNA in case it is fully complementary [44]. RISC complexes that contain Ago3 accumulate in so called "pBodies" and cause translational repression [45].

1.2.2 Gene expression regulation by microRNAs

MicroRNAs were discovered in 1993 in the labs of Victor Ambros and Gary Ruvkun by identification of lin-4 in *C. elegans* [46] [47]. They can be found in mammals, insects and plants as well (several hundred miRNAs are known today) and are often highly conserved among kingdoms and even higher grades of phylogeny [48].

The mature miRNA which causes the RNAi effect is a short non-coding dsRNA. Its targeted mRNA can be of cellular or foreign origin (e.g. viral RNA). The microRNA guide strand contains a region with complete homology to the target mRNA referred to as "seed sequence". The seed sequence covers usually the nucleotides two to seven at the 5' end of the guide strand. The remaining guide strand shows no or only partial complementarity with the target mRNA. In some cases the seed sequence is imperfect in complementarity as well. This can be complemented or compensated by 3' end pairings with the target mRNA [49].

Prediction of miRNA target mRNAs remains problematic. A single miRNA can have many target sites. Prediction tools like TargetScan [11] give sometimes more than thousand possible target sites. Furthermore a single miRNA can have several target sites in a single mRNA [50]. Each potential target site needs to be experimentally validated so far and studies have shown that single target sites can differ in strength when effecting gene expression. One reason is that sequences adjacent to miRNA target sites (so called cooperative miRNA sites), AU-rich motifs and the position of the site in the 3' UTR of the target mRNA can all influence the impact of the miRNA on the target. For the same reason the prediction of the mechanism (mRNA degradation, translational arrest)

or dimension of the RNAi effect is currently impossible [51].

MiRNA target sites in the mRNA are usually found in the 3' UTR of protein-coding genes. A recent study suggests that the 5' UTR can contain miRNA target sites in humans for human specific miRNA too. In this case especially the 3' end of the miRNA and not the 5' end seed match seems to define the target mRNA. Additionally some miRNA seem to be able to target the same mRNA in the 3'UTR and the 5' UTR with synergistic effects [53]. Previous estimations about the number of genes in mammals to be regulated by miRNAs suggest at least 20%-50% of the transcriptome [50], while some researchers believe the number to be in the range of 80%. Those are estimates made before the 5' UTRs were considered to contain miRNA target sites [52].

MicroRNAs are mostly transcribed from introns, intergenic regions or transcribed as antisense sequences of coding genes. Often miRNA pseudo-genes are found in clusters in the genome too [54] - [56]. In the case of intronic location it has been reported that miRNAs can negatively regulate its own host gene [57] and that they can be expressed without correlation to the expression of their host gene [58].

DdR Pol II transcribes primary miRNAs (pri-miRNAs, see fig. 1.2, right hand, [59]) which can differ tremendously in length and measure in some cases up to several kilo-basepairs (kbp). The pri-miRNA folds due to internal complementarity into complicated secondary structures. A single pri-miRNA contains at least one, often several units of pre-miRNA (ranging from copies to totally different sequences) [60].

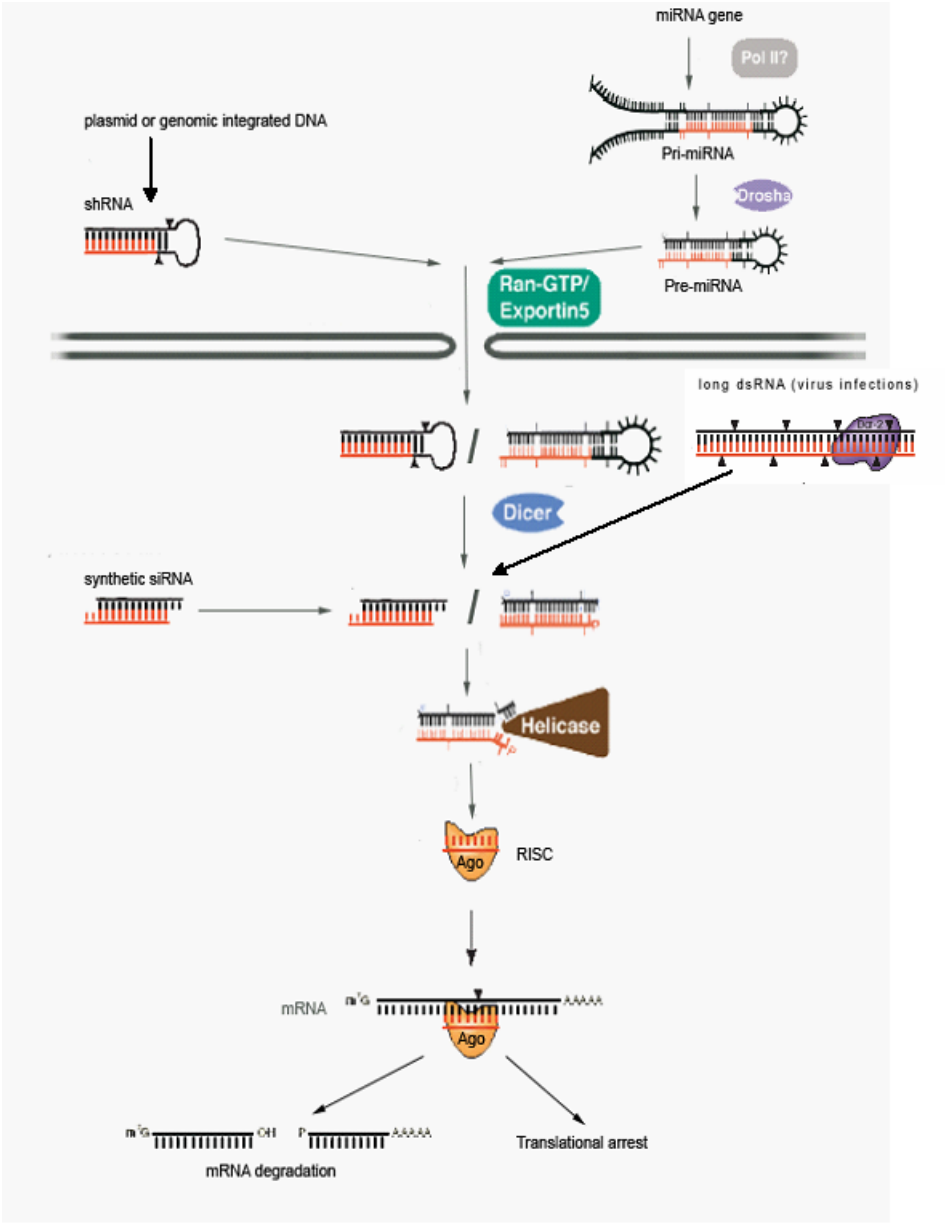

Figure 1.2: Schematic overview of artificially (left side) and naturally (right side) triggered RNAi pathways at the post-transcriptional level, modified after [62]

A pre-miRNA is around 65-75 bp long. It is characterized by a double stranded "stem" with more or less imperfect complementarity. Both strands of the stem are connected at one end by a "loop" sequence with non-complementary nucleotides. Drosha processes the pre-miRNA out of the pri-miRNA (see chapter 1.2.1.1). The pre-miRNA is then exported via Exportin-5 into the cytosol [61]. In the cytosol the pre-miRNA is further processed by Dicer (see chapter 1.2.1.2). The resulting mature miRNA is - depending on

the length of the pre-miRNA and the organism the process takes place - 21 to 28 bp long [62]. The mature miRNA is incorporated into RISC and hybridizes with its target RNA (see chapter 1.2.1.3). So far miRNAs with perfect complementarity to the target site have been found only in plants. That would rule out Argonaute mediated mRNA cleavage in other species [44]. Nevertheless mRNA degradation mediated by miRNA loaded RISCs can be observed even with imperfect complementarity between miRNA and target site (e.g. in *C. elegans,* [63]). Instead of mRNA cleavage, de-capping and de-adenylation are suggested to cause these effects [64].

In most cases miRNA mediated RNAi seems to base on inhibited translational initiation and repression of ongoing translation (fig. 1.2, right). Inhibition of translation initiation seems to happen via interference with the eukaryotic initiation factor 4E and the cap or disruption of the PolyA tail in *H. sapiens* for example through let-7 [66] [67]. Studies have shown that translation initiation by viral IRES sequences (internal ribosome binding sites, allow cap independent translation initiation) is blocked by miRNA too. Thus at least in some cases the cap based translation initiation can not serve as the interference point for miRNAs. Instead translational competent ribosomes could lead to a drop off of the holo-ribosome [68]. Other models suggest a co-translational protein degradation [69].

Translational repression is characterized by a decrease in target protein levels while the mRNA levels of a gene can remain stable. But translational repression can go along with de-adenylation or de-capping as well and the decreased mRNA stability leads to mRNA degradation too. Especially in *D. melanogaster* both mechanisms (mRNA degradation and translational repression) seem to happen in parallel [64]. In *C. elegans* starvation seems to uncouple the mRNA degradation caused by the miRNA lin-14 from the lin-14 based translational repression. This indicates that not only the mRNA target, the miRNA and their complementarity decide which pathway is active [70].

In fission yeast, *C. elegans* and plants the process of miRNA based gene expression regulation is amplified by an additional mechanism. In those organisms RNA-dependent RNA polymerases (RdRP) use the target mRNA as template to created an extended dsRNA. This fuels DICER and produces further gene specific and fully complementary miRNAs. In contrast fly and vertebrate genomes apparently do not to code for RdRP homologous proteins [71].

1.3 Regulation of gene expression by artificially triggered RNA interference

The possibility of artificially triggered gene expression regulation by RNAi was demonstrated in 2001 in *D. melanogaster* by the use of synthetic small non-coding dsRNAs in the lab of Thomas Tuschel [72].

Today artificial RNAi can be mediated by two types of dsRNAs referred to as "small interfering RNAs" (siRNAs) and "small hairpin RNAs" (shRNAs, fig. 1.2, left). Artificially triggered RNAi shows two differences compared to endogenous RNAi: siRNAs and shRNAs cause site specific mRNA cleavage [44]. And they are mostly designed to target the CDS but rarely the UTRs. This allows targeting of specific splice variants. If siRNAs or shRNAs target the promoter they can cause (pre-) transcriptional gene silencing or activation [73] [74].

1.3.1 siRNAs

Small interfering RNAs are short chemically synthesized dsRNAs which have a 2bp overhang at least at one of its 3' ends. The relatively expensive synthesis of siRNAs is one of the disadvantages of the platform type. The design algorithms for siRNAs are still improved and varying success rates in predicting effective siRNAs make it necessary to test several sequences [75] [76]. Commercially available libraries of siRNAs are mostly not functionally validated (personal communication Dr. D. Becker, MPIIB). Some validated siRNA sequences can be found in publications or online databases [5].

As siRNAs are not transcribed in the cell they need to be delivered from the environment into the cytosol. Current delivery systems for siRNAs are advanced *in vitro* but the transfection efficacy is often highly cell type dependent (e.g. rates can vary between 2% transfection efficacy up to nearly 100% with the same reagent for example in case of Lipofectamine2000 for THP-1 cells compared to HEK 293T, see chapter 4.3.2). The choice of the delivery system on the effect of the siRNA can be tremendous. High transfection efficiencies can go along with a cell toxicity or cell activation [77].

The administration of siRNA without encapsulation by a delivery system ("naked" siRNA) can cause unspecific immune responses. This occurs by binding to cellular Toll like receptors (TLR) evoking subsequent responses like activation of the interferon-α/β pathways [78]. The TLRs which can be activated by siRNAs are i) TLR3: sequence independent but length dependent, [79] [80] and ii) TLR7 and TLR8: sequence specific, [81] [82] activation. Potential activation of TLR9 by naked siRNA, maybe by chemical stabilization

by phosphorothioate backbones, is poorly understood [83] [84]. All of these TLRs are located at the cell surface or in endosomal compartments of the cell [85]. Thus in case of siRNAs administration the described unspecific effects need to be considered.

An advantage of siRNAs is the option of chemical modification. This can increase the bias of guide strand selection (in case of symmetric siRNAs with overhangs at both ends both strands can act as guide strand). The increase in strand bias decreases the chances of off target effects by incorporating the sense strand instead of the antisense strand into RISC. Especially 2'-O-methylation seems to play a role in this and even abrogates TLR7 and 8 (but not TLR3) activation [86] [87]. Meanwhile many other modifications have been developed and tested as well [88].

Kinetics of dose, response and degradation of siRNAs can be determined relatively well [89]. The low half life in serum and cell plasma due to RNase activity can be influenced by chemical modifications and encapsulation [90].

Small interfering RNAs do not need to be processed by the cell anymore. Because of this siRNAs can be delivered in high amounts without causing interference with the nuclear miRNA processing pathway or saturation of Exportin-5 export of miRNAs into the cytosol. RNAi effects are dose dependent thus a delivery of high siRNA numbers favors strong knockdown effects [92]. Availability of Ago2 seemed for some time to be the only limiting factor for siRNA mediated RNAi and overexpression of Ago2 improves the knockdown effect [91]. But recent studies indicate that the usually transfected siRNA (or mature miRNA) doses can have global off target effects on the gene expression pattern [93]. Smaller siRNA doses seem to decrease these effects [75].

The above described problems (or therapeutically useful effects) can lead to false interpretation of study data [94]. Meanwhile several cases of reported RNAi effects (even in clinical trials) have been determined as off target effects but not target specific RNAi [95]. Especially in case of siRNA application the differentiation between a direct target specific RNAi effect and off target effects has thus become a critical point of review.

1.3.2 shRNAs & artificial miRNAs

The second artificial trigger for RNAi is based on shRNAs. Those dsRNAs consist of a fully complementary stem of 20-22 basepairs whose strands are connected via a non-pairing loop of varying length which are usually derived from miRNA loops. The shRNA is transcribed from a DNA template that is either temporarily or permanently transferred into the cellular nucleus. Minimal templates consist of Polymerase III promoters like U6 or H1 and with terminators which enclose the shRNA coding region for sense, loop and

antisense (or the other way around). Those minimal templates can even be PCR fragments [96]. Because Pol III promoters drive the expression of many small non-coding RNAs like tRNAs these promoters were believed to drive miRNA expression too. Therefore mostly Pol III promoters were used for shRNA expression. Comparisons between these have shown that the U6 promoter is more effective than the H1 promoter *in vitro* and *in vivo* [97]. At the same time the strong transcription of shRNAs driven by U6 leads to an interference with the endogenous miRNA processing pathway and causes *in vivo* toxicity in mice [98]. A lower shRNA expression seems to be favorable for the stability of transduced primary human lymphocyte cell populations as well [99]. Activation of interferon pathways (see chapter 1.4.2) as off target effect is stronger in U6 driven expression than in H1 driven constructs. Furthermore interferon response induction seems to depend on length and sequence of the expressed shRNA [100].

Meanwhile it is known that in most cases miRNAs are expressed from Pol II promoters as intron derived sequences or from intergenic regions [59]. This discovery led to the more recent approach, the so called "artificial miRNAs" (amiRNA). Artificial miRNAs are transcribed not just as small hairpins with subsequent Dicer processing but transcribed as pre-miRNA structure. The transcript is processed the similar to an endogenous miRNA. In an amiRNA the mature miRNA sequence is replaced by a shRNA stem. Because the stem region of the artificial miRNA is fully complementary with its target mRNA (contrary to endogenous miRNA) the amiRNA causes slicing of the mRNA. Mediation of RNAi effector pathways of endogenous miRNAs have not been reported for amiRNA or shRNAs.

It could be shown that the Pol III driven shRNA approach is more effective in knockdown than artificial miRNA. The most likely reason is the considerably higher expression level of shRNAs [101]. But amiRNA do not compete with each other for capacity of the RNAi pathway while shRNAs do [103]. This allows less interference with endogenous miRNA pathways (if not avoids it) and multi-knockdown constructs [102]. Additionally, the cell viability of cells expressing amiRNAs is higher *in vitro* and *in vivo* [104]. Furthermore the expression of the same sequence as amiRNA from Pol II promoters abrogates the toxicity of expression as shRNA while retaining knockdown efficacy *in vivo* [105].

Since shRNAs and amiRNA can't co-localize with TLR's [85] the risk of interferon-α response activation is much lower compared to siRNAs.

1.4 Delivery systems for artificial RNAi platforms and side effects

Delivery of siRNAs or shRNA-coding DNA templates can be achieved by several different methods:

a) transfection with transfection reagents i) cationic lipo-complex systems like Lipofectamine (Invitrogen), iii) other cationic complex systems like polyethylenimine [106] and ii) non-ionic systems: calcium phosphate-nucleic acid crystal formation or Dextran based delivery [107];

b) transfection by physical means i) electroporation: application of a single high voltage pulse of defined shape [108]) or ii) Nucleofection: similar to electroporation but the pulse follows a more complex pattern (Amaxa GmbH);

c) transfection via recombinant proteins e.g. antibody-protamin systems which allow targeted delivery [109];

d) recombinant bacteria i) delivery of eukaryotic expression plasmids [110] or ii) by expression of the siRNA from plasmids in RNaseIII knockout strains to prevent siRNA degradation [111];

Additionally shRNA or amiRNA coding templates can be packaged into viral vectors which will be discussed in detail.

1.4.1 Viral delivery systems for RNAi platforms

Viral vectors used to delivery RNAi constructs in most cases are based on adenoviruses (AV), adenoassociated viruses (AAV), retroviruses (often Molony murine leukemia virus MMuLV) or lentiviruses (HIV, SIV).

The advantage of AV based systems is the bio-safety level 1 classification (BSL 1, any laboratory). The risk of mutational effects is low *in vivo* since the delivered DNA does usually not integrate into the chromosome. Random integration can never be excluded and happens in up to 1% of cells [112]. AV derived vectors which can actively integrate into the genome are currently in development [113]. AV based vectors can lead to strong inflammatory reactions *in vivo* as very high titers need to be administered for transduction. The temporary transduction makes regular re-administration or the use of replication competent particles necessary [113] [114]. Retrovirus-based platforms are BSL 1 classified systems too but integrate naturally thus allowing permanent expression. The most common retrovirus based vectors which have been used in clinical trials and even gene therapy in humans are of the γ subclass. Unfortunately they have a quite high

carcinogenic potential [115]. Lentivirus based systems are classified BSL 2 due to their origin from human (HIV) or simian (SIV) immuno-deficiency virus. They show no carcinogenic integration pattern. Immune responses *in vivo* after viral transduction proved to be directed at the transgene expressed by antigen presenting cells (APC) rather the virus itself [116]. The stability of transgene expressing cells *in vivo* could be strongly enhanced by cell specific miRNA mediated down regulation in APC. [117] The greatest advantage of lentiviral based systems is the ability to transduce even non-dividing cells at a reasonable level [118].

The transduction efficacy of a virus particle is influenced by the affinity of the particle ligand to the cell membrane or surface molecule. The surface molecule responsible for the attachment can be chosen independently of the virus origin and determines its "pseudotype". Lentiviral vectors are commonly pseudo-typed with VSV-G. VSV-G is a protein complex built of three 64 kilo Dalton (kDa) glycosylated monomers and originates from the vesicular stomatitis virus (VSV). The VSV-G surface molecule binds to the cell membrane component phosphatidylserin thus needing no cell specific surface receptor [119]. After endocytosis the pH decrease leads to conformational changes in the VSV-G trimer which cause membrane fusion [120].

Another factor that influences the transduction efficacy is the ratio of cell number to virus particle used (multiplier of infection, MOI). The MOI should be kept low otherwise the virus can be toxic. E.g. high amounts of VSV-G can lead to giant cell formation by fusion) and activation of the OAS-1 interferon pathway [135]. This and the time consuming and complex production of vector particles are the reasons why lentiviral particles tend to be used in low MOI leading to single copy integration into the cell [121].

1.4.2 Possible effects of viral infections on gene expression regulation in eukaryotic cells

Viral dsRNAs can be degraded by Dicer into dsRNA of 21 to 23 bp length too. After incorporation into RISC these can cause mRNA degradation of complementary host mRNA [122]. Furthermore viruses can encode miRNAs as well thus regulating viral and cellular gene expression [123].

Additionally long dsRNA (for example viral genomic dsRNAs) can be detected by the members of the cytosolic eukaryotic Rig-I like receptor (RLR) family [124]. Members of the RLR family (fig. 1.3) are the proteins RIG-I (retinoic acid-inducible gene-I), MDA-5 (melanoma differentiation-associated antigen 5) and LGP-2 (laboratory of genetics and physiology 2). All members have a RNA helicase domain, MDA-5 and RIG-I one to

two CARD domains at the N-Terminus and RIG-I and LPG-2 a RD domain (repressor domain) at the C-Terminus [125] [126].

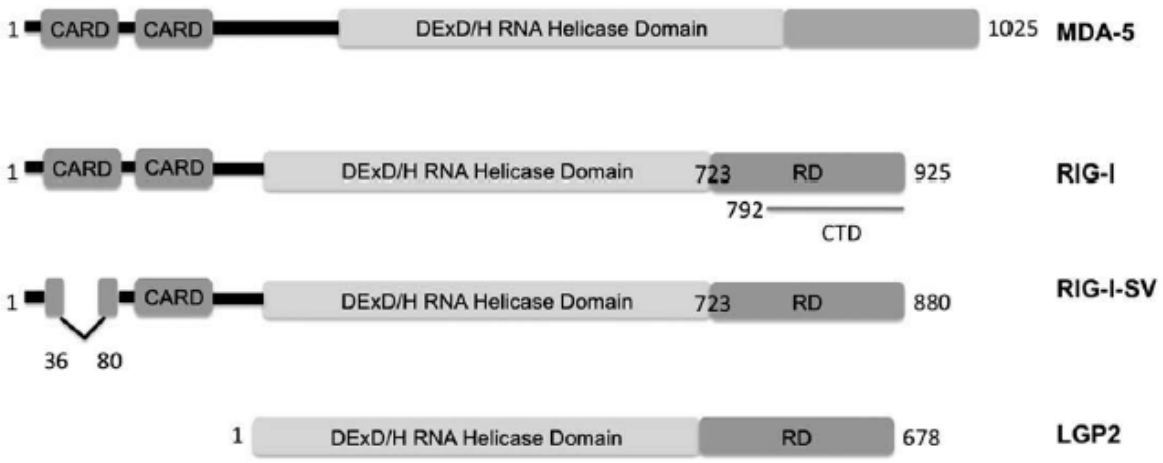

Figure 1.3: Domains of RLR family proteins, figure from [127]

Not every viral RNA is recognized by every RLR member, for example the Hepatitis C virus (HCV) genome is a single stranded RNA and bound by RIG-I and LPG-2 but not MDA-5. Furthermore the binding affinity of MDA-5 to dsRNA is significantly weaker than the one of RIG-I and LPG-2 [128].

RIG-I allows the discrimination between self (5' end capped mRNA) and foreign RNA (triphosphate at 5' end of RNA) [129]. It exists in resting cells in a monomeric stage and upon RNA binding undergoes conformational changes. The subsequent formation of a multimeric complex leads to the unmasking of the CARD domain by RD [126]. The CARD domain can thus interact with the outer membrane of mitochondria [130] which leads to recruitment of adaptor molecules of the TRAF family to activate TBK1, IKKi (ε), and the IKK$\alpha/\beta/\gamma$ complex with subsequent signaling cascades [131] [132]. The thus induced immune response is referred to as interferon β response of the cell [78]. Since overexpression of a RIG-I mutant lacking a CARD domain as well as overexpression of wildtype LPG-2 in cell culture acts as dominant inhibitor of signaling, LPG-2 is today assumed to be the natural IFN-β inhibitor of the cell at least in viral infections [125]. Another pathway, the OAS-1 pathway (OAS-1: 2'-5' oligoadenylate synthetase) seems to play a role in several natural viral infections and can be activated in lentiviral vector triggered RNAi as well [133] - [135].

These pathways need to be considered when designing viral RNAi platforms.

1.5 Tuberculosis

1.5.1 Causative agent

Tuberculosis (TB) is a disease which is primarily caused by the bacterium *Mycobacterium tuberculosis.* It was first described by Robert Koch in 1882 [136]. This discovery was rewarded with the Nobel Price in 1905.

The bacterium has, due to some of its properties, an exceptional position among eubacteria. It is classified as acid-fast and Gram-positive even though it can not retain staining with crystal violet. Instead it remains in that category because it has - like other Gram-positive bacteria - no outer membrane. It is nonmotile and aerobic but can switch to a status of dormancy under anaerobic conditions [137]. *M. tuberculosis* is rod-shaped and 2-4 micrometers long. Its genome of 4.4kBp contains about 4000 genes and has a high G/C content [138] [139].

All member of the genus *Mycobacterium* share the same unusual cell wall properties. The cell wall is much thicker compared to most bacteria. It contains a high abundance of mycolic acids leading to its hydrophobic, waxy texture. Furthermore this causes the impermeability to Gram staining. An unusually high amount of the genome seems to code for enzymes involved in lipogenesis or lipolysis. One division cycle of *M. tuberculosis* takes around 15-22 hours, for some mutants even longer. This is much longer than other bacteria need, *E. coli* for example divides in 20-30 minutes [140].

There is a strong phenotypic variety among different clinical isolates and long term *in vitro* passaged *M. tuberculosis* strains regarding virulence and infectivity. This led to the separation in *M. tuberculosis* Rv (virulence) and Ra (attenuated) strains and seems to be caused by genetic variety. Even among Rv strains phenotypes can differ a lot and re-sequencing and genetic fingerprinting has become an important tool to identify the different sub strains [141].

One of the major problems of working with *M. tuberculosis* is that 48% of its predicted protein coding genes can not be linked to functions yet. A relatively high number are not even homologues to other eubacterial genes (375 putative proteins) [139]. As *M. tuberculosis* is prone to mutations on one hand but resilient to standard eubacteria knockout protocols on the other hand, dissection of the genetic functions remains difficult. The most common strategy used today is homologous recombination from plasmids or linear DNA fragments to replace the gene of interest with an antibiotic resistance cassette. This plasmid can be introduced via electroporation or packaging in Mycobacteria phages [142]. Another widely used approach is the TraSH (transposon site hybridization) method which

was specifically developed for research on *M. tuberculosis*. With this method huge libraries of clones are generated by random integration of so called "Mariner transposon". Those transposons can integrate in thousands of locations in the *M. tuberculosis* genome. They are usually delivered via Mycobacteria phages and their integration causes frame read shifts in the coding sequence [143]. Analysis of enriched clones under chosen selection criteria is performed by microarrays [142] [143].

The functional validation of essential genes is unfortunately impossible with these methods. And their application in case of multi-cistronic genes is problematic if downstream CDS are affected. Furthermore both methods are time consuming. It takes at least several months for the homologous recombination for example. And selection of clone enrichment criteria can be very complicated in case of TraSH.

1.5.2 Diagnosis and pathology

Infection with *M. tuberculosis* usually occurs by inhalation of aerosols produced by coughing from other patients [152]. The first contact with the immune system is usually phagocytotic cells like dendritic cells (DC) and macrophages. DCs are potent activators of the acquired immune system of the body [144]. Binding of *M. tuberculosis* to their surface receptor DC-SIGN seems to suppress those functions [145]. Macrophages are considered the primary target of *M. tuberculosis* which they phagocytose [146]. The bacteria arrest phagosome maturation e.g. by expression of TDM (trehalose 6,6 dimycolate, the "cord factor"). The infected macrophages form the center of a structure referred to as "granuloma". The center is surrounded by a layer of activated $CD4^+$ T cells which later on is surrounded by a $CD8^+$ T cell ring. Over time this center turns into a caseous texture as the macrophages become necrotic and the bacteria switch into dormancy [147] [148].

Sometimes there is a structure similar to a secondary lymphatic organ associated with the granuloma. This structure is supposed to prime the immune system against the infection [149]. The granuloma tends to be encapsulated later on by a fibrous capsule and finally calcifies over the following years. Those granulomas can be located all over the body but are usually found in the lung due to the aerosol infection [148] [147].

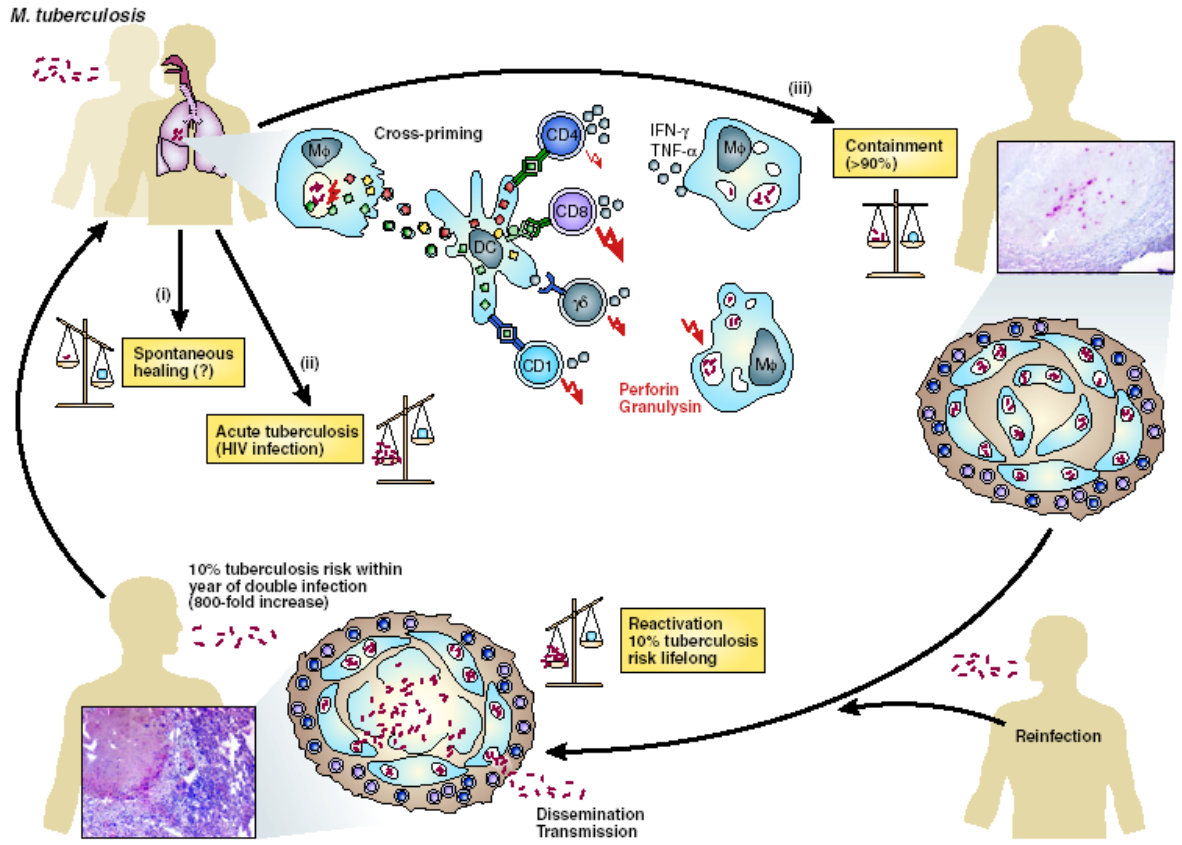

Figure 1.4: Tuberculosis infection cycle (figure from [148]).

If the containment inside a granuloma is effective the infection is asymptomatic and called "latent tuberculosis" (latent TB, [150]). If the granuloma breaks down for example because of immunodeficiency, the TB becomes symptomatic (active TB) [148]. So far no biomarkers exist to detect if there is a latent TB infection. Thus it is unclear if treatment in case of active TB truly eradicates the *M. tuberculosis* infection once symptoms disappear or if the status just switches back to latent TB. Because of this those cases as well as household contacts of patients with active TB are considered as latently infected [151].

Only patients with active TB are contagious. There can be several episodes of active TB. The first disease onset is called primary (active) TB (fig. 1.4). This onset can be much later than the time point of infection. According to estimations 90% of latently infected people seem never to develop an active TB [147]. If there is another symptomatic phase after the primary TB it is called "secondary" or "post-primary" TB. A post-primary TB usually occurs on reactivation after long years of latency. Depending on the location of

the symptoms of primary TB there is a differentiation between pulmonary TB and extra-pulmonary TB [153]. The majority of people develop a pulmonary TB (80% of cases in 2004 [170]). But especially with a weakened or immature immune system (AIDS or childhood) the bacterium can spread over the whole body. This is referred to as "miliary TB". In those cases every organ can be infected which leads to a large variety in individual pathologies and pathogenesis [153].

1.5.3 Vaccination and therapy

So far there exists a single vaccine strain against TB which is based on an attenuated *M. bovis* strain. This vaccine strain is called BCG ("bacillus Calmette Guerin" because of its developers Albert Calmette and Camille Guerin) and was first applied to children as oral live vaccination in 1921 in France. Since this time it has been applied world wide in large numbers. Today it is still used in endemic areas to vaccinate children [154].

The immune response against TB seems to be based on an early $CD4^+$ T cell priming against the secreted antigens ESAT-6 [155] and Ag85 [156] followed by a $CD8^+$ T cell activation later on [157]. Unconventional $\alpha\beta$ T cells are activated when they bind to cell wall glycolipids of *M. tuberculosis* which are presented on CD1b (cluster of differentiation 1b) [158]. The extent of this step seems to correlate with protection. Furthermore interferon-γ and members of the interleukin 12 family seem to play a role in TB immunity too [159]. This complex immune reaction makes the development of more effective vaccines difficult. Subunit or inactivated vaccines which have been developed for other pathogens lead mostly to the production of neutralizing antibodies. Those seem to play no or only a minor role in TB protection. At the same time the use of live vaccines in cases of co-infection with HIV is problematic. While those people face a high risk of active TB the treatment of immuno-compromised patients with the common BCG vaccine is not advisable. Currently many different approaches for TB vaccines are in development. The approaches range from subunit vaccines with specific *M. tuberculosis* proteins or glycolipids with adjuvants over naked DNA vaccines to recombinant bacterial and viral carriers that express those components including recombinant BCG. Some candidates have entered into clinical trials including a recombinant BCG strain rBCG:ΔureC:hly developed in our department (personal communication Dr. A. Nasser-Edine, MPIIB). This BCG strain has an urease C deficiency and expresses recombinant listeriolysin *from L. monocytogenes* which aims to cause membrane perforation. This recombinant strain was developed to increase escape from the phagosome and thus enhance antigen presentation to $CD8^+$ T (thymic) cells [160]. While some of the vaccines seem to improve the BCG

effect none seems to offer a solution to the problem of TB yet. Because of this a better understanding of *M. tuberculosis*, it's interaction with the host and the immune response which protects from active TB is still necessary for the development of better vaccines [148].

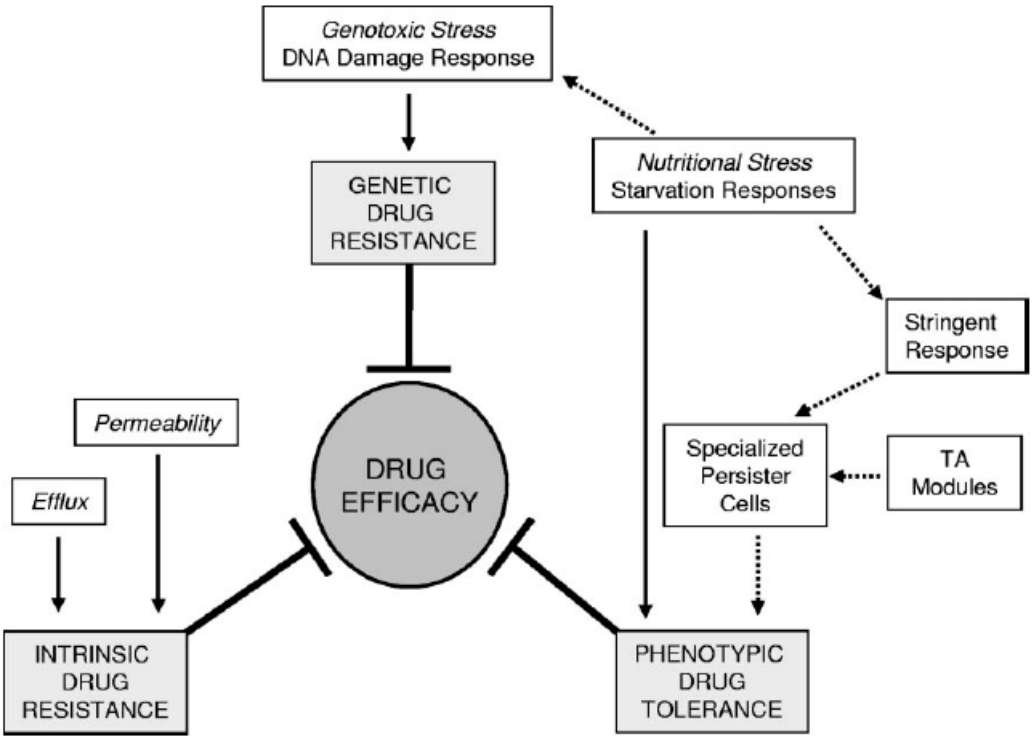

Figure 1.5: Drug resistance in M. tuberculosis infections (image taken from [161])

Therapy against *M. tuberculosis* is usually done as a multidrug therapy called DOTS (directly observed therapy, short course) which takes - contrary to its name - at least 6 months. The antibiotics used are referred to as "front-line" or "first-line" drugs and are biased towards actively dividing bacteria [161]. These drugs are isoniazid (INH, inhibiting mycolic acid synthesis), rifampicin (RMP, inhibits the prokaryotic DdR polymerase), pyrazinamide (PZA, disrupts membrane potential and -transport [162]) and ethambutol (disrupts cell wall formation). They are administered in combination for two months followed by an administration of just INH and RMP for additional four months.

Resistance against this chemotherapy can be caused in several ways. Inherited resistance due to genetic changes of *M. tuberculosis* develops spontaneously and is common. The mutation rates are well known: 1 bacterium per generation out of 10^7 develops a resistance against ethambutol, 1 out of 10^8 against INH and 1 out of 10^{10} against RMP. Because of

this the number of single-resistant bacteria per person is high and the multidrug therapy is necessary [163]. Strains which are resistant to at least INH and RMP are called MDR-TB (multi drug resistant TB). Another level of drug resistance strains is called XDR-TB (extreme drug resistant TB). According to WHO XDR-TB is defined as the strains which are not just MDR-TB but in addition resistant to "any fluoroquinolone, and to at least one of three injectable second-line anti-TB drugs used in TB treatment (capreomycin, kanamycin, and amikacin)." [4].

Phenotypic (non-inherited) antibiotic resistance plays an important role in TB too. *M. tuberculosis* can be drug susceptible per se, but due to phenotypic changes *in vivo* compared to standard *in vitro* culture (for example switching into the stage of dormancy) the antibiotics are not effective anymore [164]. Treatment with common antibiotics is not possible in those cases due to the intrinsic properties of the bacterium. The major reason is the unusual cell wall composition which makes it impermeable to most antibiotics [161].

1.5.4 Local and global importance

TB in humans can be traced by PCR back to 3000 B.C. in Egyptian mummies. It might have been even been a frequent infection in those times [165]. WHO estimates that around 30% of the world population is infected with this bacterium. Only 10% of those infected are estimated to develop an active TB. Incidence of TB cases was reported to be around 9-10 million people per year in 2002 [150] [166] [167].

Because of the difficult treatment and the high infectivity TB is subject to report by law in Germany [168] and many other countries. Contrary to public belief TB is a significant health problem in industrialized countries and higher population densities favor the incidence of TB. Reports from New Zealand about correlations with household crowding [169] fit well with regional prevalence in Germany and Great Britain which show significantly higher TB incidence for big cities compared to surrounding less densely populated areas [170] [171].

The co-incidence of HIV infection with active TB is high, increasing the problem of drug side effects as both infections are treated with multi drug regiments [172]. Furthermore, those suffering from both infections are mostly found in third world countries with little access to medical treatment. In addition the risk of obtaining active TB or not adhering to the drug treatment seems higher in people with low socio-economic background [173] - [175]. Those factors increase the chances for mortality due to TB or development of XDR-TB. The impact of ethnicity, gender, age, immigration status und genetic and social factors on TB transmission, disease outbreak, treatment or likeliness of drug resistance

makes the development of models for prediction of infection, its kinetics or treatment strategies even more difficult [176] [177].

Since 1950 with the development of chemotherapy the incidence of TB (determined by positive PPD skin tests from US soldier recruits) dropped by 10% each year and the number of cases of active TB declined as well. Despite this, since the discovery and spread of HIV the number of cases of active TB is rising (18% rise in the USA from 1985 to 1992) once more [178]. Additionally the growing number of MDR-TB and XDR-TB strains with high virulence is causing concerns too. In summary TB is on the rise again in developing as well as industrialized countries.

2 Aim of this thesis

Aim of this thesis was to develop a platform to analyze the impact of gene knockouts or knockdowns in *M. tuberculosis* infection models by RNAi.

On the prokaryotic side the usability of a proposed method should be tested and if possible the technology be optimized. This method aims at complementing the existing prokaryotic enzyme repertoire with eukaryotic RNAi components to mimic a RNAi like pathway of gene regulation in bacteria - first in *M. smegmatis*, subsequently (if successful) in *M. tuberculosis*. The aim was to build a gene validation platform based on RNAi for *M. tuberculosis* as well as the vaccine strain BCG since conventional gene knockout protocols are very time consuming in those strains and the chances of success can be extremely low depending on the target gene properties.

On the eukaryotic side an *in vitro* RNAi platform should be established for use in primary monocytes, macrophages and dendritic cells as well as comparable cell lines (THP-1 e.g.) and if possible this platform be used *in vivo* as well.

With those two approaches the goal was to:

- knock down candidate genes from literature, existing microarray data and other projects performed in the department which are supposed to play a role in *M. tuberculosis* infection, if possible not only on host side but on pathogen side as well;
- overexpress microRNAs predicted to play a role in *M. tuberculosis* infections;
- test the effects of those genetically modified cells or bacteria in *in vitro* infection models of M. *tuberculosis.*

3 Material and Methods

3.1 Material

3.1.1 Enzymes

restriction endonucleases BspEI, Eco91I, XmaI, XbaI, Esp3I, BamH1, DraII (Fermentas)
uracil deglycosylase UDG (Fermentas)
polynucleotide kinase PNK (Fermentas)
shrimp alkaline phosphatase SAP (Fermentas)
Platinum PFX polymerase (Invitrogen)
ThermoTaq polymerase (NEB)
Phusion polymerase (NEB)
MulV reverse transcriptase (Applied Biosystems)

3.1.2 Other material

1kB plus DNA ladder (Invitrogen)
Acrylamide (acrylamide:bisacrylamide = 19:1, Roth)
Ammonium persulfate APS (Sigma-Aldrich)
Biozym LE agarose (Biozym)
BSA (Serva Electrophoresis)
BUPH Tris Hepes SDS running buffer (#28398, Thermo Fisher)
Chloroform (Sigma-Aldrich)
Complete (Roche)
Complete Mini, EDTA-free (Roche)
Effectene (Qiagen)
Ficoll-Hypaque (Biocoll density 1,077, Biochrom)
Genecarrier-1 (epoch biolabs)
hGM-CSF (Active Biosciences)
hIL-4 (Active Biosciences)
Hybond N+ Nitrocellulose membrane (GE Healthcare Life Sciences)
Isopropyl-β-D-thiogalactopyranosid IPTG (Sigma-Aldrich)
Leupeptin (Serva Electrophoresis)
Lipofectamine2000 (Invitrogen)
Metafectene (Biontec)
Metafectene Pro (Biontec)
PBS (GIBCO)
PDVF membrane IMMODILON (Millipore)

Pefablock SC (Roche)
PSC-Protector solution (Roche)
Phenol (Roth)
Phenol-Chloroform-Isoamylethanol (25:24:1, Fluka)
Phorbol-12-myristate-13-acetate PMA (Cell Signaling)
PhosphoStop (Roche)
Precise Protein Gel 12% (#25222, Thermo Fisher)
SuperSignal West Pico Chemiluminescent Substrate (Pierce)
SYBR Green Fast Mix 2x (Applied Biosystems)
TaqMan RT-PCR Fast Mix (Applied Biosystems)
TEMED (IGN)
TRIzol (Invitrogen)
Trypsin/EDTA 100x (PAA)
UltraClear #344058 ultracentrifugation tubes (Beckman Coulter)
Urea (Sigma-Aldrich)

3.1.3 Eukaryotic cell lines

HEK 293T/17 (human epithelial cell line, ATCC)
HeLa (human epithelial cell line, kindly provided by Juan Patron, MPIIB)
NIH 3T3 (mouse epithelial cell line, kindly provided by Isabell Dietrich, MPIIB)
THP-1 (human pro-monocytotic cell line, ATCC)

3.1.4 Bacteria strains

RNaseIII experiments (kindly provided by Dr. S. Kushner [22], MG1655/1693 background)

- grows on Thymin-LB (50μg/ml)
- *E. coli* MG1655/1693 (wildtype)
- *E. coli* SK7622 (RNaseIII knockout, chromosomal kanamycin resistance 25μg/ml)

Argonaut2-expressing strains (kindly provided by Dr. L. Joshua-Torr [38], BL21-DE3-RIPL background)

- grows on LB
- pACYC-based plasmid (chloramphenicol resistance 34μg/ml, ~3.5 kb, expression of argU, proL tRNA)
- pSC101-based plasmid (streptomycin resistance, 4.7 kb, low copy number, expression of argU, ileU, leuW tRNA)

- pET28 (HSP90 expression, 7,8kB, Kanamycin resistance 25μg/ml)
- pGEX4T-1 (Ago2-GST or Ago2-ΔPiwi-GST expression, 7,526kB, Ampicillin resistance 50μg/ml)
- *E. coli* Ago2 and Ago2-piwi (D597A mutant)

***E. coli* DH5α**(kindly provided by I. Dietrich, MPIIB)

***E. coli* SCS110** (Stratagene)

- growth on LB, resistance for Streptomycin 30μg/ml
- deficient in *dam* and *dcm* methylases,
- endA^- version of JM110
- contains the lacIqZΔM15 gene on the F´episome

rpsL (Strr) thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44 Δ(lac-proAB) [F´traD36 proAB lacIqZΔM15]. (Genes listed signify mutant alleles. Genes on the F´episome, however, are wild-type unless indicated otherwise.)

***L. monocytogenes* EGD pNF8** (kindly provided by Dr. N. Fortinea [179])

- grows on LB
- GFPmut3 expression
- resistance to erythromycin (50μg/μl)

M. smegmatis MC2 155 pMV262-EGFP (kindly provided by J. Kowall, MPIIB)

- grows in 7H11 and on 7H9
- EGFP expression from plasmid pMV262-EGFP
- resistance to kanamycin (25μg/ml)

rBCG:ΔureC:hly (kindly provided by Dr. A. Nasser-Edine, MPIIB, Praque background)

- grows in 7H11 and on 7H9
- conditional urease C knockout
- expresses listeriolysin chromosomally integrated
- hygromycin resistance (25μg/ml)

***S. typhimurium* GFP** (kindly provided by Dr. B. Raupach, MPIIB)

- grows on LB
- expresses chromosomally integrated GFP

3.1.5 Kits

Calphos Kit (Clontech/Takara)
G-25 MicroSpin Kit (Amersham Pharmacia)
GeneAmp RNA PCR Kit (Applied Biosystems)
Human Monocyte Nucleofector Kit (Amaxa/Lonza)
miRNeasy Kit (Qiagen)
NucleoBond Kit AX500 (Macherey-Nagel)
Nucleofector Kit SG (Amaxa/Lonza)
Plasmid Maxi Purification kit (Qiagen)
RACE Kit (Invitrogen)
RNeasy MinElute CleanUp Kit (Qiagen)
Superscript III RT Kit (Invitrogen)
TagMan MicroRNA Reverse Transcription Kit (PN4366596)
TaqMan® microRNA Assays for hsa-miR-133b (#002247), hsa-miR-146a (#000468), RNU6B standard (#4373381)

3.1.6 siRNAs

- commercially available siRNAs

siGFP: gfp22 (Qiagen)

- custom siRNAs (Dharmacon)

siGFPtrans2/g_aph_hom:
Sense: 5' AGGGCCGCAGUGAGCAAGG
Antisense: 3' GGUCCCGGCGUCACUCGUU
siGFPtrans4/gfp 416:
Sense: 5' AGCGCACCAUCUUCUUCAAdTdT
antisense:3' CCUCGCGUGGUAGAAGAAGUU
siGFPprom/gro_non:
Sense: 5' GGGCAUGACCCGGUGCGGGdTdT
Antisense: 3' CCCGCACCGGGUCAUGCCCCU
siGFPtrans1/gro_ts:
Sense: 5' CGAUCCGGAGGAAUCACUUdCdG
Antisense: 3' AAGUGAUUCCUCCGGAUCGdGdG
siGFP blocked: 3'O-methylated version of siGFP
sense 5' GCAAGCUGACCCUGAAGUUCAU

antisense 5' GAACUUCAGGGUCAGCUUGCCG-OMe3'
Gmut1:
Sense: 5' AGAUGGACCAGUCCUUUUAdTdT 3'
Antisense: 3' dCdCUCUACCUGGUCAGGAAAAU 5'
siLuc95:
Sense: 5' GAGGAGUUGUGUUUGUGGAdTdT
Antisense 3' GCCUCCUCAACACAAACACCU
si fstl:
Sense 5' AUUUUGACGGCGGUGACUGdTdT
Antisense 3' dAdAUAAAACUGCCGCCACUGAC
si JTN (jagged 6-5):
Sense: 5' GGAGCACAUUUGCAGUGAAUU
Antisense: 3' UGCCUCGUGUAAACGUCACUU

3.1.7 Primer/oligomers

- Primer eGFP sequencing pMV262-eGFP in *M. smegmatis*

pMV262 Met for: 5' AGATCTTTAAATCTAGAGGTGACCACAA 3'
pMV262 Met rev: 5' AATGTAACATCAGAGATTTTGAGACACAA 3'
pMV262 nestM for: 5' cacaagctggagtacaactacaaca 3'
pMV262 nestM rev: 5' tgttgtagttgtactccagcttgtg

- Primer GFPmut3 sequencing in *L. monocytogenes*

Gmut3 fw: 5' GAGTTGTCCCAATTCTTGTTGAATT 3'
Gmut3 fwP: 5' GAAACAGCTATGACATGATTACGAATTC 3'
Gmut3 rev: 5' GGGCAGATTGTGTGGACAGG 3'

- 5' Race Primer

Adapter Oligo JVO-367: 5' ACTGACATGGAGGAGGGA 3'
reverse primer: 5' ACTTGTACAGCTCGTCCATG 3'

- Primer for RT-PCR normalization

GAPDH (human)

GAPDH H fw: 5' GCAAATTCCATGGCACCGT
GAPDH H rev: 5' TCGCCCCACTTGATTTTGG
HuPo (human acidic ribosomal protein)
HuPo RT-PCR fw: 5' GCTTCCTGGAGGGTGTCC
HuPo RT-PCR rev: 5' GGACTCGTTTGTACCCGTTG
6PBG (*L. monocytogenes* 6-phospho-beta-glucosidase)
Lm house fw: CCATCCGATGTTTTGCTAGCTAA 59°
Lm house rev: TGAACGTCGCCGCAGAA 60°
TaqMan Probe Lm 6PBG: CAGACGGAAGCAATGCACAGCCA 68°

- primer hairpin protocol

conserved reverse primer TaqMan RT-PCR
RT-PCR hp rev: 5' GTGCAGGGTCCGAGGT
TaqMan probe in hairpin
TaqManhp: 6FAM - TCG CAC TGG ATA CG - MGBNFQ (Applied Biosystems)
GFP22 siRNA detection (underlined: hybridization with siRNA)
GFP22 RT-PCR fw: 5' GCCGAACttCAGGGtCAGCtt 3'
GFP22 RT-PCR RT1:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>CGGCAAGC</u>
GFP22 RT-PCR RT2:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>CGGCAAGCTGA</u>
GFP22 RT-PCR RT5:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>CGGCA</u>
GFP22 RT-PCR RT6:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>CGGCAA</u>
detection of secondary siRNAs derived from GFP22 priming in *M. smegmatis*
hp_0_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>caagtt</u>
Fw_0_sec_gfp_smeg: cGCCGGACACGCTGAACT
hp_2_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>agttca</u>
Fw_2_sec_gfp_smeg: CGCCGGACACGCTGAA
hp_4_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>ttcagc</u>
Fw_4_sec_gfp_smeg: CTCGCCGGACACGCTG
hp_6_sec_gfp_smeg:

GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcagcgt
Fw_6_sec_gfp_smeg: CCTCGCCGGACACGC
hp_8_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgcgtgt
Fw_8_sec_gfp_smeg: GCCCTCGCCGGACAC
hp_m2_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcacaag
Fw_m2_sec_gfp_smeg: gCCGGACACGCTGAACTTG
hp_m4_sec_gpf_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgccaca
Fw_m4_sec_gfp_smeg: cCGGACACGCTGAACTTGTG
hp_m6_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACcggcca
Fw_m6_sec_gfp_smeg: gGACACGCTGAACTTGTGGC
hp_m8_sec_gfp_smeg:
GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACaacggc
Fw_m8_sec_gfp_smeg: ACGCTGAACTTGTGGCCG

- Primer pHIVx construction

1. removal of CMV-EGFP-wpre and insertion of restriction sites for intron-exon cassette (random – BamHI - BspEI – Eco91I - pHIV7 re-insert – DraII - random)
HIV reinsert fw:
CGCGACTCTAGATCATGGATCCTCCGGAGGTCACCACCGTCGACTAGCCGTACCT
HIV reinsert2 rev:
ACTATAGGGCGAATTGGGTACC

2. PCR intron-exon cassette (random – BspEI - pSM30-GFP – Eco91I – random) = pHIV8-GFP
HIV8 miR30 fw:
TATTCGCACTGGATACGATCCGGATGATTCTGTGGATAACCGT
HIV8 miR30 rev:
AATATCCTCCTTAGTTCCGGTGACCTAGAATGCAGTGAAAAAAATG

3. test pHIV8-GFP integration
reinsert test rev: 5‘ CCCAGTCACGACGTTGTA
reinsert test fw: 5‘ GCAACAGACATACAAACTAAAGAA

4. exchange GFP with Puromycin (random – XmaI – pHIV8 – Puromycin – XbaI - random) = pHIV8-Puro

Hi8DestPur fw:

5' ACTAGATCATCCCGGGATCCACCGGTCGCCACCATGACCGAGTACAAGCCCA

Hi8DestPur rev:

5' GGAACTAAGGTCTAGAGTCGCGGCCGCTTTATCAGGCACCGGGCTT

5. non-coding single base exchange in puromycin to remove Esp3I restriction site = pHIV9-Puro

Pur-REN fw: 5' TCCTGGCCACCGTCGGCGTTTCGCCCGACCACCAG

6. puromycin sequencing primer

PurTest fw: 5' ATGACCGAGTACAAGCCCA

PuTest rev: 5' TCAGGCACCGGGCTT

- Primer amiRNA ligation test pHIV

shtest fw: 5‘ TGGAAACACTTGCTGGGATT

shtest rev (pHIV8-GFP): 5‘ TTGCTCACCATGGTGGC

shtest puro rev (pHIV9-Puro): 5' GTGGGCTTGTACTCGGTCAT

- Primer amiRNA into pHIV constructs

control shLuc95 (L95 [182])

shLuc95 fw

AGCGAGAGGAGTTGTGTTTGTGGACGtagtgaagccacagatgtaCGTCCACAAACACA
ACTCCTCC

shLuc95 rev

GGCAGGAGGAGTTGTGTTTGTGGACGtacatctgtggcttcactaCGTCCACAAACACA
ACTCCTCT

shTLR2-1 and shTLR2-2 human (Hannon-Elledge database)

hTLR2-1 fw:

AGCGCccaggtaaagtggaaacgttaTAGTGAAGCCACAGATGTAtaacgtttccactttacctgga

hTLR2-1 rev mis:

GGCAtccaggtaaGgtAgaaacgttaTACATCTGTGGCTTCACTAtaacgtttcTacCttacctggG

hTLR2-2 fw:

AGCGAgccctctctacaaactttaatTAGTGAAGCCACAGATGTAattaaagtttgtagagagggcc

hTLR2-2 rev mis:
GGCAggccctcCTtacaaactttaatTACATCTGTGGCTTCACTAattaaagtttgtaAGgagggcT
shNRLP1-1 and shNRLP1-2 human (Hannon-Elledge database)
sh2NRLP1-1 H fw
AGCGACCTGATTATGTGGAGGAGAATTAGTGAAGCCACAGATGTAATTCTCCT
CCACATAATCAGGC
sh2NRLP1-1 H rev
GGCAGCCTGATTATGTGGAGGAGAATTACATCTGTGGCTTCACTAATTCTCCTC
CACATAATCAGGT
sh2NRLP1-2 H fw
AGCGCGGAGAATCGAGGACATTTAATTAGTGAAGCCACAGATGTAATTAAATG
TCCTCGATTCTCCT
sh2NRLP1-2 H rev
GGCAAGGAGAATCGAGGACATTTAATTACATCTGTGGCTTCACTAATTAAATGT
CCTCGATTCTCCG
shCard9-1 and shCard9-2 human (Hannon-Elledge database)
sh2Card9-1 H fw
AGCGCGGATCTGTTCTGTTTCACCATTAGTGAAGCCACAGATGTAATGGTGAAA
CAGAACAGATCCT
sh2Card9-1 H rev
GGCAAGGATCTGTTCTGTTTCACCATTACATCTGTGGCTTCACTAATGGTGAAA
CAGAACAGATCCGT
sh2Card9-2 H fw
AGCGCCGTTTGTTAAGCGGCACTCATTAGTGAAGCCACAGATGTAATGAGTGCC
GCTTAACAAACGG
sh2Card9-2 H rev
GGCACCGTTTGTTAAGCGGCACTCATTACATCTGTGGCTTCACTAATGAGTGCCG
CTTAACAAACGG
Raf-1 human (RT-PCR primer and shRaf1-1 (Dharmacon raf-1 SMARTPOOL) and shRaf1-2 (Hannon-Elledge database))
Raf1 fw: 5' AGAATCGGAGAGCCGGT
Raf1 rev: 5' TTACACCTAAATTTAATTTATTTTATTAAAATAAC
shRaf1-1 H fw: AGCGACAAAGAACATCATCCATAGAGTAGTGAAGCCACAGATG-
TACTCTATGGA
TGATGTTCTTTGC
shRaf1-1 H rev: GGCAGCAAAGAACATCATCCATAGAGTACATCTGTGGCTTCAC-
TACTCTATGGAT
GATGTTCTTTGT

shRaf1-2 H fw: AGCGACCAGATCTTAGTAAGCTATATTAGTGAAGCCACAGATG-TAATATAGCTTA

CTAAGATCTGGG

shRaf1-2 H fw: GGCACCCAGATCTTAGTAAGCTATATTACATCTGTGGCTTCAC-TAATATAGCTTAC

TAAGATCTGGT

DC-Sign (based on si11 from [183])

shCD209 fw:

AGCGAAAGGCTGCAGTGGGTGAGCTTtagtgaagccacagatgtaAAGCTCACCCACTGCAG

CCTTC

shCD209 rev: GGCAGAAGGCTGCAGTGGGTGAGCTTtacatctgtggcttcactaAAGCT-CACCCACTGCAGC

CTTT

3.1.8 Antibodies and blocking solution

TLR2 antibody (APC) (ab24996, abcam)

FcR Blocking Reagent, human (130-059-901, Miltenyi)

Mouse IgG2a-APC (130-091-836, Miltenyi)

total Raf-1 (rabbit, #9422 Cell Signaling)

phosphorylated Raf-1 (Ser338, rabbit, #56A6 Cell Signaling)

goat anti rabbit antibody labeled with horseradish peroxidase (DOD, kindly provided by M. Staeber, MPIIB)

GAPDH (6C5, Calbiochem)

3.1.9 Antibiotics and Fungicides

ampicillin (Sigma-Aldrich)

chloramphenicol (Sigma-Aldrich)

erythromycin (Sigma-Aldrich)

gentamycin (Sigma-Aldrich)

hygromycin (Sigma-Aldrich)

kanamycin (Sigma-Aldrich)

100x penicillin/streptomycin (PAA)

penicillin G (Sigma-Aldrich)

puromycin (Sigma-Aldrich)

3.1.10 Buffer and solutions

Cell culture medium

Medium HEK 293T/NIH 3T3 ("Complete", 500 ml)

- 500 ml DMEM (Gibco)
- 10 % v/v FCS (Gibco)
- 1x L-Glutamine (PAA)
- 1x penicillin/streptomycin (PAA)
- 100 mM Sodium pyruvat (Biochrom)
- 10 mM HEPES (PAA)

Medium THP-1/monocytes (500 ml)

- 500 ml RPMI (Gibco)
- 10% v/v FCS (Gibco)
- 1x L-Glutamine (PAA)
- 1x penicillin/streptomycin (PAA)
- 100 mM Sodium pyruvat (Biochrom)
- 10 mM HEPES (PAA)
- 0.1 % v/v β-Mercaptoethanol (Gibco)

Bacterial culture medium

7H9-Medium (1l)

- 4,9 g 7H9 Middlebrook (Difco)
- 10 % v/v ADC (Difco)
- 0,5 % v/v Glycerol (Sigma-Aldrich)
- 0,5 % v/v Tween 80 (Fluka)
- filter sterile at 0,22 μm pore size (Millipore)

Luria-Broth (LB) medium and - agar (Roth)

Buffers used in molecular biology

TfbI pH 5,8

- 30 mM Potassium acetate (Sigma-Aldrich)
- 100 mM Rubidium chloride (Sigma-Aldrich)
- 10 mM Calcium chloride (Sigma-Aldrich)
- 50 mM Manganese chloride (Sigma-Aldrich)

- 15% v/v Glycerol (Sigma-Aldrich)

Tfb II pH 6,5

- 10 mM MOPS (Roth)
- 10 mM Rubidium chloride (Sigma-Aldrich)
- 75 mM Calcium chloride (Sigma-Aldrich)
- 15 % v/v Glycerol (Sigma-Aldrich)

A10G (1l)

- A. dest.
- 10 % v/v Glycerol (Sigma-Aldrich)

HS-Buffer (ice cold, pH 7.0)

- 1mM HEPES pH 7.0 (PAA)
- 0.5M Sucrose (Sigma-Aldrich)
- A. dest.
- filter sterile at 0,22μl pore size

shRNA Annealing-Buffer

- 100 mM Tris (Sigma-Aldrich) pH 7,4
- 10 mM Magnesium chloride (Sigma-Aldrich)

GIMe buffer

- 4M Guanidinium isothiocyanat (4 month storable at room temperature, Fluka)
- 1 % v/v beta-Mercaptoethanol (add fresh, Gibco)

2x RNA Loading solution

- 95 % v/v Formamide (Sigma-Aldrich)
- 0.03 % v/v Bromophenol Blue (Sigma-Aldrich)
- 0.03 % v/v Xylene Cyanol FF (Sigma-Aldrich)
- 0.03 % v/v SDS (Sigma-Aldrich)
- 0.5 mM EDTA (Sigma-Aldrich)

- A. dest

10X TBE (Tris borat EDTA buffer)

- 0.9 M Tris base (Sigma-Aldrich)
- 0.9 M Boric acid (Sigma-Aldrich)
- 0.5 M EDTA (Sigma-Aldrich)
- A. dest.

20x SSC (sodium chloride/sodium citrate)

- 3 M Sodium chloride (Sigma-Aldrich)
- 300 mM tri-Sodium citrate dihydrate (Sigma-Aldrich)
- A. dest.
- adjust pH to 7.0 with hydrochloric acid (Sigma-Aldrich)
- autoclave

20x SSPE (Sodium chloride/Sodium phosphate/EDTA)

- 3 M Sodium chloride (Sigma-Aldrich)
- 20 mM EDTA (Sigma-Aldrich)
- 0.20 mM Sodium di-hydrogen phosphate (Sigma-Aldrich)
- adjust to pH 7.4

100x Denhardt's solution

- 10 g Ficoll 400 (Sigma-Aldrich)
- 10 g polyvinylpyrrolidone (Sigma-Aldrich)
- 10 g BSA (fraction V, Sigma-Aldrich)
- adjust with A. dest. to 500 ml
- filter sterilize
- store at –20°C in 25-ml aliquots

Prehybridization solution

- 6x SSC
- 10x Denhardt's solution
- 0.2 % Sodium lauryl sulfate

Hybridization solution I

- 6x SSC
- 5x Denhardt's solution
- 0.2 % Sodium lauryl sulfate

Hybridization solution II

- 7% Sodium lauryl sulfate
- 0.2M di-Sodium hydrogen phosphate
- adjust to pH7.0

Washing buffer

- 2x SSPE
- 0.1% Sodium lauryl sulfate

3.1.11 Software & tools

CLC DNA Workbench v3, v4, v5 (CLC Bio, Aarhus N, Denmark)
ABI SDS 2.2.2 (Applied Biosystems)
FCS Express 6.33 (De Novo Software, Los Angeles, CA, [6])
preGAP, GAP4 and Phrep [185]
REST-MCS [7]
mfold 3.2 [8]
NetPrimer 3 [9]
Oligo Melting Temperature [10]

3.2 Potential new method to establish gene knockdown and knockout in prokaryotes

3.2.1 Design of siRNAs

If sequences were not available already in literature or available commercially, siRNAs were designed by the use of an in-house developed algorithm [182].

3.2.2 Cellular extract generation

Three different protocols have been used for the generation of cellular extracts.

The first protocol was developed in house. For this three T75 flasks (175cm^{2}area) confluent with HeK 293T and NIH 3T3 cells were trypsinized or cells scraped with a cell scraper, both cell suspensions mixed and washed three times with sterile PBS (centrifugation at 300xg for 5 minutes at room temperature). Afterwards the pellet was resuspended in 700 μl PBS and 10 μl of a protease inhibitor mix was added (100 μl Complete 25x (Roche), 200 μl Pefablock SC (stock 12.5 M, Roche), 50 μl Leupeptin stock (10 mg/ml, Serva) add adjusted with A. dest to 500 μl). Half volume of the suspension was lysed by freeze-thaw lysis (four times in liquid nitrogen freezing, four times at 37°C water bath thawing). The other 500 μl of the suspension was lysed by mechanical lysis by pushing and pulling twenty times through an insulin syringe needle. Than both fractions were mixed again and centrifuged at 20,000xg and 4°C centrifuged. 25 μl aliquots of the supernatant were frozen in liquid nitrogen and immediately stored at -80°C. The supernatant was freshly thawed on ice prior to each experiment and never frozen again.

For the second protocol the amount of protease inhibitor was increase by preparing 1.25 ml 4x protease inhibitor mix from 5 μl Leupeptin (2 mg/ml stock solution), 5 mg Pefablock SC Plus 250 μl PSC-Protector solution (Pefablock SC Plus kit, Roche), and 1ml Complete Mini, EDTA-free (1 pill in 1.5 ml A. dest, Roche). After the cell suspensions had been mixed and washed with PBS as described above the pellet was resuspended in 750 μl PBS and 250 μl 4x Protease inhibitor mix. The following protocol was the same as described above.

The third protocol was published recently [184] and has been used widely for *in vitro* RNAi experiments.

3.2.3 Electrocompetent *M. smegmatis*

To make *M. smegmatis* electrocompetent pre-cultures of 5ml 7H9 medium were incubated over night at 37°C while shaking at 90 rpm. Next morning the pre culture was added to 300 ml 7H9 medium in Erlenmeyer flasks and further incubated at 37°C shaking 90 rpm until an optical density at wavelength of 600 nm (OD600) of 0.5 - 1 was reached in photometer. Afterwards the culture was incubated 1-2 hours on ice and than centrifuged at ca. 6000xg and 4°C for 15 minutes. Every following step was performed on ice with material chilled in advance to 4°C.

The supernatant was discarded and the pellet washed three times by repeated resuspension

and centrifugation in 50 ml A10G buffer.

After this the pellet was resuspended in 4ml A10G and 200 μl Aliquots in 1.5ml reaction tubes either directly used or frozen in liquid nitrogen and stored at -80°C. In the later case after thawing the bacteria on ice they were washed once with A10G or diluted with 100 μl A10G.

3.2.4 Electrocompetent *L. monocytogenes*

Pre-cultures of 5 ml LB medium were inoculated with *L. monocytogenes* from glycerol stocks and incubated over night at 90-120 rpm and 37°C. Next morning the pre-culture was transferred into 300 ml LB and incubated further at 37°C shaking until optical density at wavelength 600 nm reached between 0.18 to 0.25. 90 μl 10 mg/ml PenicillinG was added and two more hours incubated shaking at 37°C. Afterwards the bacteria culture was centrifuged at 4°C and 6000xg for 10 minutes and the supernatant discarded. The pellet was resuspended in 35 ml HS-buffer and transferred into 50ml conical tubes. The centrifugation step was repeated and the supernatant once more discarded. This time the pellet was resuspended in 10 ml HS-buffer, the suspension once more centrifuged and the supernatant discarded. Afterwards the pellet was resuspended in 2.5 ml HS-buffer and aliquots of 100 μl frozen in liquid nitrogen and stored at -80°C.

3.2.5 Electrocompetent *E. coli*

2,5 ml *E. coli* overnight pre-culture was transferred into 300 ml LB and incubated at 37°C until an optical density at wavelength 580 nm or 600 nm of 0.5-0.7 was reached. Cells were then incubated on ice for 15 minutes or up to 1 hour. This was followed by 15 minutes centrifugation at 4°C and 5000xg and the supernatant was discarded. Afterwards the pellet was resuspended in 250 ml ice cold A. dest, centrifuged once more and the supernatant discarded again. Now the pellet was resuspended in 50 ml A10G and the suspension again centrifuged and the supernatant discard. Afterwards the pellet was resuspended in 4 ml A10G and aliquots of 100 μl directly used or after shock freezing in liquid nitrogen stored at -80°C.

3.2.6 Electrocompetent BCG

Electrocompetent BCG was produced like electrocompetent *M. smegmatis*, except that every step was performed at room temperature instead of incubation on ice or pre-cooling of liquids/buffers.

3.2.7 Electroporation of Prokaryotes

1μg DNA, 100pmol of siRNA and 1μl cellular extract were adjusted to 25 μl with A. dest. and incubated for 5-10 minutes at room temperature. Afterwards the mix was added to 100μl electrocompetent bacteria, the suspension mixed on ice (except for BCG) and electroporated in 0.2cm cuvettes pre-cooled on ice.

For *Mycobacteria* parameters of electroporation were: 2,5kV, resistance of 1000Ohm and 25μFD capacity. Time constant should be around 19-22ms.

For *L. monocytogenes* electroporation parameters have been: 1 kV, 400 Ohm, 25 μFD. Time constant should be around 5ms.

After electroporation 1ml of medium (LB in case of *L. monocytogenes*, 7H9 in case of *Mycobacteria*) was added immediately and the suspension moved to sterile reaction tube and incubated at 37°C for 2-3 hours. Afterwards the bacteria suspension was added into 5ml of medium with antibiotics (50μg/ml Erythromycin (Sigma) in case of *L. monocytogenes*, 25μg/ml Kanamycin (Sigma) in case of *M. smegmatis)* and incubated over night at 37°C while shaking.

3.2.8 Culturing and clone testing (PCR, Fluoroscan)

Every day 10μl bacteria culture were mixed with 990μl fresh medium with antibiotics and 100μl platted on agar plates with antibiotics (LB agar plates in case of *L. monocytogenes*, 7H11 in case of *M. smegmatis).* The remaining 900μl were used to inoculate 5ml fresh medium with antibiotics and incubated again over night.

This was repeated until day 5-7 after electroporation.

GFP expression and Optical density were measured every 24 hours. Optical density of culture was measured at 600nm wavelength excitation (in case the optical density was above 1, the bacterial culture was diluted 1:10 and the measurement repeated). GFP expression of 1ml undiluted culture was measured in a Fluoroscan Ascent (Thermo Fisher) with filter pairs at 485nm and 510nm.

3.2.9 Purification of mRNA

TRIzol (Invitrogen, Carlsbad, USA) was used according to manufacturer's protocol for purification of mRNA from *E. coli* and mammalian cells. For *L. monocytogenes* or *M. smegmatis* the protocol was modified (all steps at 4°C and material pre-cooled on ice) as followed:

50ml of bacteria culture was centrifuged at 3800xg for 7 minutes at 4°C and the supernatant discarded by decantation. The pellet was slowly resuspended in the remaining supernatant on ice and mixed with 1-2 ml TRIzol (depending on pellet size) and immediately further processed or with a delay of maximum 10-15 minutes frozen in -80°C. 1ml of the TRIzol/bacteria suspension was transferred into 2 ml FastPrep Tubes Lysis Matrix B (Qbiogene) and shaken at maximum speed setting (6.5) 20 seconds in a FastPrep shaker (Qbiogene), incubated 3-5 minutes on ice to cool the tubes and the shaking process repeated. Afterwards the tubes were centrifuged for 10 minutes at 18.000xg or higher at 4°C and the supernatant transferred into a new tube without carrying any pellet remains over. 350 μl of chloroform was added and the whole mix was shaken vigorously 10 seconds by hand. Afterwards the suspension was incubated at room temperature for 5 minutes and than centrifuged at 18.000xg or higher and 4°C for 15 minutes. The upper phase without the interface was carefully transferred into a new tube and 0.7 times the volume of isopropanol, 0.2 times the volume of 5 M ammonium acetate and 1/60 times the volume of glycogen (Ambion) was added. The tube was than centrifuged at 15 minutes and 4°C at 18.000xg or higher and the supernatant discarded with remaining liquids carefully removed by pipetting. The same volume of 75% Ethanol was added and the tube once more centrifuged for 8 minutes to desalt the precipitated nucleic acids. Afterwards the supernatant was completely discarded and the pellet dried by air contact. The pellet was solved in 60 μl RNase-free A. dest and digested with RNase-free DNase: 4 μl DNase (Roche, 10 U/μl) was mixed with 40 μg RNA (around 20 μl usually), 10 μl 100 mM manganese sulfate and the volume adjusted to 100μl with RNase-free A. dest. This solution was incubated at 37°C for 20 minutes and the DNase and buffer removed by phenol-chloroform extraction: the volume was adjusted to 400 μl with RNase-free A. dest and 400 μl of Phenol (Roth) was added. Afterwards the solution was shaken vigorously by hand for 1 minute and centrifuged for 5 minutes at 4°C and 18.000xg or more. The aqueous phase was removed carefully and transferred into a new tube and adjusted to 400 μl again if necessary. Than the same step was repeated twice, this time with 400 μl phenol-chloroform-isoamylethanol (v/v ratio 25:24:1) and repeated three more times with 400 μl chloroform-isoamylethanol (v/v ratio 24:1). The RNA quality was assessed on Bioanalyzer RNA Chips (Applied Biosystems).

3.2.10 siRNA re-purification after electroporation into bacteria

1ml of *M. smegmatis* or *L. monocytogenes* culture was mixed with 1 ml GIMe buffer and centrifuged (10 minutes, 4500xg, 4°C) and the pellet washed two times in growth media. The pellet was than resuspended in 1ml Trizol and transferred into a FastPrep

Tube Lysis Matrix B (Qbiogene). Afterwards the tube was shaken 3 times for 30 seconds with 2 minutes cooling breaks on ice in a FastPrep (Qbiogene). Following this the tubes were inverted gently for 5 minutes at 4 °C and 1 minute centrifuged at maximum speed and 4°C. The supernatant was mixed with 300 μl Chloroform, shaken vigorously for 15 seconds and mixed 2 minutes with gentle shaking. Thereafter a centrifugation step for 10 minutes at maximum speed and 4°C followed and the aqueous phase was transferred into a new tube, Ethanol-precipitated and once more centrifuged. The pellet was washed twice in 70% Ethanol and solved in 350 μl RNase-free Water (Invitrogen). Afterwards the Qiagen miRNeasy Kit protocol was applied and after addition of 450 μl 100% Ethanol to the flow-throw and vigorous mixing the solution was added to a MinElute column (RNeasy MinElute CleanUp Kit, Qiagen). Subsequent steps were performed according to manufacturer's protocol.

3.2.11 Northern Blot for small RNAs after delivery into prokaryotes

A 12.5% denaturing polyacrylamide gel was made from 62.5 ml 20% acrylamide in 7 M urea/1xTBE, 37.5 ml 7 M urea/1x TBE, 200 μl 10% fresh w/v ammonium persulfate and 60μl TEMED with an one hour polymerization.

Chambers were assembled and the gel pre-run for 30 minutes at 30mA to pre-heat the gel. Afterwards 10 μl small RNA purified from bacteria after the electroporation was mixed with 30 μl loading buffer (7 M urea, 0.1 % SDS, bromophenol blue und xylene cyanol) and incubated for 2 minutes at 95°C and cooled down on ice. The solution was than loaded into the gel pockets and bands separated by electrophoresis at 30 mA for 100 minutes or until the bromophenol blue band had reached the end of the gel.

Then the RNA was transferred on a Hybond N+ membrane (GE Healthcare Life Sciences) at 0.55 A for 1.5 hours in 1x TBE with continuous cooling of the gel chamber on ice. The transferred nucleic acids were cross linked to the membrane by UV light (120 mJ) and afterwards the membrane incubated at 80°C for one hour.

For the hybridization the labeled probe was added to 30 ml Hybridization solution II, incubated for 2 minutes at 95°C and cooled on ice and the membrane incubated with the hybridization mix over night at 35°C. Afterwards (if necessary to remove background) the washing buffer was preheated to 35-37°C and the membrane washed up to three times at 35°C.

3.2.12 Isolation of low copy plasmid DNA (*M. smegmatis*)

To obtain plasmids from *M. smegmatis* the Qiagen Maxi Preparation kit was used with a modified protocol. At first 1 l of 7H9 medium was inoculated with 3 ml 7H9 *M. smegmatis* pre-culture and incubated while shaking (90 rpm) at 37°C for 24 hours. Afterwards chloramphenicol was added (160 mg/l final concentration) and the culture further incubated. The bacterial culture was centrifugation for 15 minutes at 6000xg and 4°C and the pellet resuspended in 40 ml ice cold P1 Resuspension Buffer with RNase A. Lysozym was added (Sigma-Aldrich, 10 mg/ml final concentration) and incubated at 37°C over night. Afterwards 40 ml P2 Lysis Buffer was added, the solution mixed vigorously and incubated for 15 minutes at room temperature. This was followed by addition of 40 ml ice cold P3 buffer, mixed vigorously and 30 minutes incubated on ice. Following centrifugation for 30 minutes at 4°C with 20.000xg the supernatant was transferred into a new tube, once more centrifuged and the supernatant mixed with 84 ml of isopropanol. The centrifugation step was repeated and the pellet resuspended in 500 μl A. dest and 11.5 μl equilibration buffer QBT added. The subsequent steps were analogous to the Qiagen Maxi Preparation Kit protocol for ultra low copy plasmid preparations.

3.2.13 Sequencing and direct methylation analysis

Sequencing was performed at the laboratory of Dr. Martin Meixner, SMB, Technical University Berlin with ABI BigDyes. Direct methylation analysis in inter sequence comparison was performed with preGAP and GAP4 according to a published protocol [185]. Briefly in preGAP files to process were added and in the "configure modules" tab the following items were activated:

- estimate base accuracy
- trace format conversion
- initialize experiment files
- augment experiment files
- quality clip
- gap4 shotgun assembly

Than a new database item was created and preGap started for processing. Afterwards GAP4 was used to open the specified database, alignment bar was right clicked and the contig edited. The Diff-button was used to read trace differences. Phred was used to calculate trace diff data (area under curve) to look for significant triplet motif changes indicating methylation by intra-sequence comparison.

3.2.14 Search for potential restriction sites/motifs adjacent to the deleted regions

Ten and twenty bps upstream and downstream of the sequenced deletion start site search for known restriction enzymes was performed. Additionally those sequences were split into each possible 4, 5, 6, 7 and 8 bp long fragments and with CLC DNA Workbench 3 a consensus search performed.

3.2.15 Overexpression of human Ago2-GST in *E. coli*

Functional human Ago2-GST and its PIWI domain mutant [38] was expressed in *E. coli* BL21-DE3-RIPL strains with a modified protocol as previously reported [187]. Briefly LB culture with Kanamycin (25μg/ml) and Ampicillin (50μg/ml) were grown at 37°C while shaking until it reached an optical density at 600nm (OD600) of 0.3. Due to toxicity of Ago2 at this time point the culture was further grown at 22°C while shaking until the OD600 reached a value of 0.6. During incubation at 22°C Ago2 expression was induced by addition of 0.5mM IPTG. At this time point bacteria were made electrocompetent and used without IPTG or decreased temperature for subsequent experiments.

3.2.16 Proof of principle experiments for proposed new method to cause sequence specific mutations by endonucleolytic activity of FEN-1 or Polymerases

Several different Oligomer designs were drafted to test the hypothesis of obtaining sequence specific gene deletions by endonucleolytic activity. Design B and C were tested.

Design B contains for each genomic strand a complementary 20-24 bp long DNA Oligomer with a Thymin or Adenine base at position 3 of the 3' end that was replaced by Uracil. The phosphodiester bonds between bases 1-3 at the 3' end and 1-2 and 4-6 at the 5' end were replaced by phosphorothioates to block Oligomer degradation by FEN-1 endonuclease and intracellular exonuclease activity.

Design C had the same target sequence in the genomic DNA as B but instead of chemical modification the DNA Oligomers were extended on the 3' end with a hairpin folding sequence which has been described to act as template for 5' endonuclease activity of eubacterial polymerases [188].

The DNA-Oligomers for B were treated with UDG (Uracil Deglycosylase, Fermentas) by mixing 20μl of 100pmol/μl Oligomer with 2μl UDG, 3μl 10x UDG Buffer and 5μl A. dest. The mix was incubated for one hour at 37°C to create an abasic site in the Oligomer by

removing the Uracil base to activate FEN-1. In case of heat inactivated UDG first 2μl UDG were combined with 3μl 10x UDG Puffer and 5μl A. dest. and incubated for 20 minutes at 95°C before 20μl (100pmol/μl) single Oligomer were added and incubated for one hour at 37°C.

The Oligomer was than added to freshly prepared electrocompetent BCG. 1.5μl untreated Oligomer of design C or 2,2μl UDG-treated Oligomer of design B (so 150pmol of each Oligomer) was used. The mix was incubated for 5-10 minutes at room temperature and electroporated. Every sample was plated on 7H11 plates with and without hygromycin as selecting antibiotic. After three weeks of incubation at 37°C the plates were checked for culture growth and replica plated from 7H11 plates without selection on plates containing hygromycin. Clones without growth on hygromycin agar were tested by PCR for deletion in the targeted region of the hygromycin gene. As control for the electroporation efficacy pUC8 plasmid was used.

In later experiments the distance between both Oligomers was extended and compared to the effects of adjacent Oligomer binding sites. As the lethality of Oligomer electroporation proved extremely high with the original protocol in later experiments the samples were treated only with 1:10 diluted DNA-Oligomers. The setup for the first proof of principle was the following:

1. 2,2μl FEN B prom fw (67pmol/μl), 2,2μl FEN B prom rev (67pmol/μl)

2. 2,2μl FEN B stop fw (67pmol/μl), 2,2μl FEN B prom rev (67pmol/μl)

3. 1,5μl FEN C prom fw (100pmol/μl), 1,5μl FEN C prom rev (100pmol/μl)

4. 1,5μl FEN C stop fw (100pmol/μl), 1,5μl FEN C prom rev (100pmol/μl)

The setup for the final experiment was:

1. 2,2μl FEN B prom fw (6,7pmol/μl), 2,2μl FEN B prom rev (6,7pmol/μl) with UDG treatment

2. 2,2μl FEN B prom fw (6,7pmol/μl), 2,2μl FEN B prom rev (6,7pmol/μl) with heat inactivated UDG

3. 2,2μl FEN B stop fw (6,7pmol/μl), 2,2μl FEN B prom rev (6,7pmol/μl) with UDG treatment

4. 2,2μl FEN B stop fw (6,7pmol/μl), 2,2μl FEN B prom rev (6,7pmol/μl) with heat inactivated UDG

5. 2,2μl FEN B prom fw with UDG treatment

6. 2,2μl FEN B prom rev with UDG treatment

7. 2,2μl FEN B stop fw with UDG treatment

3.3 Hairpin Protocol

3.3.1 Hairpin reverse transcription und TaqMan RT-PCR

For detection of siRNA delivery and quantification with our modified primer and probes Reverse Transcription was performed using GeneAmp RNA PCR Kit with final concentrations of 1x PCR buffer, 5.5mM Magnesium Chloride, 250μM per dNTP and 3.33U/μl MuLV Reverse Transcriptase. Additionally final concentrations of 0,25U/μl RNasin (Fermentas) or RNaseOut (Applied Biosystems), 1:100 diluted snRNA sample and 50nM hairpin primer were added. The final volume was 15μl. The protocol was performed as published previously [189]. Briefly the reverse transcription profile used was 30 minutes incubation at 16°C, 30 minutes at 42°C and 5 minutes at 95°C. Afterwards TaqMan RT-PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) in a final concentration of 1x, 1.5μM forward primer, 0.7μM reverse primer, 0,2μM TaqMan probe and 0.67μl of the reverse transcription reaction in a final volume of 10μl. The TaqMan RT-PCR used was 10 minutes incubation at 95°C and 40 cycles of 15 seconds denature at 95°C and 1 minute extension at 60°C.

For detection of overexpression of hsa-miR-133b, hsa-mir-146a and RNU6B (small nuclear RNA U6B, standard) TagMan MicroRNA Assays and TagMan MicroRNA Reverse Transcription (PN4366596) kits were purchased from Applied Biosystems and used according to manufacturers protocol.

3.3.2 primer and TaqMan probe design

Reverse transcription hairpin primer were designed with five, six, eight and eleven base-pairs overhang at the 3' end of the stem complementary with the 3' end of the antisense siRNA strand. The remaining stem-loop structure was taken from the published material section for the method [189].

Forward primer was designed to be as closely located at the 5' end of the antisense siRNA strand as possible while having a melting temperature of around 65°C using Primer3 output with Breslauer's enthalpy and entropy from the Online Oligo Melting tool at Weizman Institute, Israel [10]. Forward primers were designed so they would never overlap with the reverse transcription hairpin primer. If the melting temperature of the remaining antisense siRNA strand was too low the forward primer was extended on 5' with random sequences.

TaqMan probe was shifted to hybridize only with the conserved part of the hairpin primer with no more overlap with the snRNA that should be detected.

3.4 Establishing a RNAi platform on host side

3.4.1 Purification of primary human monocytes from buffy coat and differentiation into dendritic cells

Buffy coat was obtained from Deutsches Rotes Kreuz Berlin-Brandenburg, spread over two 50ml conical tubes (Sarstedt) and adjusted with room temperature PBS to a volume of 50ml. The tubes were centrifuged at 20°C for 20 minutes at 390xg with slow deceleration. The serum supernatant was removed carefully up to 1cm above the pellet. The pellet with the remaining supernatant (around 10-15ml) was then adjusted to a volume of 105ml with room temperature PBS. In three more conical tubes 15ml room temperature Ficoll-Hypaque (Biocoll, density 1.077) was pipetted and 35ml of the cell suspension carefully layered on top of the Ficoll without mixing of both phases. The tubes were then centrifuged for 25 minutes at 20°C and 390xg with slow deceleration. Afterwards the supernatant was carefully removed without disturbance of the interphase followed by transfer of around 10ml interphase and upper phase into a new conical 50ml tube. The cell suspension was adjusted with room temperature PBS to 50ml and centrifuged at 200xg and room temperature for 5 minutes. This step was repeated performed at least twice or until only few platelets remained (determined under the microscope). The cell suspension was stored on ice and the cell density determined. Cell suspension was centrifuged again at 200xg for 8 minutes at 4°C and the supernatant discarded. $2*10^8$cells were resuspended in 300μl MACS buffer (Miltenyi Monocyte Isolation Kit II) and monocytes purified according to manufacturers protocol. 10^7cells/ml was the final cell density. Cells were grown in 75cm^{2}cell culture flask with 20ml THP-1 media. Differentiation into Dendritic cells (DC) was performed by addition of 800U/ml hGM-CSF and 500 U/ml hIL-4 (Active Biosciences) and incubation for 7 days at 37°C and 5% CO_2.

3.4.2 Nucleofection of primary human monocytes or THP-1 cells

In case of single well Nucleofection 200 ng Plasmid DNA or 50-100 pmol siRNA was mixed with 100 μl room temperature, supplemented Human Monocyte Nucleofector Solution (mix of 0.5 ml Supplement and 2.25 ml Human Monocyte Nucleofector Solution, Amaxa) and a 24 well plate loaded with 1ml Human Monocyte Nucleofector Medium (Amaxa, enriched with 2 mM Glutamine und 10 % FCS) per sample and well and pre-incubated at 37°C and 5 % CO_2 for 30 minutes. 10^6 primary human monocytes or THP-1 cells were resuspended in the DNA-Nucleofector solution mix and transferred immediately into Nucleofector cuvettes (Amaxa). Cells were nucleofected with protocol Y-01 and

immediately and very carefully 500μl of the pre-warmed supplemented Human Monocyte Nucleofector Medium added and the cells suspension carefully transferred into the 24 well plate.

In case of 96 well shuttle Nucleofection 2 μl siRNA (10-100μM stock) or 200 ng plasmid were plated into each well of 96 well round bottom plates (NUNC) and 80 μl THP-1 medium plated into each well of a second 96 well round bottom plate and 900 μl THP-1 medium was plated into each well of a 24 well plate and incubated at 37°C and 5 % CO_2 for 30 minutes. Cells were aliquoted at 5*10^5cells per sample and centrifuged at 200xg at room temperature for 10 minutes with slow deceleration and the supernatant discarded. The pellet was resuspended in 20 μl Nucleofector Solution (Amaxa, Kit SG) and carefully transferred into the 96 well plate with the nucleic acid aliquots. Cells were nucleofected at protocol DN100 and immediately 80 μl of the pre-warmed THP-1 medium added. The cell suspension was than transferred into the 24 well plate with the pre-warmed THP-1 medium and incubated at 37°C and 5 % CO2.

3.4.3 Luciferase assay

48 hours after cell transfection or Nucleofection cells were resuspended in the medium by careful pipetting and centrifuged at 300xg and room temperature for 5 minutes. The supernatant was removed carefully and the cells washed with 1ml room temperature PBS and centrifuged again. The pellet was resuspended in 100 μl Passive Lysisbuffer (Promega) and the solution incubated while gently shaking for 20 minutes at room temperature. The samples were immediately measured or snap frozen in liquid nitrogen and stored at -80°C. Measurement was performed in a Victor Luminometer (Perkin Elmer).

3.4.4 Reagent based THP-1 cell transfection methods

THP-1 cells were split one day in advance and washed before transfection two times with 15 ml serum free Optimem (Invitrogen). Cells were centrifuged at room temperature and 400xg for 10 minutes in 50 ml conical tubes (Sarstedt). Transfections were performed in 24 well plates except in case of Calcium phosphate protocol, plasmid transfected was pEGFP-N3 (Clontech). Each method was performed according to the manufacturer's protocol with the following additions or modifications:

For Metafectene (Biontec) 2.5*10^5cells were seeded in 500 μl THP-1 media, 0.5 μg plasmid mixed with 12.5 μl OptiMem I, 12.5 μl OptiMem I mixed with 2 μl Metafectene, both components mixed followed by 20 minutes incubation at room temperature.

For Metafectene Pro (Biontec) 0.5*10^5cells were seeded 24 hours before transfection in 500μl THP-1 media, 0.5 μg plasmid mixed with 30 μl OptiMem I, 30 μl OptiMem I mixed with 3.7 μl Metafectene, both components mixed followed by 15 minutes incubation at room temperature and added to the cells. Cells were incubated for 5 hours at 37°C 500 μl THP-1 medium was added.

For Lipofectamine (Invitrogen) method **I** 4*10^5cells were seeded in 200μl OptiMem I, 0.4μg plasmid mixed with 25μl OptiMem I, 20μl OptiMem I mixed with 5μl Lipofectamine. Both components were mixed followed by 15 minutes incubation at room temperature, addition of 150μl OptiMem I and afterwards incubated for 5 hours at 37°C that was followed by addition of 400μl THP-1 medium with 20% FCS and no antibiotics.

For Lipofectamine (Invitrogen) method **II** 4*10^5cells were seeded in 200 μl OptiMem I, 0.4 μg plasmid mixed with 25 μl OptiMem I, 15 μl OptiMem I mixed with 10 μl Lipofectamine. Both components were mixed followed by 15 minutes incubation at room temperature, addition of 150 μl OptiMem I and added to the cells. Cells were incubated for 5 hours at 37°C that was followed by addition of 400 μl THP-1 medium with 20 % FCS and no antibiotics.

For the Lipofectamine (Invitrogen) method **III** 4*10^5cells were seeded in 200 μl OptiMem I, 0.4 μg plasmid mixed with 25 μl OptiMem I, 4 μl Plus reagent added and the mix incubated for 15 minutes at room temperature. 24 μl OptiMem I were mixed with 1μl Lipofectamine. Both components were mixed followed by 15 minutes incubation at room temperature, addition of 150 μl OptiMem I and added to the cells. Cells were incubated for 5 hours at 37°C that was followed by addition of 400 μl THP-1 medium with 20 % FCS and no antibiotics.

For the Lipofectamine/Protamine (Invitrogen) method **I** 4*10^5cells were seeded in 200 μl OptiMem I, 0.4 μg plasmid mixed with 25 μl OptiMem I with 1μg/μl Protamine sulfat and 15 μl OptiMem I mixed with 10 μl Lipofectamine. Both components were mixed followed by 15 minutes incubation at room temperature, addition of 150 μl OptiMem I and added to the cells. Cells were incubated for 5 hours at 37°C that was followed by addition of 400 μl THP-1 medium with 20 % FCS and no antibiotics.

For the Lipofectamine/Protamine (Invitrogen) method **II** 5*10^5cells were seeded 24 hours before transfection in 200 μl OptiMem I, 0.4 μg plasmid mixed with 25 μl OptiMem I with 1 μg/μl Protamine sulfate and 15 μl OptiMem I mixed with 10 μl Lipofectamine. Both components were mixed followed by 15 minutes incubation at room temperature, addition of 150 μl OptiMem I and added to the cells. Cells were incubated for 5 hours at 37°C that was followed by addition of 400 μl THP-1 medium with 20 % FCS and no

antibiotics.

For Lipofectamine2000 (Invitrogen) $6*10^5$cells were seeded in 200 μl OptiMem I, 0.8 μg plasmid mixed in 50 μl OptiMem I and incubated for 15 minutes at room temperature. 48 μl OptiMem I was mixed with 2μl Lipofectamine. Both components were mixed followed by 15 minutes incubation at room temperature and added to the cells. Cells were incubated for 5 hours at 37°C which was followed by addition of 500 μl THP-1 medium with 20 % FCS and no antibiotics.

For Genecarrier-1 (epoch biolabs) 1-5 10^5cells were seeded 24 hours before transfection in 200 μl OptiMem I, 0.25 μg plasmid was mixed with 25 μl OptiMem I and 25 μl OptiMem I was mixed with 0.125 μl, 0.25, 0.5, 1 or 1.5μl Genecarrier-1. Both components were mixed followed by 15 minutes incubation at room temperature and added to the cells. Cells were incubated for 5 hours at 37°C which was followed by addition of 250 μl THP-1 medium with 20 % FCS and no antibiotics.

For Calciumphosphate (Calphos Kit, Clontech) method $2*10^6$ cells were seeded in 15 ml THP-1 medium (100 mm plate). See 3.4.10 for method, 15 μg of plasmid was used.

For Effectene (Qiagen) $1.75*10^5$cells were seeded 24 hours before transfection in 600 μl OptiMem I. 0.2 μg DN mixed with 37.5 μl EC buffer and 1.6 μl Enhancer and incubated for 5 minutes at room temperature. After combining both components and 10 minutes incubation at room temperature 300 μl THP-1 medium was added.

3.4.5 Construction of pHIV8-GFP and pHIV9-Puromycin

In pHIV7-GFP the CMV-GFP-wpre cassette was replaced with the CMV-Exon1-Intron(pre-mir30)-Exon2(GFP) cassette from pSM30-GFP [186]. The resulting transgene plasmid was termed pHIV8-GFP. For puromycin selection the GFP Exon in pHIV8-GFP was replaced with puromycin CDS from the pDest Super plasmid (kindly provided by Dr. T. Joeris, MPIIB) and a single silent basepair exchange performed to remove an Esp3I restriction site in the puromycin CDS. The resulting vector was termed pHIV9-GFP. The plasmids were sequenced and tested for transgene expression (fluorescent microscope for GFP expression, puromycin resistance for puromycin) and shRNA processing (Northern Blot and TaqMan RT-PCR for GFP-expression constructs, TaqMan RT-PCR for puromycin selected constructs expressing endogenous miRNAs).

3.4.6 Design and cloning of amiRNA constructs

AmiRNAs were designed from validated siRNAs described in literature with the antisense strand extended at the 3' end according to target structure in case the strand was shorter than 22 bps. If no validated siRNA sequence was available the predictions of DSir ([180] [2], ranked as one of the best predictors or siRNA tools available in a comparison study [181]) and the Hannon-Elledge database [1] for miR30 based amiRNAs were compared with the predictions of Origene [3] and RNAScout [182]. This order of prediction weighting was used and compared with the location of validated siRNAs from the Qiagen database (sequence not revealed) to define the most likely hit. Extension of the antisense was performed according to target sequence if necessary to reach 22 base pairs of antisense strand. Than the sense strand was designed accordingly with full complementarity except the first base at 5' end being replaced (A in case of G or C, G in case of T or A) to reach the common miR30 three-dimensional structure.

Between both strands a pre-mir30-modified loop was placed according to the recommendations of the Hannon-Elledge database (TAGTGAAGCCACAGATGTA, [1]) to maximize processing efficacy independent of the complementary stem sequence. On the 5' end of the forward DNA oligomer the remainder of the pHIV backbone digest was added (AGCG) and on the reverse DNA-Oligomer on 5' CCGT. So the resulting sequence of the forward Oligomer was

5' AGCG- *modified base in sense* - *modified miR30 loop* - *antisense*

and the reverse oligomer (italic sequences in full complementary to each other)

3' modified base in sense - *modified miR30 loop* - *antisense* - TGCC.

For each target two amiRNAs were designed to counter the prediction failure rate.

The two oligomers were synthesized by Thermo Fisher and PAGE purified and solved in A. dest. For the annealing step 10 μl forward oligomer (100 pmol/μl) and 10 μl reverse oligomer (100 pmol/μl) were mixed with 5 μl shRNA Annealing-Puffer, 5 μl 1 M sodium chloride and 20 μl A. dest. and incubated for 5 minutes at 90°C. In parallel around 150 ml water were heated in a micro wave to 90°C and the oligomer mixture incubated in this water bath for one hour while letting the water cool down.

Afterwards the annealed double stranded oligomer (ds-oligomer) was stored at -20°C or phosphorylated: 2 μl of the ds-oligomer were mixed with 1 μl T4 Polynucleotide Kinase PNK (Fermentas), 1 μl 10x T4 PNK Buffer A, 1μl ATP 10mM (Fermentas) and 5 μl H_2O, incubated for 30 minutes at 37°C and the kinase inactivated by incubation at 70°C for 10 minutes.

The thus phosphorylated ds-oligomer was ligated into the pHIV backbone which was digested with Esp3I restriction enzyme (Fermentas) and 0.2 mM DTT in 1x Tango Buffer in a volume of 20 μl for one hour at 37°C. The reaction was stopped by heat inactivation at 65°C for 20 minutes.

For the ligation 0.4 μl of T4 DNA ligase (Fermentas) was mixed with 2 μL 10x T4 DNA ligase Buffer, 4 μL phosphorylated ds-oligomer, 50 ng Esp3I digested pHIV backbone, 1 μl 10 mM ATP and the volume was adjusted to 20 μl with A. dest.

Afterwards the mix was incubated at an oscillating thermo cycler protocol (Applied Biosystems) of 30 seconds 10°C and 30 seconds 30°C for 8 hours and the reaction stopped by incubation at 70°C for 10 minutes.

The ligation was than transformed into heat competent *E. coli* by incubation of 100 μl ice-thawed bacteria with 10 μl of the reaction mix on 42°C for 45 seconds and incubation on ice for 5 minutes. Than 1ml of LB medium was added and the bacteria suspension incubated with little shaking for 1 hour at 37°C. Afterwards medium was replaced with 1ml fresh LB medium by centrifugation for 7 minutes at 5,000xg and discarding of the supernatant.100 μl of the suspension were each plated on LB Agar plates with ampicillin as selecting antibiotic (50 μl/ml). The remaining bacteria suspension was centrifuged once more, the supernatant discarded except 100μl and the pellet resuspended in these remaining 100 μl. That volume was plated on a second LB Agar plate with ampicillin selection (50 μl/ml) too. Both plates were incubated over night at 37°C.

The next morning colonies were tested for successful ligation of the amiRNAs into the backbone by colony PCR with ThermoTaq polymerase (NEB) and primers shtest fw and shtest rev (for the GFP construct) or shtest puro rev (for the Puromycin construct) in a PCR with a bit of the clones from the plate as direct PCR templates (table 3.1). Steps 2-4 were repeated 30 times.

Table 3.1: colony PCR temperature protocol to test for amiRNA insertion into pHIV backbone

94°C	94°C	57°C	72°C	72°C
5 minutes	20 seconds	30 seconds	30 seconds	7 minutes

If the PCR showed positive results (bands size increase by 40 bp) and sequencing confirmed the unmutated sequence, pre-cultures of the colony were used for plasmid preparation.

3.4.7 Northern Blot for snRNA detection

To analyze the expression of small RNAs in mammalian cells RNA was purified using TRIzol (Invitrogen) according to manufacturer's protocol. Afterwards an polyacrylamide gel was made (table 3.2)

Table 3.2: Denaturing polyacrylamide gel mixture for 12% and 15% gels.

12%	15%	component
7.2 g	7.2 g	Urea
1.5 ml	1.5 ml	10x TBE
4.5 ml	5.6 ml	40 % acrylamide (acrylamide:bisacrylamide = 19:1)
adjust to 15 ml		Nuclease-free A. dest.
stir and add		
75 μl	75 μl	10 % Ammonium persulfate
15 μl	15 μl	TEMED

The solution was mixed briefly and immediately poured. Afterwards equal amounts of RNA (20 μg) were mixed with 2x gel loading buffer, the samples heated for 5 minutes at 95°C and cooled on ice. Meanwhile the gel was pre-run for one hour at 300V to reach the necessary electrophoresis temperature. Then the samples were loaded on the gel and the electrophoresis run at 300 V until the bromophenol blue dye front had migrated 1.5cm before the bottom of the gel chamber.

The membrane (Hybond N+, GE Healthcare Life Sciences) was than soaked in 2x SSC and the membrane, sponges and Whatman paper afterwards soaked in 0.5x TBE. The chamber was assembled, the transfer chamber filled with 0.5x TBE to cover all of the membrane and run at 4°C and 400 mA for 1 hour. Cross-linking was performed under UV by administration of 2x 600 mJ. Prehybridization took place for 30 minutes at 42°C in prehybridization buffer.

During gel electrophoresis and blotting the radioactive probe labeling and purification was performed. 2μl of 10 μM of the 20 bp long DNA-oligomer/probe was mixed with 2μl of 10x T4 PNK Buffer (Fermentas), 2μl $^{32}P\gamma$-ATP (>7000Ci/mmol, Amersham), 13μl dH_2O, 1μl T4 Poly-Nucleotide Kinase (Fermentas) and incubated at 1 hour at 37°C. Reaction was stopped by incubation at 68°C for 10 minutes. Afterwards 30μl of water was

added and the probe purified from unincorporated radioactive ATP with G-25 MicroSpin Columns (Amersham Pharmacia) according to manufacturers protocol. Centrifugation steps were performed at 735xg. This was followed by an examination with a Geiger counter set at 100x, column to labeled probe should be each around 50% of radioactivity. The radioactive labeled probe was than added to the Hybridization buffer and Hybridization performed over night at 42°C. Next day the membrane was washed with Wash solution I (6x SSC, 0,1% SDS) at 42°C for 10 minutes followed by another washing step with Wash solution II (2x SSC, 0,1% SDS) at 42°C for 10 minutes. Afterwards the membrane was wrapped in plastic wrap and exposed to the film. To remove background signals the membrane could be washed at 42°C for 10 minutes with Wash III (0.1x SSC). Stripping (if necessary) was performed by washing the membrane at 80°C for 10-30 minutes with Stripping Buffer (0.1% SDS, 0.1x SSC).

3.4.8 Rubidiumchlorid-competent *E. coli* (heat shock competence)

3 ml LB medium were inoculated over night as pre-culture and next morning 100 ml LB medium inoculated with 1ml of the over night culture. At an optical density of 0.4-0.6 (best 0.5) at 600 nm wavelength cells were incubated for 15 minutes on ice and centrifuged for 15 minutes at 5,000xg and 4°C. Afterwards the pellet was resuspended in 100 ml ice cold TfbI and incubated for 15 minutes on ice. This was followed by another centrifugation step for 15 minutes at 5,000xg and 4°C and the pellet resuspended in 10 ml ice cold Tfb II. Aliquots of 100 μl were used immediately or stored at -80°C.

3.4.9 Plasmid purification

Plasmid purification was performed from 300 ml culture in log phase with Plasmid Maxi Kits from Qiagen or Macherey-Nagel according to the manufacturer's protocols for high copy plasmids.

3.4.10 Virus vector packaging

One day in advance 150 cm tissue culture grade plates (Sarstedt) were seeded with $2.5*10^6$ HEK 293T/17 cells (ATCC) in 15 ml "complete" medium and cells incubated at 37°C and 5% CO_2. The next morning medium was gently replaced with fresh medium and 2-3 hours later the packaging mix added. The packaging mixture was prepared by combining per plate 35 μg of pCMV-G plasmid, 25 μg of pCMV-rev, 25 μg of pCHGP-2 plasmid with 35 μg of transgene coding plasmid each solved in A. dest with 374.4 μl 2M $CaCl_2$ and

adjusted to 3 ml with A. dest. and mixed thoroughly. The mix was than added slowly drop wise (under constant maximum air ejection from a pipette boy (Thermo Fisher) through a filter Pasteur pipette to cause strong liquid agitation) into 3ml of 2xHBS buffer (Calphos Kit, Clontech). After both volumes had been combined the liquid agitation was kept for 20 more seconds without interruption to improve the size and distribution of the forming calcium phosphate nucleic acid crystals. Afterwards the solution was incubated without disruption at room temperature for 20 minutes and than added drop wise over the whole 150 cm-plate area with seeded with HEK 293T/T17. The plate was gently shaken to optimize the crystal distribution and incubated for 5-6 hours at 37°C and 5% CO_2. Afterwards the medium was once more gently exchanged without detaching the cells but this time 20 ml of medium added to each plate.

3.4.11 Virus vector harvest und purification

Virus was harvested 3-4 times (minimum 48 hours and maximum 72 hours after medium packaging mix transfection or last harvest time point). For this the supernatant was carefully aspirated and centrifuged in 50 ml tubes at 4°C around 3,800xg for 10 minutes with slow deceleration to remove cell debris. If another time point of harvest was still planned 20 ml of fresh medium was carefully added on the plate and the plate further incubated. The 32-40 ml of the centrifuged supernatant were then transferred into single-use ultra centrifugation tubes (UltraClear #344058, Beckman Coulter) and the supernatant centrifuged at 25,000rpm (around 104,000xg) and 4°C for 90 minutes in a SW32 Ti rotor. Afterwards the supernatant was carefully discarded (decantation) and the pellet resuspended in 200 μl of "complete" medium or THP-1 medium depending on intended use. Aliquots of 500 μl virus supernatant were immediately used for target cell transduction or stored at -80°C and thawed on ice previously to experiment.

3.4.12 Target cell transduction

For the evaluation of transducing units (TU, infectious particles per ml of virus suspension) at day one 3x10^5 HEK 293T/T17 cells were seeded per well (12 well plate). For suspension cells no seeding prior to transduction was performed. At day two medium was exchanged and cells adjusted to a density that would sustain cell culture for at least 2-3 days during continuous culture (2x10^5 cell/ml for THP-1 cells). 8 μg/ml Polybrene was added per well/sample to mask cell membrane charges. Purified lentiviral particles were added at an appropriate MOI (1:10 TU for THP-1, 1:10 up to 1:20 TU for primary cord blood or buffy coat derived CD34+ cells) while keeping the final volume as small as

possible. In case the cells were suspension cells "spin down transduction" was performed by centrifugation of cell-virus suspension for one hour at room temperature and 300xg. For adherent cells no further steps were necessary. Cells were incubated at 37°C and 5% $CO_2$for 24 hours. Afterwards the medium was exchanged to remove remaining virus particles and decrease cell mortality due to the VSV-G surface protein exposure.

3.4.13 FACS

Cells were analyzed in a FACS Canto (Becton Dickinson). Medium was removed by aspiration, cells were detached by pipetting if not suspension cells and around 10^6cells washed with PBS by centrifugation at 4°C and 300xg for 8 minutes. In case of TLR2 measurement the cell pellet were resuspended in 100μl PBS and incubated for 10 minutes on ice with human 10μl FcR Blocking Reagent (Miltenyi Biotec). Afterwards either 10μl anti-TLR2 IgG2 antibody labeled with APC (2B4A1, abcam) or 10μl Mouse IgG2a-APC Isotype control (Miltenyi Biotec) were added and cell suspension incubated on ice and in the dark for 20 minutes. TLR2 expression was measured using the APC channel. For GFP expression cells were not labeled with antibody but used directly. FITC channel was used for analysis. 10^5 cells were counted. FCS Express V6.3 (De Novo Software) was used to analyze the data and calculate the percentage of GFP positive or TLR2 positive cells.

3.4.14 Cell sorting

Cell sorting was performed 5-7 days after cells had been transduced with the virus particle in the cell sorter facility. GFP positive cells were sorted after gating for cells with normal morphology by forward/sideward scatter dot plot.

3.4.15 Puromycin selection

To identify the minimal concentration at which a cell type or cell lines would be killed efficiently a Puromycin (Sigma-Aldrich) dilution series was added to the cell medium ranging from concentrations of 0.1 to 5μg/ml. The cell viability was assessed under the microscope for 5 days each day in parallel to cell division capability by expansion in puromycin free medium. The concentration which killed 100% of the cells without any puromycin resistance cassette after 3-4 days was used as selection concentration. For primary monocytes and THP-1 cells this concentration was 0.5μg/ml, for HEK 293T 1μg/ml was used.

3.4.16 Reverse transcription and SYBR Green RT-PCR

For reverse transcription Superscript III kit (Invitrogen) was used according to manufacturer's protocol. Briefly 2-3 μg mRNA, 1 μl 50 μM oligo(dT) Primer, 1μl 10 mM dNTP mix, 2 μl 10x RT buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, 1 μl RNaseOUT, 1 μl Superscript III were mixed and adjusted to 20 μl with A. dest. followed by incubation at 5 minutes for 65°C for denaturation and placed on ice for 1 minute. Reverse transcription reaction was carried out by incubation at 50°C for 50 minutes followed by 5 minutes incubation at 85°C. Afterwards the cDNA was diluted 1:10.

For the RT-PCR 5 μl diluted cDNA was mixed with 0.15 μl gene specific 100 μM forward primer, 0.15 μl gene specific 100 μM reverse primer, 10 μl 2x SYBR Green FastMix (Applied Biosystems) and adjusted to 20μl reaction volume with A. dest. Every sample was prepared in duplicates or triplicates. The RT-PCR was carried out on a ABI Prism 7900H in a Fast Cycle block, the software used was ABI SDS 2.2.2 (Applied Biosystems). The reaction was performed using a two step protocol: one cycle of 95°C for 20 seconds and forty cycles of 95°C 1 second and 60°C 20 seconds. Analysis was performed by REST-MCS (Relative Expression Software Tool, multi condition software) [7].

3.4.17 Western Blot

Cells were lysed in complete Lysis-M buffer with phosphatase (PhosphoStop, Roche) and protease inhibitors (Mini Protease Inhibitor Cocktail, Roche). Bradford assay was used to measure the protein concentrations. This was followed by denaturation of 30μg of protein in Laemmli buffer the protein separated in a 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12% Precise Protein Gel, Thermo Fisher #25222). Afterwards proteins were transferred to a polyvinylidene difluoride membrane (IMMODILON, Millipore) and the primary antibodies for phosphorylated Raf-1 (dilution 1:500) and GAPDH added and the blot incubated over night at 4°C. Antibody binding was detected by adding horseradish peroxidase labeled corresponding secondary antibodies (DOD, kindly provided by M. Staeber, MPIIB) and SuperSignal West Pico Chemiluminescent Substrate (Pierce). Chemoluminescence was measured with a the Fujifilm LAS-3000 (FUJIFILM Life Science).

4 Results

4.1 Analysis of a potential new method for gene knockdown and knockout in bacteria

Since the genetic manipulation of several bacteria including *Mycobacteria* and *Listeria* is difficult, time consuming and often very inefficient, the idea of translating RNAi pathways into these bacterial genera had been developed. Bacteria do not produce proteins homologues to the minimal eukaryotic RNAi machinery. Thus the idea was to test whether complementation with eukaryotic RNAi components could overcome this problem. As a first experiment eukaryotic cell extracts were prepared from cells that have been extensively used for *in vitro* RNAi experiments. The extracts were incubated with siRNAs targeting chromosomal or plasmid expressed genes and electroporated into the bacteria. First results seemed promising to achieve gene expression regulation. But the low efficiency and unexpected long terms required dissection of the underlying mechanisms. We investigated if any RNAi effects play a role and, if it is possible, to improve the efficacy to develop a usable tool in research.

4.1.1 Development of a modified TaqMan RT-PCR system

For the analysis of the proposed method we needed to be able to test delivery and stability of siRNAs into bacteria for several different siRNAs and small scale set ups. A recently published protocol by Applied Biosystems to quantify small non-coding RNAs in eukaryotes [189] was chosen due to its sensitivity. Design and delivery of custom kits from Applied Biosystems proved very time consuming (for the ordered kit for the siRNA GFP22 this took over two month) and expensive. Furthermore each RNA sequence to be detected needed a single kit which was available only in unnecessary big scales. To overcome these problems we modified the published TaqMan probe design and tested factors for detection sensitivity. To avoid the need for individual TaqMan probes for each siRNA or miRNA the published TaqMan probe location was shifted to bind fully to the conserved hairpin primer sequence. For the reverse transcription we constructed several hairpin primers with five, six, eight and eleven bp overhang that could pair with the 3' end of the antisense strand of the siRNA GFP22. Dilution series ranging from 0 to 10^7 molecules per RT-PCR in 1:10 dilution steps were analyzed in TaqMan RT-PCR. As template the double stranded siRNA as well as the single antisense strand was used. The sensitivity was best with a five or six basepair overhang (10^3molecules per reverse transcription was the detection threshold, fig. 4.1, same results for antisense strand of

GFP22, data not shown).

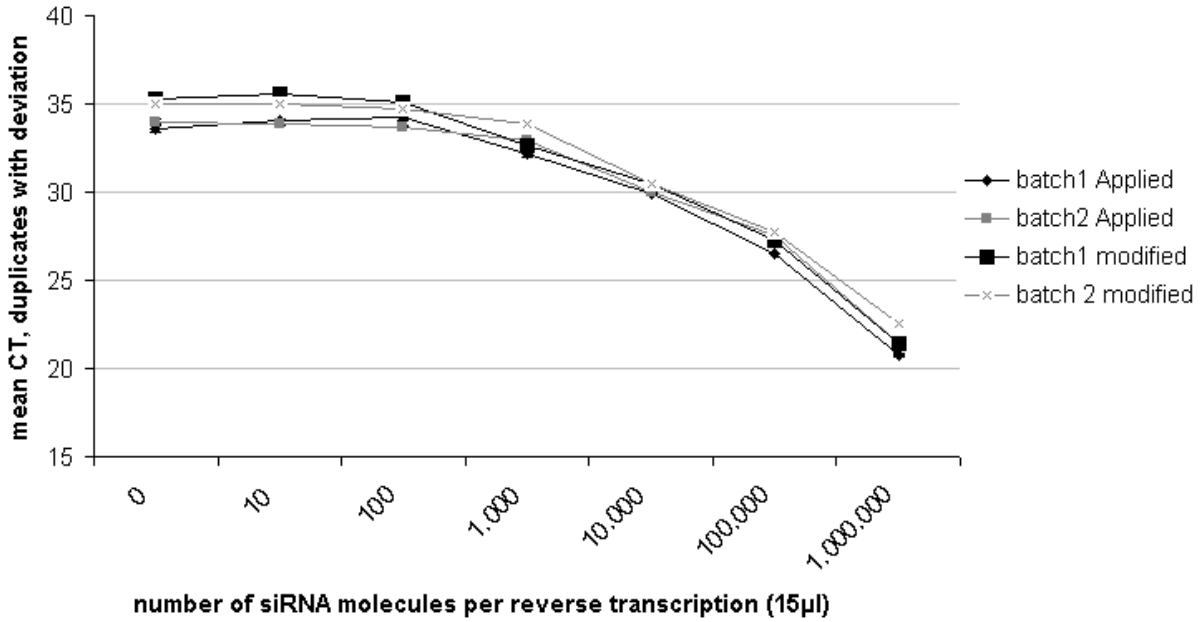

Figure 4.1: Comparison of TaqMan RT-PCR sensitivity in dilution curves for 2 batches of GFP22 siRNA (Qiagen) with our modified primer design (modified) and the custom kit supplied by Applied Biosystems (Applied)

The sensitivity of our design with the custom kit from Applied Biosystems was tested with two different batches of siRNA (fig. 4.1). There was no difference in sensitivity detectable with the commercially available system. Unexpectedly the sensitivity of both designs was more than 100 fold lower than the sensitivity reported previously [189]. In a published study on effects of siRNA modification after transfection into primary human T cells our modified primer/probe design was used too. The sensitivity of our design for the tested siRNAs against the T cell surface marker CD4 was measured in dilution series as well and proved to be similar to the one measured previously for GFP22 [190].

4.1.2 Confirmation of delivery of siRNAs into bacteria by TaqMan RT-PCR

We tested if incubation of the siRNAs with cell extract has an impact on siRNA stability. Furthermore the reliability of siRNA transport into the bacteria by electroporation needed to be demonstrated. For this we used TaqMan RT-PCR on mRNA which was purified 0 and 4 hours after the electroporation. As the *L. monocytogenes* gene 6-phospho-beta-

glucosidase has been well defined as constitutively expressed gene [191] it was used for normalization. The TaqMan probe and reverse transcription hairpin primer were designed according to our modified protocol. We showed that the siRNA targeting GFP was stable when incubated with extracts even without 3'O-methylation of the siRNA ("siGFP (blocked)", to abrogate RNase activity, fig. 4.2 left). The extensive washing steps performed prior to the RNA purification did not seem to completely remove the siRNA in unpulsed samples from the purified mRNA, but the amount detected with pulse was clearly higher (fig. 4.2).

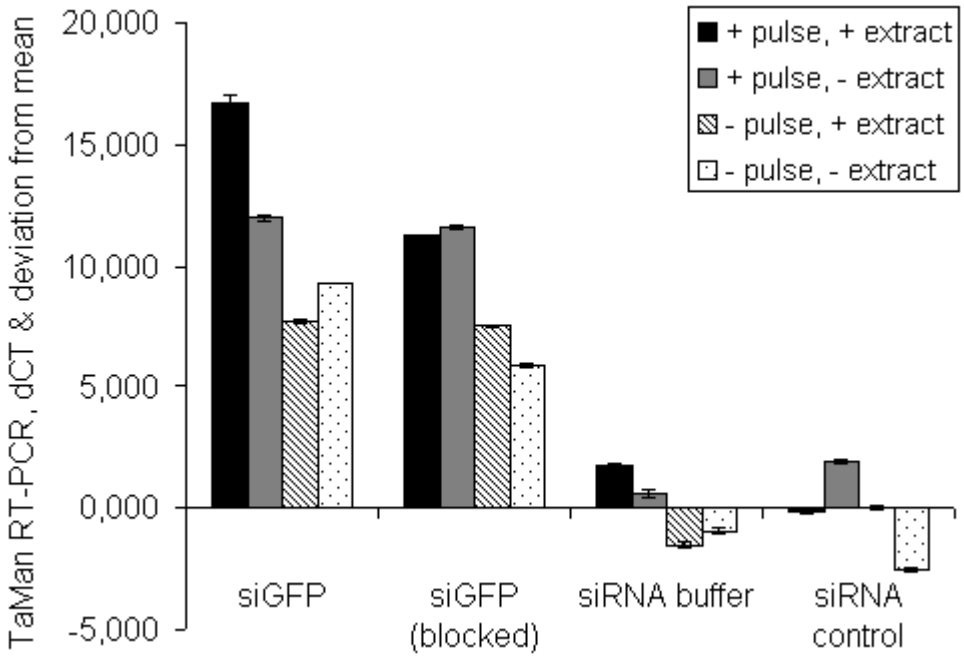

Figure 4.2: Delivery of siRNAs into *L. monocytogenes* with and without cell extract, TaqMan RT-PCR from RNA purified 4 hours after electroporation, *L. monocytogenes* housekeeping gene is 6-phospho-beta-glucosidase, siGFP blocked is siGFP with 3'O-methylation at antisense strand, siRNA control is siJTN.

We tested the same in *M. smegmatis* including absolute quantification with an siGFP dilution series and could confirm that unpulsed samples still had a detectable siRNA amount in the purified RNA which was much smaller than the pulsed samples (fig. 4.3). Furthermore there was a clear siRNA loss detectable 4 hours after electroporation (fig. 4.3). Detected siRNA numbers in siRNA control (siRNA against eukaryotic jagged-1 gene, siJTN) and siRNA buffer were at the detection threshold demonstrated for the hairpin TaqMan RT-PCR protocol (fig. 4.1).

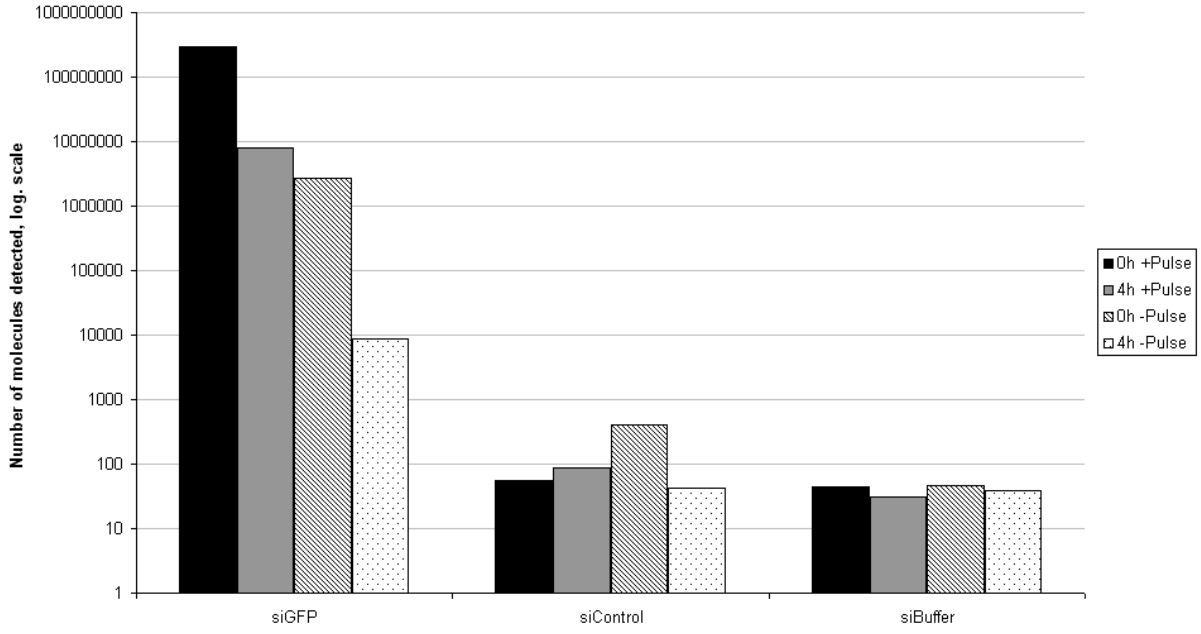

Figure 4.3: Delivery of siRNAs into *M. smegmatis* with cell extracts directly after electroporation (0h) and after 4 hours (4h), TaqMan RT-PCR from purified RNA, absolute quantification based on siGFP dilution series (0-10^7 molecules/reverse transcription, 1:10 dilution steps).

4.1.3 Search for mRNA slicing and secondary siRNAs

In some clones in one experiment in *M. smegmatis* and one experiment in *L. monocytogenes* (both performed with the same cell extract batch) obtained after electroporation we detected a persistent lack of GFP expression. GFP was expressed from a plasmid (pMV262-eGFP). To understand the reason for these long term effects we proposed RNAi based slicing as underlying mechanism in combination with the generation of secondary siRNAs by RdRPs as reported for plants. This could explain the longevity of the effects with the decline in primary siRNA detectable already after 4 hours. 5'RACE (detection of mRNA slicing) was performed as well as Northern Blot and TaqMan RT-PCR (detection of secondary siRNAs with radioactive respective TaqMan probes). There was no slicing in mRNA or mRNA degradation detectable and no secondary siRNAs could be found in Northern Blots and RT-PCR (data not shown).

4.1.4 Epigenetic and genetic analysis of clones with stable lack of GFP expression

We hypothesized that the reason for the persistent lack in GFP expression could be epigenetic based expression silencing by methylation [192]. In plants siRNA can cause *de novo* methylation of genes [193]. We tested methylation patterns in the promoter and CDS region by sequencing based direct methylation detection.

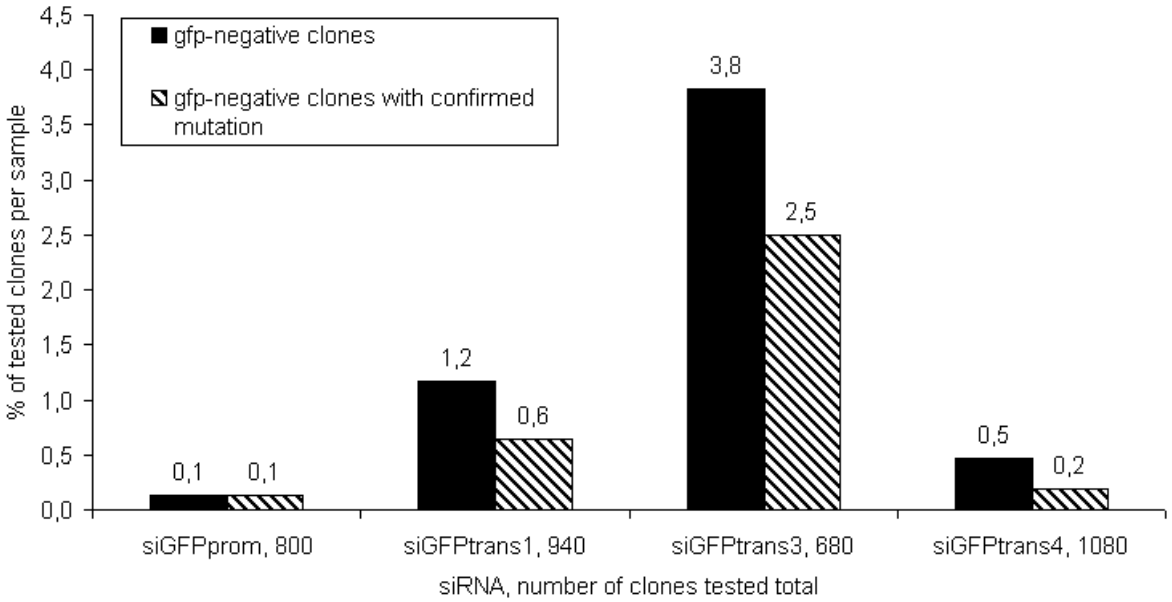

Figure 4.4: GFP negative clones in *M. smegmatis,* percentage of clones lacking GFP expression with confirmed deletion (sequencing or fragment length analysis after PCR) or without confirmed mutation (no PCR fragment/plasmid detectable even with second primer pair or temporary phenotype) over time course of one week post siRNA/extract electroporation

No obvious changes in methylation patterns could be found. Instead, for all the clones with persistent loss of GFP expression, mutations in the GFP expression cassette were demonstrated by sequencing or PCR (fig. 4.4). The percentage of clones which were sequenced to confirm mutations varied with the siRNA used (fig. 4.4). In *M. smegmatis* out of twenty-five sequenced clones twenty-three proved to have developed a deletion and one had developed a three bp insertion. The deletions were found even in sequence parts that were not covered by the siRNA. None of the mutations could be attributed to the

location of the siRNA (fig. 4.5) or sequence motifs in the DNA.

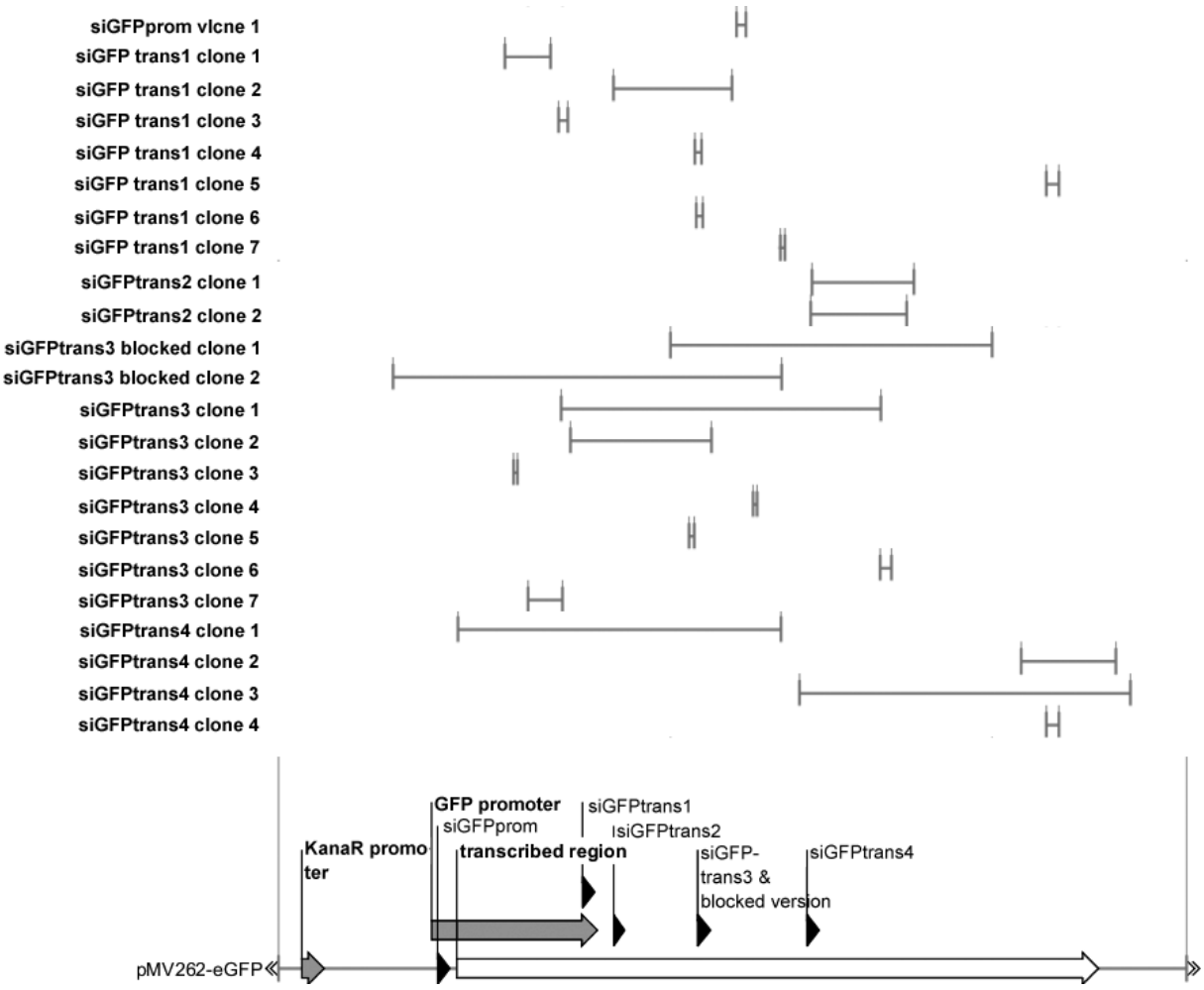

Figure 4.5: Location and size of deletions of sequenced *M. smegmatis* clones after electroporation of cell extract and different siRNAs targeting GFP. Name and position of the siRNAs (black arrows) are depicted on the promoter (dark grey) and CDS (light grey) region of the GFP expression plasmid pMV262-eGFP.

Four, five and six bps surrounding the mutations were analyzed for potential consensus motifs to indicate possible restriction enzyme sites, but no consensus sequence could be found. Furthermore we tested if complementary regions in the GFP expression cassette would cause any typical structures adjacent to the deleted regions. Modeling of the deleted regions or surrounding regions by the RNA/DNA folding program mfold 2.3 [8] did not yield any typical structure.

In case of *L. monocytogenes* GFP knockout clones had mutated transcribed regions too but the mutations found were solely base exchanges instead of deletions.

4.1.5 RNase III effects and low efficacy of method

RNaseIII is known to regulate dsRNA decay and processing in *E. coli* [194]. In order to rule out RNaseIII activity as mechanism of the observed temporary effects an *E. coli* wildtype (WT) and a RNaseIII knockout (RNaseIII KO) strain were compared. No RNaseIII dependency of the siRNA based knockdown effects could be detected (fig. 4.6).

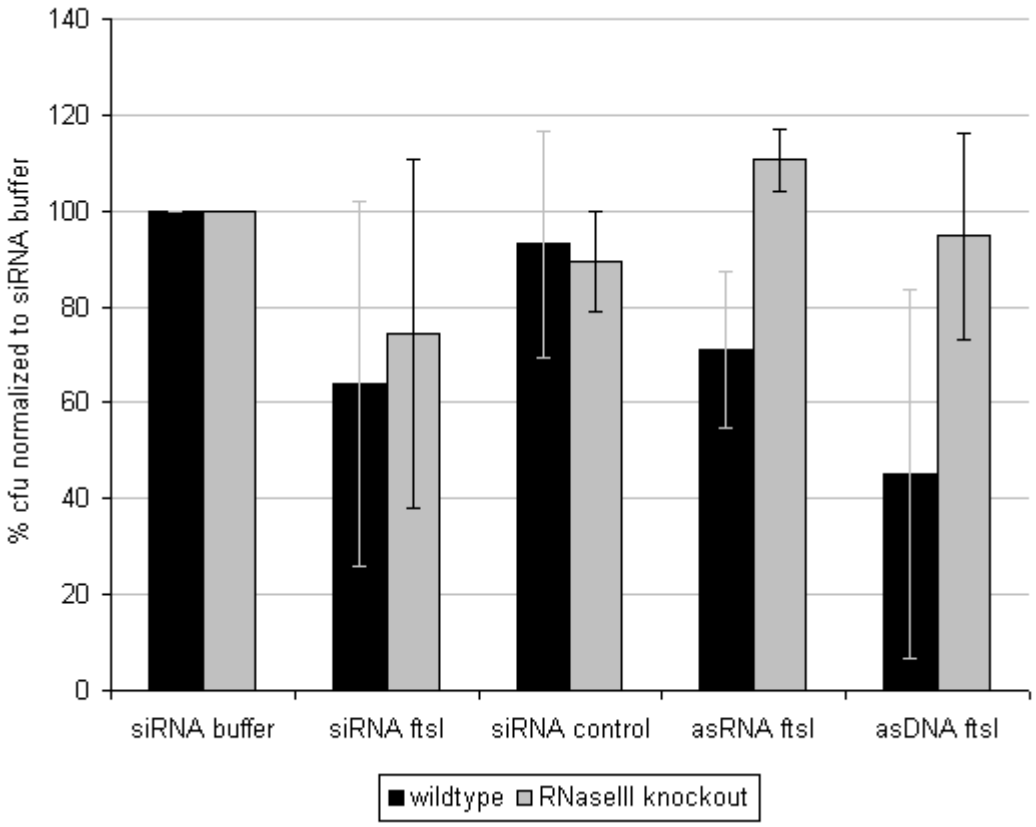

Figure 4.6: Impact of RNaseIII wildtype and RNaseIII knockout on *E. coli* growth. The number of colony forming units (cfu) on LB agar 3 days after electroporation of cell extract with siRNA buffer, siRNA targeting the essential *E. coli* gene *ftsl* (siRNA ftsl), *ftsl* antisense RNA (asRNA ftsl), *ftsl* antisense DNA-Oligomers (asDNA ftsl) or a siRNA control (siGFP targeting GFP). Mean cfu with mean variation, 3 experiments (only two included asDNA ftsl).

Antisense effects of asRNA and asDNA depending on RNaseIII expression that have been reported before [195] could be confirmed (fig. 4.6).

Electroporation of *E. coli* strains expressing functional human Ago 2 or the Ago2 PIWI domain mutant D597A [196] with 100pmol siRNA or asRNA against the essential *E. coli* gene *ftsl* did not yield significant decreases in cfu (fig. 4.7). Unexpectedly the control asRNA targeting GFP decreased cfu. Addition of extracts did not cause cfu decreases when electroporating ftsl siRNA or asRNA either (data not shown).

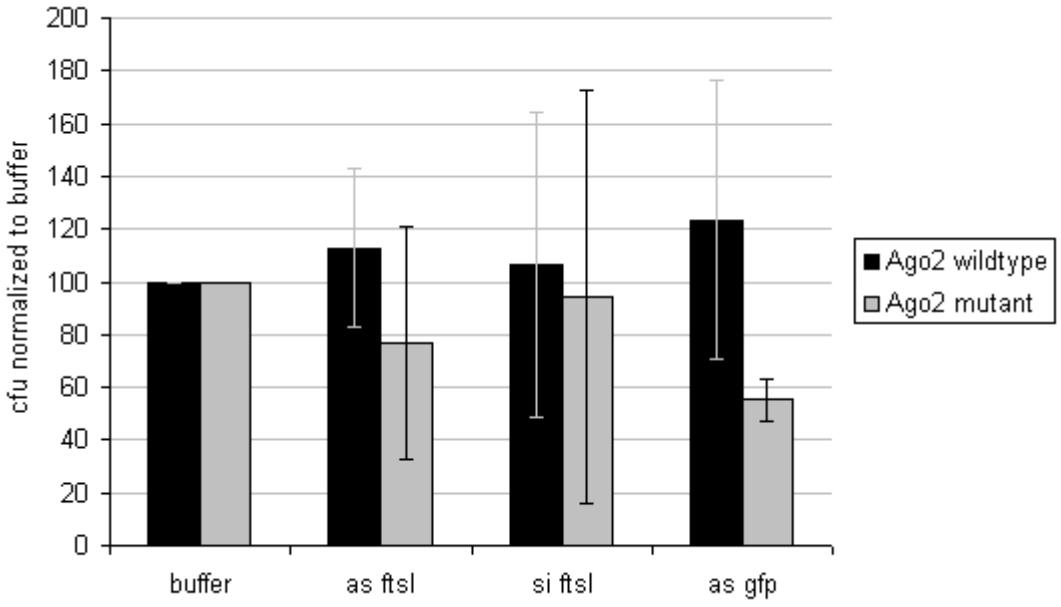

Figure 4.7: Effect of functional human Ago2 wildtype or mutant (piwi domain inactive) expression in *E. coli* one day post electroporation of 100pmol siRNA or antisense RNA against *ftsl*. Antisense RNA against GFP (as gfp) acts as antisense control. Cfu with standard deviation shown, normalized to buffer, 7 experiments for Ago2 wildtype, 4 experiments for Ago2 mutant.

The method efficacy in general proved very unreliable and most of the time the effects - if any - were low (fig. 4.6 for *E. coli*, fig. 4.8 for *M. smegmatis*). Similar results for *L. monocytogenes* and *S. typhimurium* (data not shown).

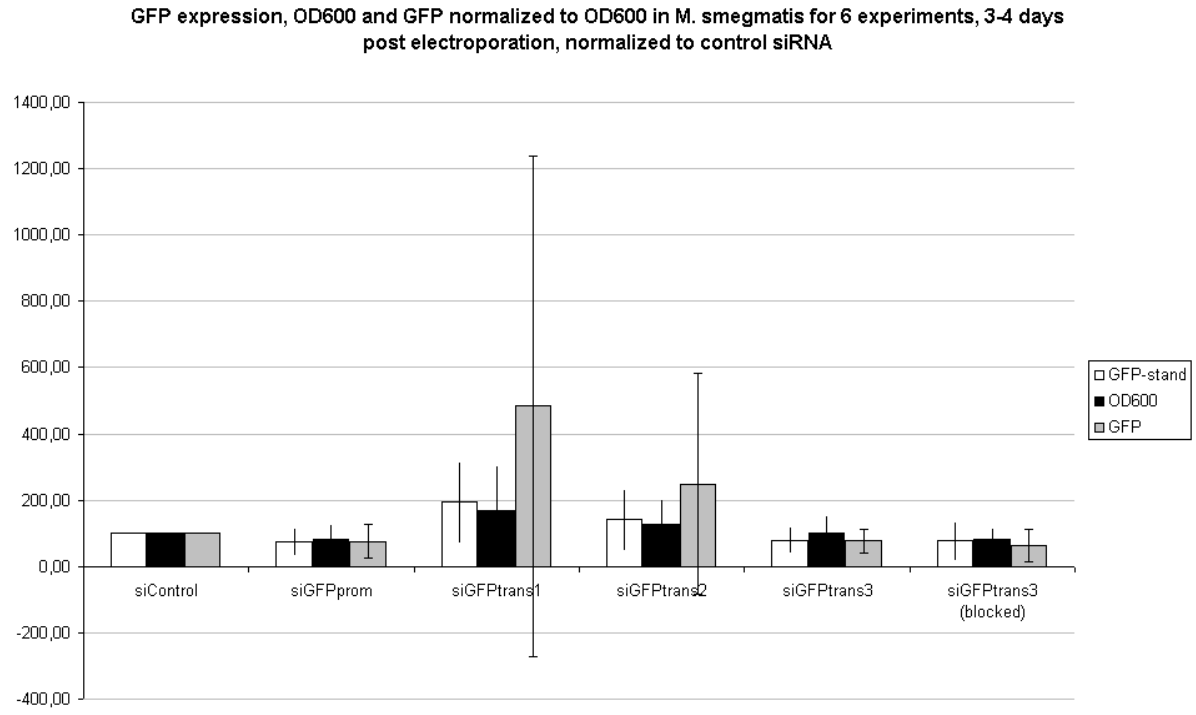

Figure 4.8: GFP expression and growth rate (culture density) of *M. smegmatis* 3-4 days post electroporation of cell extracts and 100pmol siRNAs for 6 experiments with control siRNA (siControl L1137) or siRNAs targeting either untranscribed (siGFPprom) or transcribed regions (siGFPtrans) of the GFP expression cassette on the plasmid pMV262-GFP. Relative values normalized to siControl with standard deviation. Blocked: 3'O methylation of siRNA. Legend: "GFP" - GFP fluorescence in culture; "OD600" - optical density of culture measured at 600nm; "GFP-stand" - GFP to OD600 ratio.

4.1.6 Hypothesis for a modified method (FEN-1 activation) based on mutations

Since RNAi as well as RNaseIII effects could be excluded and no sequence motifs or 3D structure patterns could be identified to explain any of the mutations a new model was developed to explain the latter ones. A repair mechanism well known in bacteria and eukaryotic cells is the flap endonuclease (FEN-1) which cuts DNA single strands in case of Okazaki fragment generation during replication. Additionally FEN-1 is activated in case of single base exchanges and UV caused abasic site generation in the DNA [197] and has been shown to cause double strand breaks in DNA [198]. Repair of double strand breaks

in *Mycobacteria* can lead to mutations, deletions are especially found in 3' overhangs [199]. Based on this a new mechanism was proposed that could have led to the observed deletions in *M. smegmatis* after electroporation of cell extracts and siRNAs (fig. 4.9).

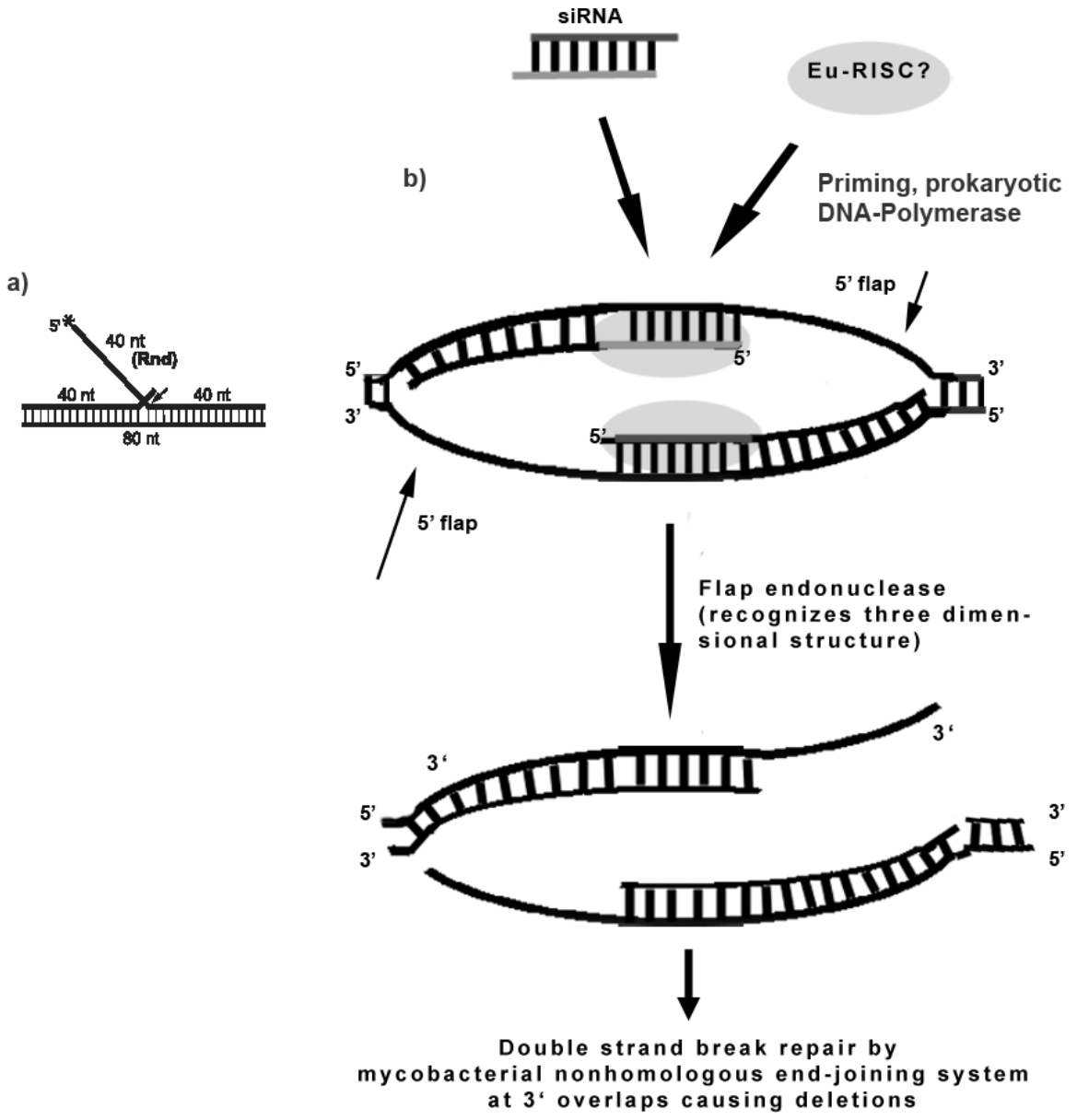

Figure 4.9: Hypothetical mechanism underlying mutation generation in M. smegmatis experiments, a) experimental confirmed FEN-1 activating constructs and FEN-1 cut site (image from [197]), b) schematic for proposed mechanism causing deletions in *M. smegmatis.*

This hypothesis lead to a new protocol which is based on chemically modified DNA oligonucleotides (oligomers) that have a defined abasic site after UDG treatment. Adjacent phosphate esters in the backbone are replaced with phosphorothioates to stabilize the oligomer against exonucleolytic and endonucleolytic repair mechanisms. The oligomers were placed in a way that would - contrary to common design for primers - allow only priming and polymerase based extension upstream and downstream external from the binding site (fig. 4.10).

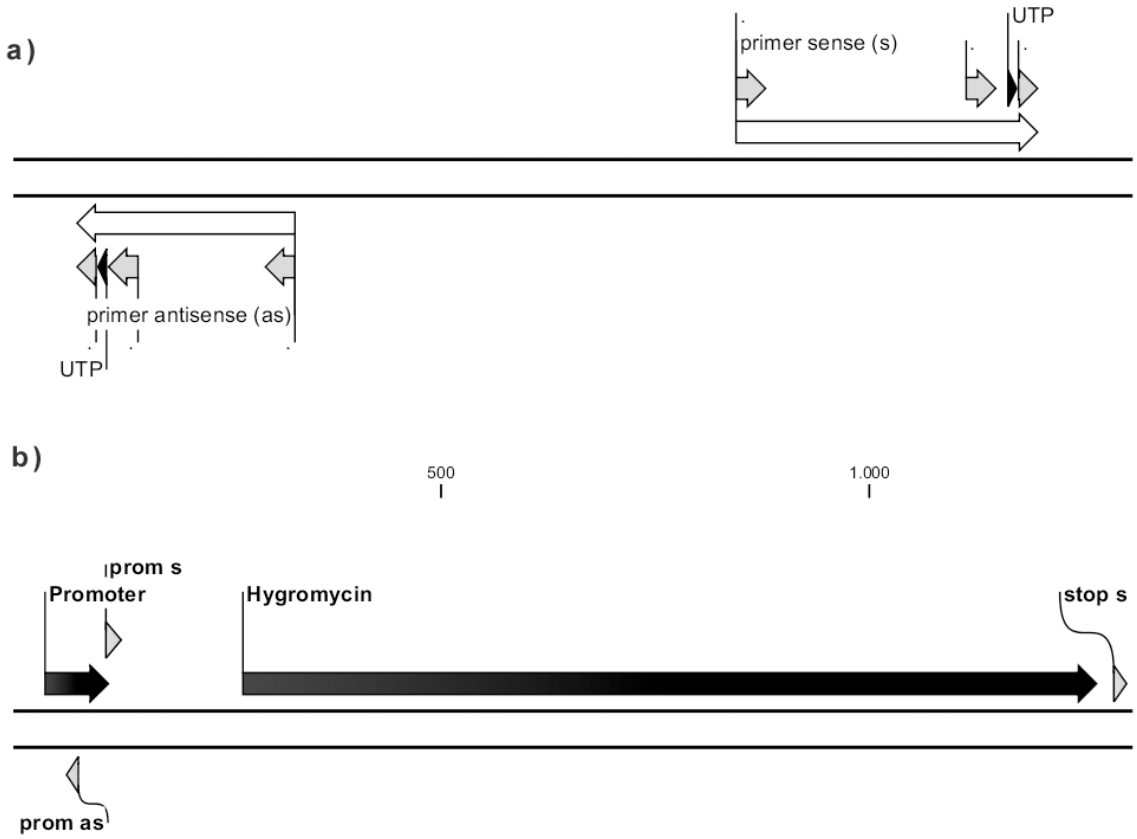

Figure 4.10: Schematic design and location of DNA-oligomers used in FEN-1 method, a) oligomer details, arrows: 5' to 3' orientation, white - oligomer, grey - phosphorothioate backbone regions, black - exchange of deoxy adenine or desoxy thymine against deoxy uracil, b) location of oligomers on the genomically integrated hygromycin resistance cassette.

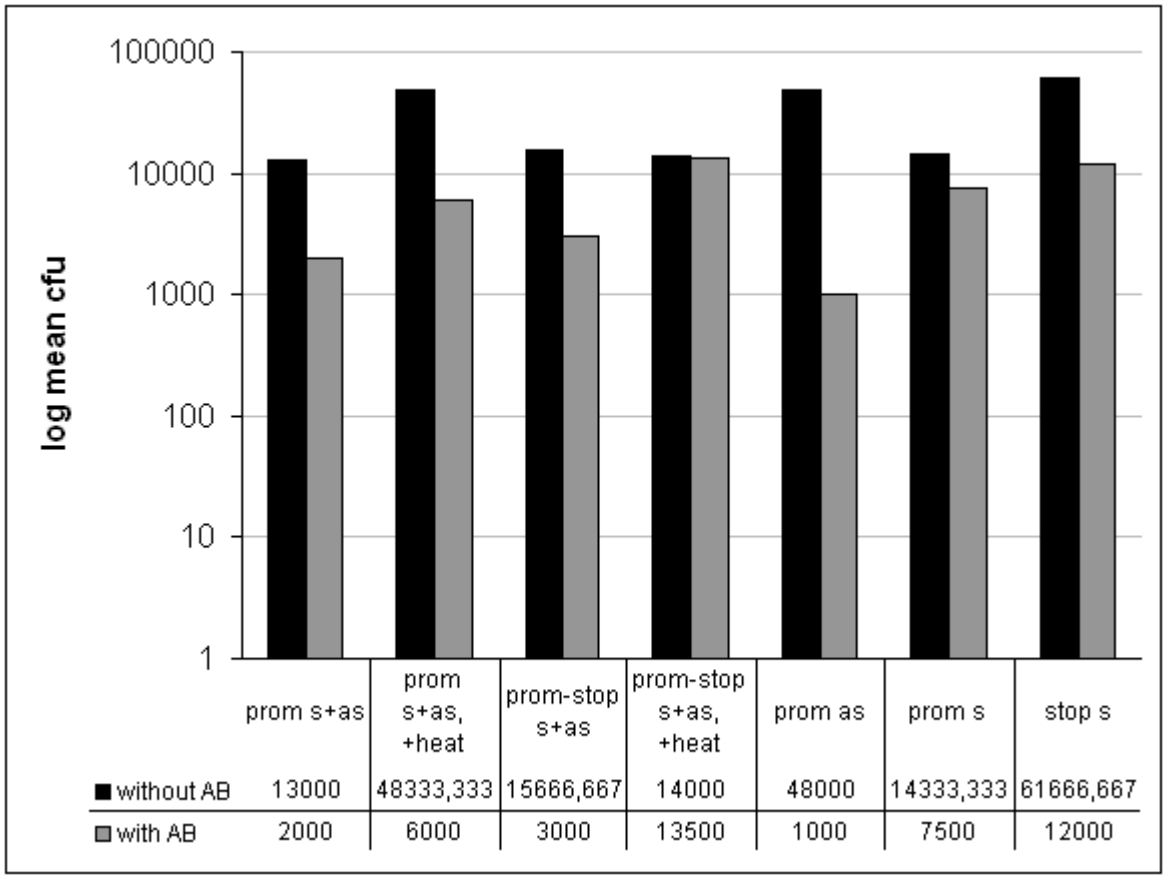

Figure 4.11: Effects of FEN-1 method, mean cfu after electroporation, one out of two experiments shown for the final protocol with 14.74 pmol per oligomer per sample used (thus half oligomer content in single oligomer samples), AB: antibiotics hygromycin

With rBCG:ΔureC:hly a recombinant BCG strain with genomic integrated hygromycin resistance was electroporated with one or two oligomers. For each oligomer 14,74 pmol were used, thus the samples with a single oligomer were electroporated with the half amount compared to samples electroporated with two oligomers. Cfu were plated on 7H9 agar plates with and without hygromycin diluted 10^3, 10^4 and 10^5 fold for each sample and cfu counted after 3 week of incubation at 37°C. As control a sample was treated with pUC18 plasmid to determine bacterial viability and hygromycin resistance after electroporation.

There was a strong reduction in cfu for each oligomer used compared to the pUC18 control. Treatment of oligomers with heat inactivated UDG instead of active UDG abrogated the hygromycin resistance loss in case of the oligomer pair located 1207 bps apart and covering the promoter - stop region; but not in case of the 30 bps apart located promoter

region covering oligomer pair. Without heat inactivation both showed similar decrease in hygromycin (81% versus 88% less hygromycin resistant clones). In case of single oligomers electroporation which were used in the same amount as used in the oligomer pair samples the antisense oligomer in the promoter showed the strongest cfu decrease (98% less hygromycin resistant clones), but the sense oligomers alone located in the stop region caused a decrease in hygromycin resistance as well (80% less hygromycin resistant clones) while the oligomer located after the stop codon had less effect (48% less hygromcyin-resistant clones).

If higher amounts of oligomer than 14.74 pmol per sample were used either the cfu number obtained was extremely low with only very small, slow growing colonies forming or there was no strong difference between the number of clones found on plates with or without hygromycin (decrease in hygromycin resistant clones 22-28%, data not shown). Experiments with oligomer design based on [188] with the same oligomer binding sequence but an hairpin attached at the 5' end and none of the chemical modifications showed no substantial effect on hygromycin resistance (9-10%, data not shown).

We performed PCR on purified genomic DNA but could in no case detect any deletion. Replica plating for one experiment showed clones with no hygromycin resistance but PCR performed on genomic DNA could not detect any differences. Six weeks after the electroporation the clones had grown in culture to provide enough genomic DNA for direct methylation detection by sequencing but at this time point there was no hygromycin sensitivity detectable anymore. Manual transfer of clones from original plates without hygromycin six weeks after the electroporation for another experiment confirmed those results.

4.2 Summary of results on protocols for gene knockdown or knockout in prokaryotes

In conclusion it could be confirmed that electroporation of siRNAs into L. monocytogenes and M. smegmatis is possible. The siRNAs remain stable at least 4 hours post electroporation even when incubated with eukaryotic cellular extracts. Detection of the siRNA delivery with a modified TaqMan RT-PCR protocol using hairpin primers with a conserved TaqMan probe design and hairpin primers complementary to the 5 bp of the 3' end of a small non-coding RNA yields the same sensitivity as the kits supplied from Applied Biosystems. Nevertheless the sensitivity seems to be sequence dependent and was found to be around 100 fold lower than the sensitivity reported originally for the microRNA let-7 [189] in case of designs for siRNAs targeting CD4 and GFP.

After electroporation of siRNAs and cell extracts into prokaryotic cells no mRNA slicing or generation of secondary siRNAs could be found. Analysis of DNA target sequences and methylation status revealed deletions in case of M. *smegmatis* in one case and inversions in case of *L. monocytogenes* in one case but no differences in methylation. No consensus motifs could be found adjacent to the deleted region and no typical three dimensional folding behavior of the adjacent sequences was detected. The protocol of electroporation of siRNAs and cell extracts into prokaryotes proved very unreliable and with extremely low efficiency for temporary knockdown effects as well. Overexpression of functional human Ago2 and comparison with its PIWI domain mutant in *E. coli* did not yield any detectable effect for the gene targeted essential gene with siRNA or asRNA. Instead the control antisense RNA caused significant decrease of the asRNA in case of the PIWI domain mutant.

A proposed method aiming to activate FEN-1 by electroporation of chemically modified DNA oligomers led to significant phenotypic effects in preliminary experiments for sense as well as antisense RNAs. Contrary to the underlying hypothesis no mutations could be found and only temporary effects were observed. In general electroporation of extremely low amounts of the chemically modified oligomers is necessary to avoid toxic effects.

4.3 Platform for eukaryotic RNAi based gene validation in *M. tuberculosis* infection

M. tuberculosis infects mostly macrophages and dendritic cells (DCs) which can both differentiate from monocytes [146]. Thus we aimed at those cell types in the development of our RNAi based gene validation platform. To our knowledge there exists no human DC line. The human pro-monocytotic cell line THP-1 can be differentiated by PMA stimulation to macrophage like cells which is a common *in vitro* model in tuberculosis research [200]. To understand what platform works to trigger RNAi in THP-1 cells and primary human monocytes after infection of *M. tuberculosis* different approaches were tested.

4.3.1 Temporary siRNA based knockdown and promoter activity in primary human monocytes

To understand the kinetics of RNAi in primary human monocytes purified from peripheral blood we tested knockdown kinetics of siRNAs on plasmid expressed luciferase. Nucleofection of 400 ng of plasmids expressing firefly luciferase under control of herpes simplex

virus thymidine kinase promoter (TK, plasmid pRL-TK) or the SV40 promoter (pGL2-control) resulted in no detectable luciferase activity in monocytes as well as THP-1 cells (data not shown).

In further experiments only cytomegalie virus immediate early promoter (CMVie promoter) driven constructs lead to detectable gene expression in primary human monocytes and THP-1 cells.

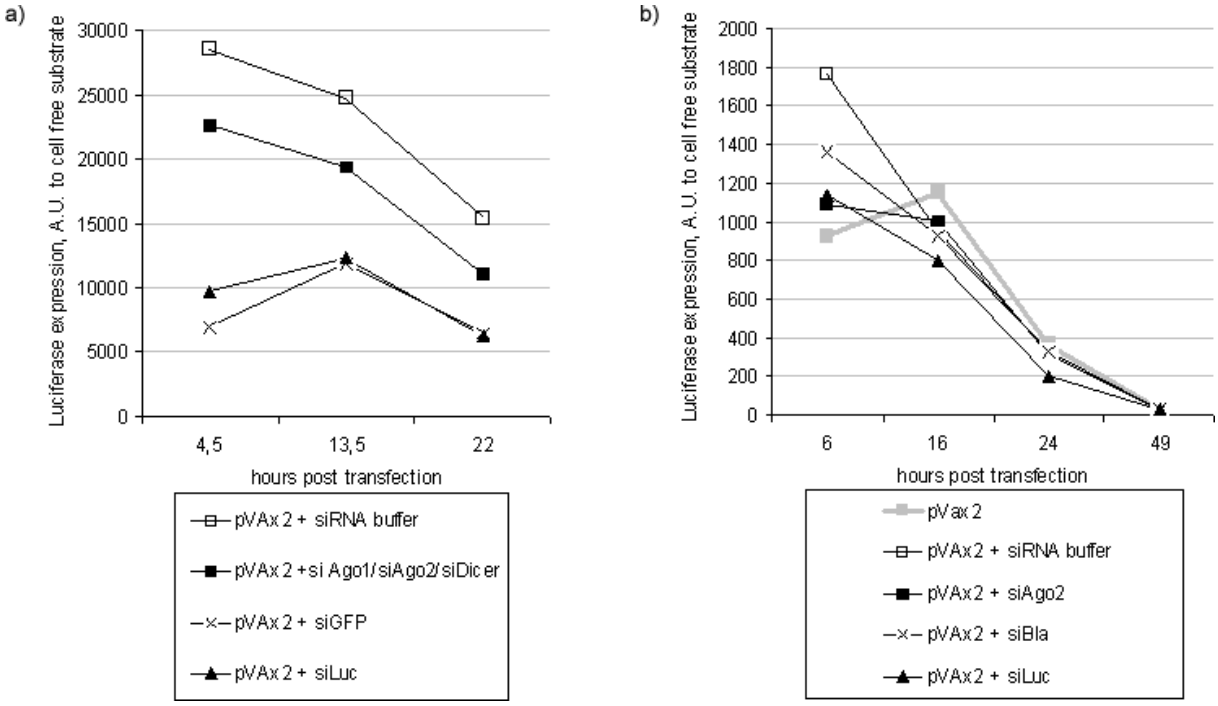

Figure 4.12: Time course of luciferase expression in a) THP-1 cells and b) primary human monocytes purified from peripheral blood after Nucleofection of luciferase expression plasmid (pVAx2, CMVie promoter) and siRNA buffer or siRNAs targeting Ago2 (siAgo2), mixture of siRNAs targeting Ago1/Ago2 and Dicer (siAgo1/siAgo2/siDicer), control siRNAs targeting GFP (siGFP) or *E. coli* gene *bla* (siBla) and siRNA targeting luciferase (siLuc). 200ng pVax2 and 5pmol siRNA were nucleofected into 10^6 cells

Nucleofection of plasmid and siRNAs or siRNA buffer in THP-1 cells showed a decreased expression of Luciferase with siRNAs targeting Luciferase (siLuc) but siRNA transfection (siGFP and siAgo2) in general seemed to have an impact on luciferase expression (fig. 4.12 a). There was a marked decrease of Luciferase activity over a time course of 22 hours post transfection. Luciferase expression and knockdown in primary human monocytes over a

time course of 49 hours after transfection showed a strong natural decline in luciferase expression and RNAi effect as well (fig. 4.12 b). Furthermore there was a negative impact of siRNA co-transfection (and even siRNA buffer transfection) by Nucleofection on the gene expression detectable.

4.3.2 Comparison of delivery methods in efficiency and toxicity

To understand if the detected loss of gene expression and RNAi effect based on cell toxicity caused by the Nucleofection transfection method optimization protocols for THP-1 Nucleofection were used to test the impact of different pulses. As cell viability was already decreased by incubation of cells with naked plasmid DNA (visual inspection by microscopy, data not shown) only 200ng of pEGFP were nucleofected into 10^6 THP-1 cells. Viability and transfection efficiency were analyzed by FACS after 24 and 72 hours. Viability proved to decline rapidly over 72 hours with pulses that had transfection efficiencies above 30 %. Ratio of transfected to untransfected cells increased over the time course (fig. 4.13).

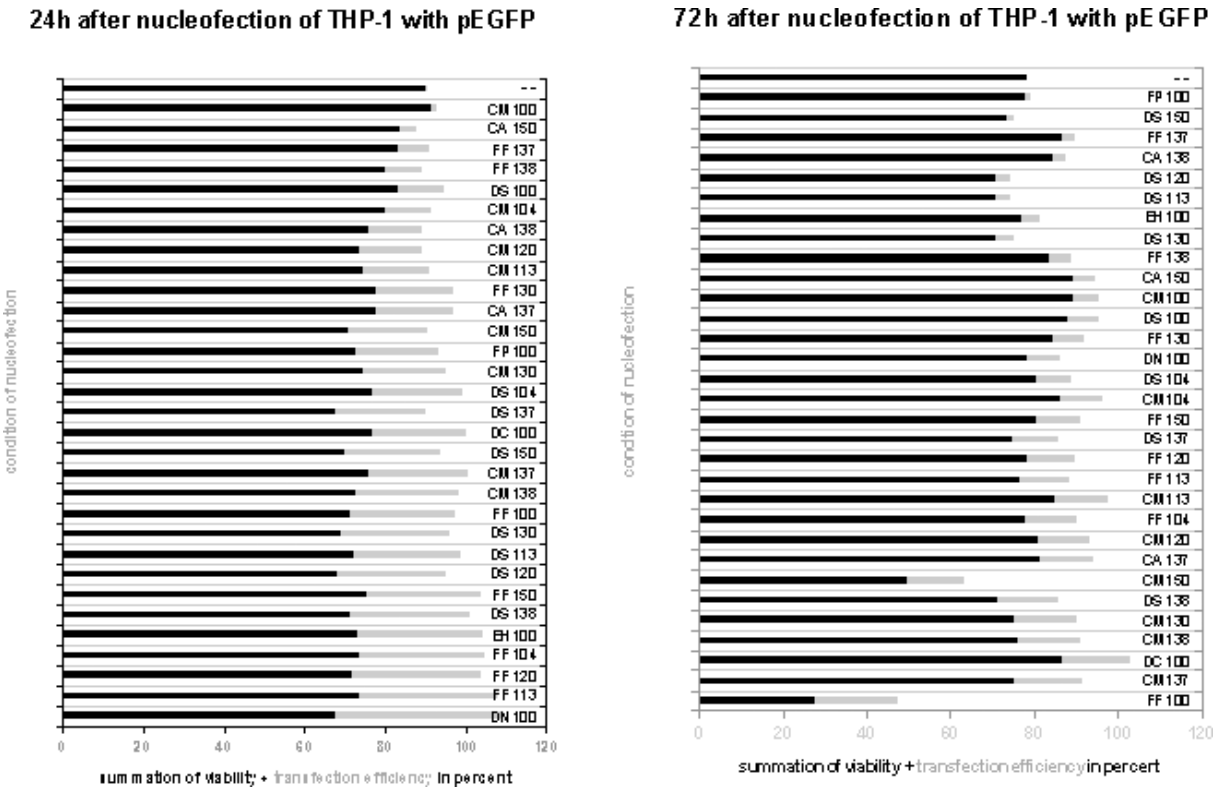

Figure 4.13: Transfection efficiency and viability of THP-1 24 hours and 72 hours after Nucleofection with pEGFP for different pulse protocols

To test if other transfection methods would be more efficient and less toxic, twelve other non-viral transfection methods were tested to deliver pEGFP including lipo-complex based

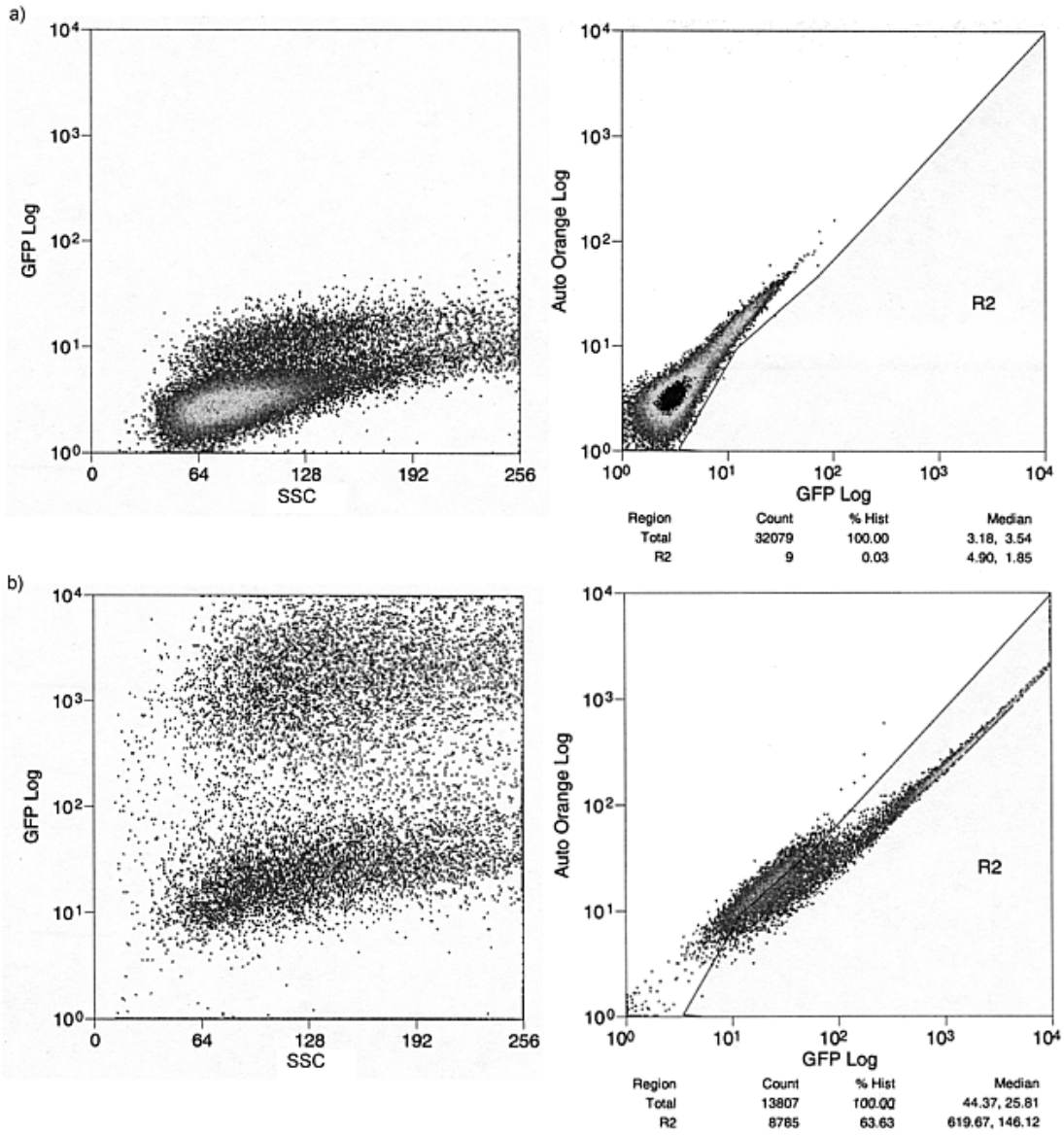

Figure 4.14: Lentiviral transduction efficacy in THP-1 cells in FACS analysis measured by GFP expression 5 days post transduction, a) untransduced cells, b) transduced cells with pHIV7-GFP at MOI 1:10, left: FL1 channel (GFP fluorescence) and side ward scatter (SSC), right: FL2 channel (orange auto-fluorescence "auto orange") and FL1 channel (GFP fluorescence)

systems like Lipofectamine, Lipofectamine 2000, Metafectene, Metafectene-Pro, Effectene, Genecarrier-1 with and without Protamin addition and two Calciumphosphate-Precipitation

protocols. None of the protocols achieved transfection efficacies above 5% but cells transfected with the complexed DNA showed only small declines in viability compared to untransfected cells (data not shown).

Transduction of THP-1 cells with lentiviral particles based on pHIV7-GFP ([201]) yielded 5 days after transduction an efficacy of around 64% (fig. 4.14). There was a certain auto fluorescence going along with transduction but GFP positive cells formed a distinct population.

Transduction of human primary macrophages derived from peripheral blood was tested once with the same protocol as used for THP-cells. Transduction efficiency was very low (fig. 4.15).

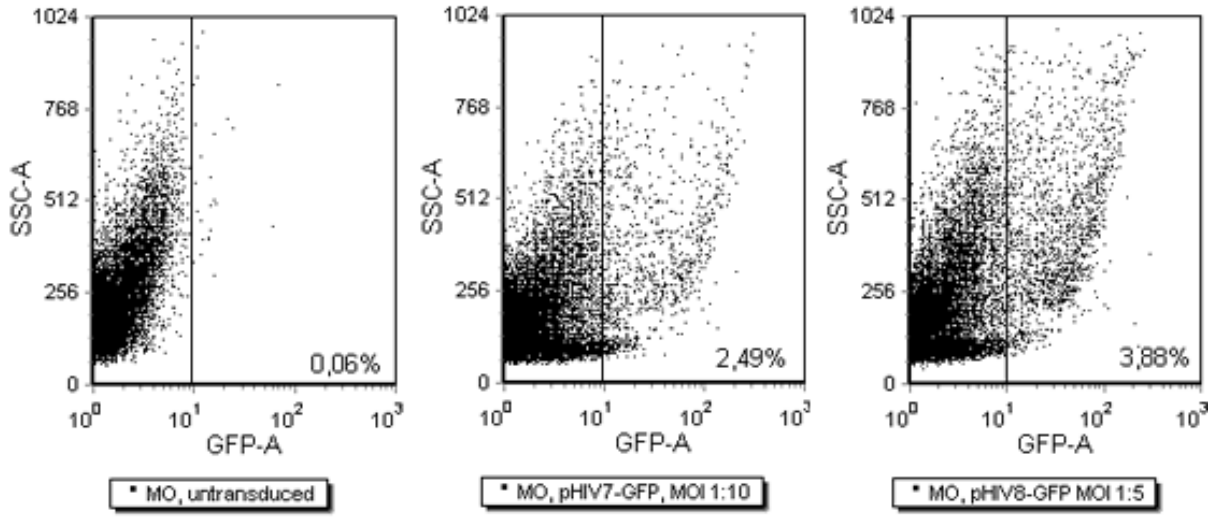

Figure 4.15: Lentiviral transduction efficiency in primary macrophages (MO) derived from peripheral blood, GFP expression measured by FACS 5 days post transduction. pHIV7-GFP: original GFP expressing transgene plasmid without any RNAi construct (kindly provided by H. Li, Rossi lab, CoH), pHIV8-GFP: new transgene plasmid with intron (amiRNA) - exon (GFP) expression cassette.

In primary human DC derived from peripheral blood we obtained 12.2% transduction efficacy with the new pHIV8-GFP transgene particles (no amiRNA expressed, fig. 4.16) while the efficacies with the original pHIV7-GFP particles were lower even with the double

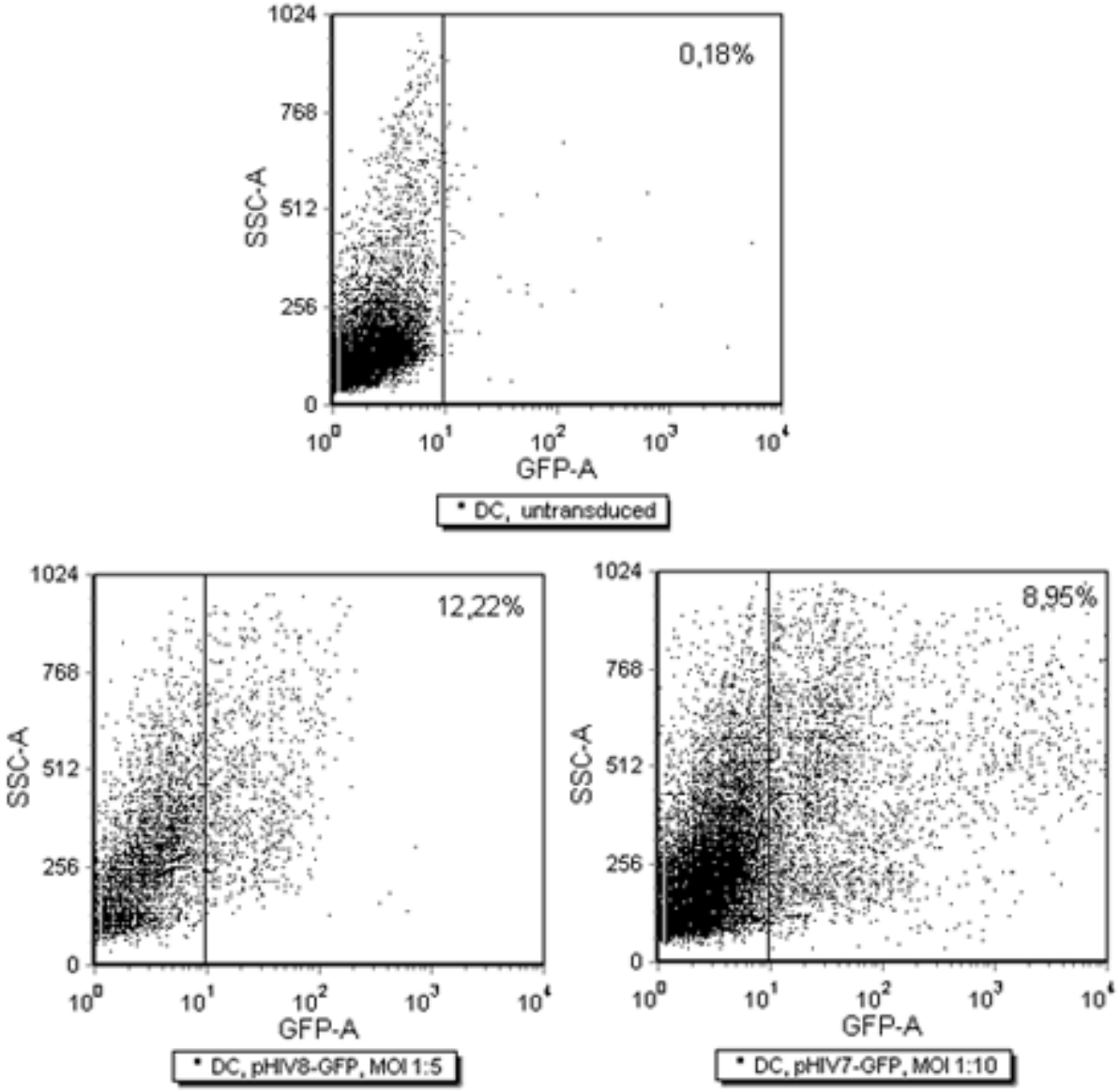

Figure 4.16: Lentiviral transduction efficiency in primary DC derived from peripheral blood, GFP expression measured by FACS 5 days post transduction. pHIV7-GFP: original GFP expressing transgene plasmid without any RNAi construct (kindly provided by H. Li, Rossi lab, CoH), pHIV8-GFP: new transgene plasmid with intron (amiRNA) - exon (GFP) expression cassette.

MOI. We repeated the transduction of $CD14^+$cells (negatively selected with Miltenyi depletion kit) which were then differentiated into DCs twice with new batches of buffy coat. In both cases we used several batches of amiRNA expressing constructs (based on

pHIV8 as well as pHIV9 background) as well as basic constructs without amiRNAs. All of the cells died 2-3 days after the transduction. The same virus batches used in the latter two transduction experiments had performed well in THP-1 cells and primary $CD34^+$ cord blood derived DC with the same protocol.

GFP expression intensity was around one log smaller in primary human macrophages and DC than in THP-1 cells.

4.3.3 Expression of artificial miRNAs from a combined intron-exon-approach

Because of the points discussed in detail in chapter 1.3.2 and 5.3.2 we decided to not use a commercially or freely available construct but cloned our own lentiviral platform based on the recently published cassette. [186] As lentiviral packaging system we chose a previously published system ([201]).

Expression of the transgene cassette (GFP expression and amiRNA processing) was tested for three different constructs by Northern Blot after transgene plasmid transfection in HEK 293T cells. The GFP expression was high (fig. 4.17 a). Processing of the artificial miRNAs (22-24 bp) from the pre-miRNA backbone (63-320 bp) was efficient and independent of the amiRNA sequence (fig. 4.17 b). The probe for TLR2-3 (TLR2: human Toll like receptor 2, TLR2-3 is a mismatch control) detected an unspecific RNA of 150 basepair length as well even though there was no target found by BLAST against the human genome.

THP-1 cells were transduced with the TLR2-1 and TLR2-2 constructs analyzed in fig. 4.17 and analyzed in FACS for GFP and TLR2 surface expression. Despite confirmed amiRNA expression, processing and GFP expression no knockdown of the TLR2 target gene could be found (data not shown).

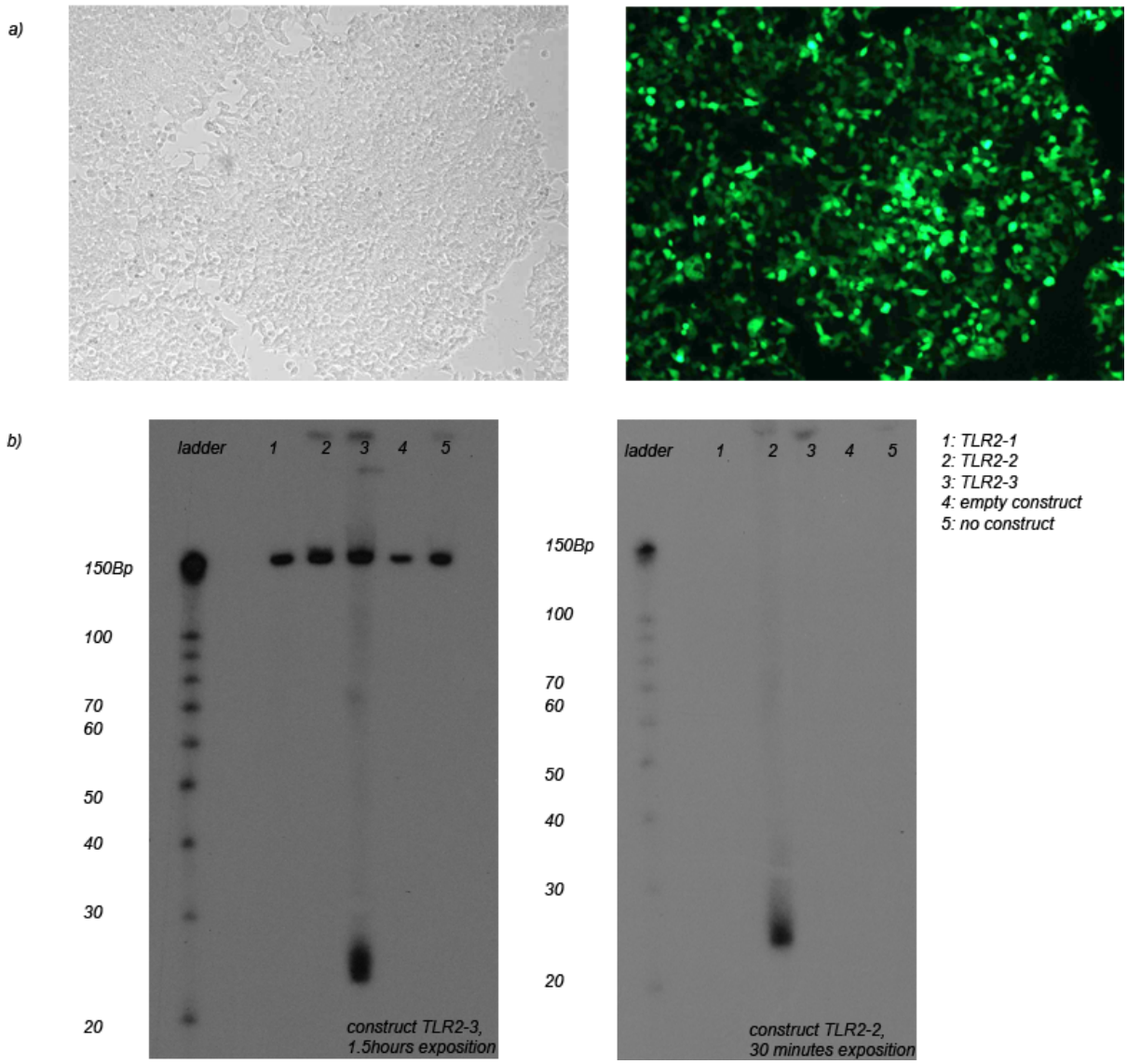

Figure 4.17: HEK 293T 24h after Lipofectamine 2000 transfection of either 800ng pHIV8-GFP-TLR2-1/2/3 or pHIV8-GFP ("empty construct") or without construct a) representative microscopic image for overview and GFP fluorescence b) Northern Blot from 20μg RNA for two different constructs

4.3.4 Gene knockdown in eukaryotic cell lines and primary CD34+ derived DC

Raf-1 (v-raf-1 murine leukemia viral oncogene homolog 1) is a serine/threonine-specific protein kinase in the MAPK/ERK pathway and sometimes referred to as c-raf too. Phos-

phorylation of Raf-1 (pRaf) mediates a downstream signaling cascade leading to NF-kB subunit p65 activation when TLR is activated [202]. Preliminary results obtained in our department indicate that Raf-1 might regulate via DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin) the expression of PD-L1 (Programmed Death Ligand-1) as well. We thus tested the knockdown efficiency of GFP based transgene constructs in cord blood $CD34^+$progenitor cell derived primary human DC on mRNA level and in HEK 293T cells on protein level.

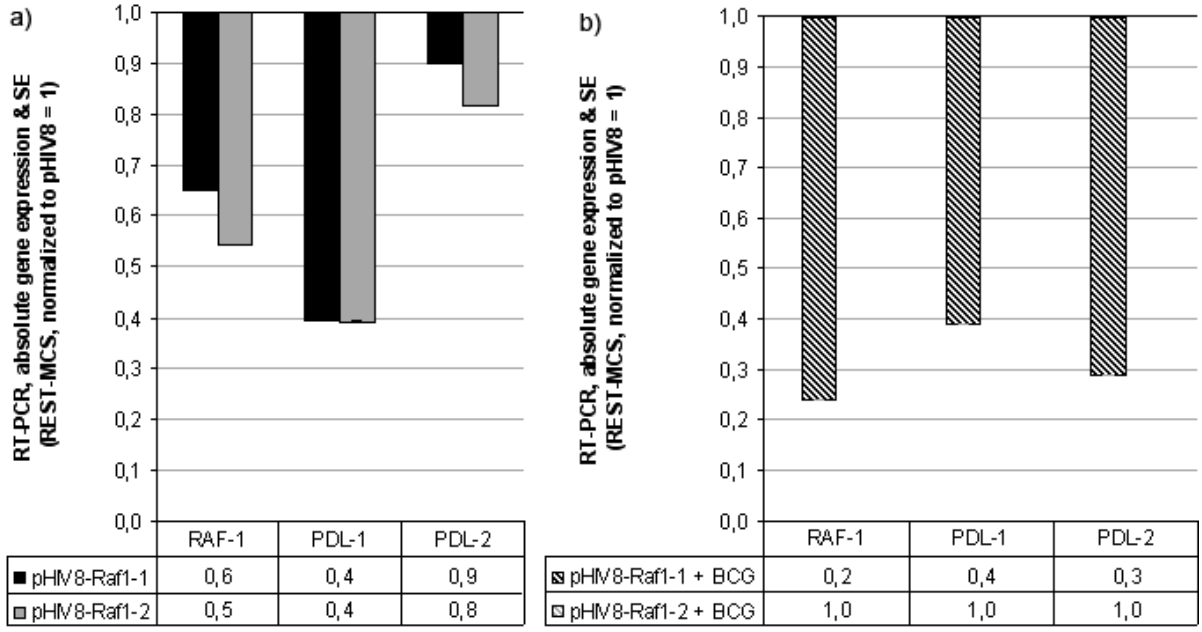

Figure 4.18: Expression of Raf-1 mRNA and predicted regulated downstream genes with and without BCG infection. Primary human $CD34^+$ derived DC 7 days after lentiviral transduction and sorting for GFP expression. SYBR Green RT-PCR 12 hours post BCG infection (+ BCG, MOI 1:10) or without infection, analyzed by REST-MCS [7], one experiment. No amiRNA (pHIV8) or amiRNAs targeting Raf-1 (pHIV8-Raf1-1 and pHIV8-Raf1-2) expressed.

Without infection both constructs caused measurable knockdown effects on the Raf-1 mRNA level. Infection with BCG increased the knockdown effect of pHIV8-Raf1-1 in CD34+ derived primary human DC (fig. 4.18) while pHIV8-Raf1-2 transduction did not yield any knockdown in infected cells. PD-L1 and PD-L2 as potential Raf-1 regulated genes showed a knockdown on mRNA level too when Raf-1 was decreased (fig. 4.18).

Infection with *M. tuberculosis* confirmed the downregulation of Raf-1 and PD-L1 when cells were transduced with pHIV8-Raf1-1 compared to pHIV8-luc control construct (fig. 4.19 a). Furthermore DC-SIGN expression was decreased in pHIV8-Raf1-1 transduced cells compared to pHIV8-luc control treated cells. Direct knockdown and downstream effects were lower in *M. tuberculosis* infected cells (fig. 4.19 a) compared to BCG infection (fig. 4.18).

GFP expression level was low thus stringent sorting in FACS for GFP expression led to only 10% of transduced cells recovered as GFP positive (data not shown). Since Raf-1 is an ubiquitous gene but expression levels are low and $CD34^+$ derived primary human DC are expensive we decided to test knockdown effects on protein level in HEK 293T. It was not possible to yield knockdown on protein or mRNA level in HEK 293T 48 hours and 24 hours after transfection without Raf-1 activation (data not shown) but activation of Raf-1 by PMA treatment [203] led to a reproducible knockdown on the protein level with pHIV8-Raf1-1 by 69% (fig. 4.19 b). Those data confirm a lack of Raf-1 knockdown for the pHIV8-Raf1-2 construct already found on mRNA level in primary human DC (fig. 4.18) when Raf-1 expression is induced by infection with BCG.

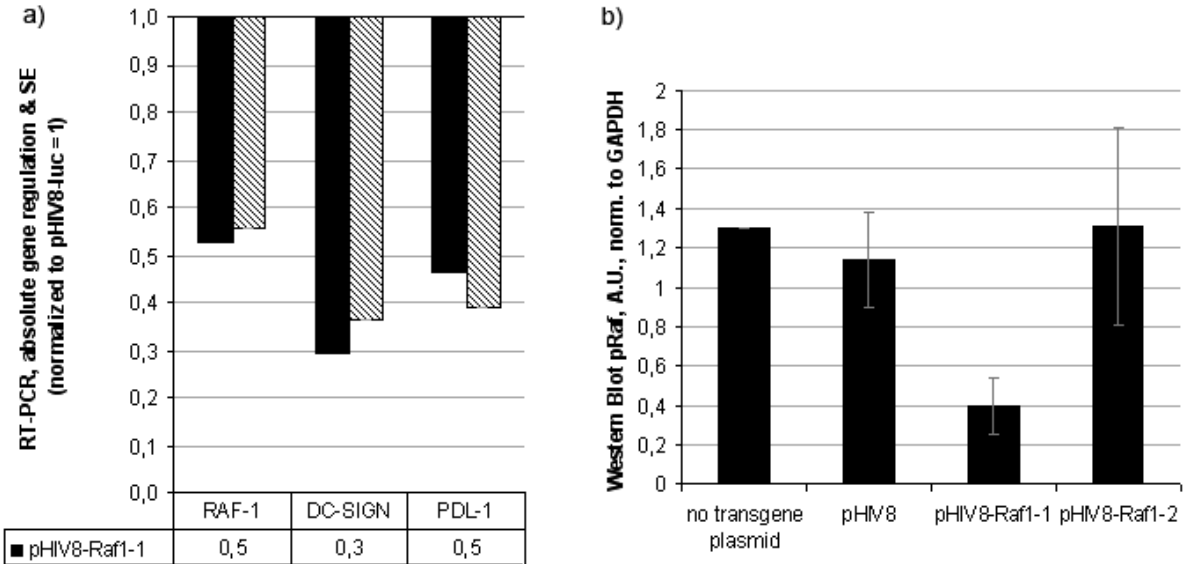

Figure 4.19: Knockdown of Raf-1 and predicted regulated downstream genes with and without *M. tuberculosis* infection. a) Primary human $CD34^+$ derived DC 7 days after lentiviral transduction and sorting for GFP expression. SYBR Green RT-PCR 12 hours post *M. tuberculosis* infection (+ TB, MOI 1:5) and without infection, analyzed by REST-MCS [7], one experiment. b) Western Blot densitometry for pRaf in HEK 293T, two experiments, 48 hours after transgene plasmid transfection (Lipofectamine2000) and 24 hours post PMA stimulation. No amiRNA (pHIV8), amiRNA targeting Raf-1 (pHIV8-Raf1-1, pHIV8-Raf1-2) or luciferase (pHIV8-luc) expressed.

Two other genes were knocked down in THP-1 cells: CARD9 (caspase recruitment domain family, member 9) and NALP1 (NACHT-LRR-PYD-containing protein-1). Both seem to play a role in *M. tuberculosis* infections *in vitro* (personal communication A. Dorhoi, MPIIB, [204] [205]). For both target genes two amiRNA constructs were designed based on the pHIV9 background (pHIV9-CARD9-1, pHIV9-CARD9-2, pHIV9-NALP1-1, pHIV9-NALP1-2). Stable THP-1 cell lines were produced and kept under constant puromycin selection. RT-PCR was performed from the cell lines in comparison to a cell line stably expressing an amiRNA against luciferase (pHIV9-luc). Three constructs (pHIV9-CARD9-2, pHIV9-NALP1-1, pHIV9-NALP1-2) yielded a knockdown of the target gene mRNA below 20% compared to the pHIV9-luc control (fig. 4.20). One construct (pHIV9-CARD9-1) caused only a knockdown of 10% on the mRNA level. Since no antibodies are currently

available for the target genes no Western Blot was performed.

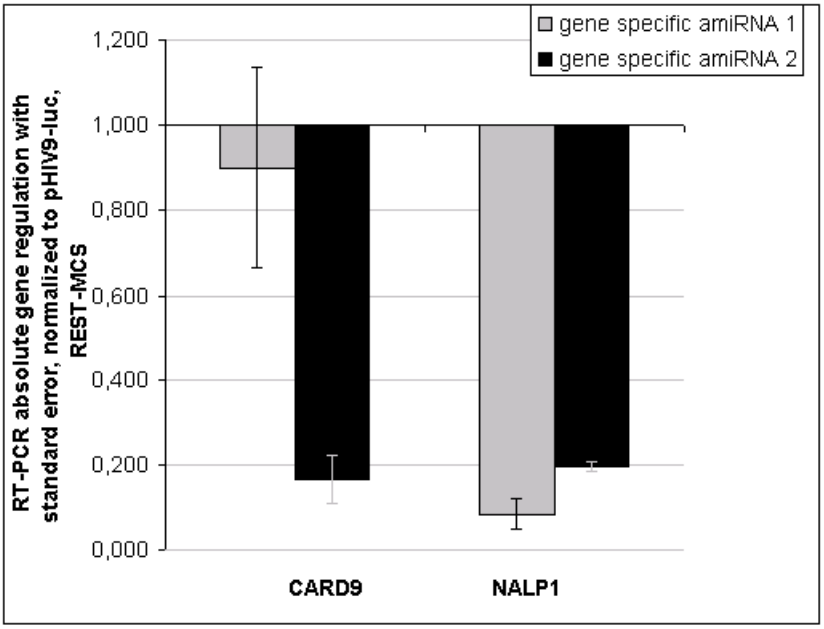

Figure 4.20: Expression of CARD9 and NALP1 mRNA in stably transduced THP-1 cells after Puromycin selection with two different amiRNA constructs per target gene (gene specific amiRNA 1 and 2). SYBR Green RT-PCR normalized to GAPDH, analyzed by REST-MCS, expression ratio relative to cell line expressing an amiRNA against luciferase.

4.3.5 microRNA overexpression in HeLa and THP-1 cell lines

To test whether the transgene construct can be used to overexpress endogenous miRNA with similar effects compared to the transfection with pre-synthesized mature miRNA we first transfected HeLa with two expression plasmids for human miR-133b and miR-146a and measured the expression level of each miRNA by TaqMan RT-PCR (fig. 4.21 a). Both miRNAs were overexpressed well. The induction of miR-146a in HeLa cells when transfecting the expression plasmid for miR-133b is not caused by the transgene construct but happens with the transfection of pre-synthesized mature miR-133b (Ambion) too (data not shown, personal communication J.P. Patron, MPIIB).

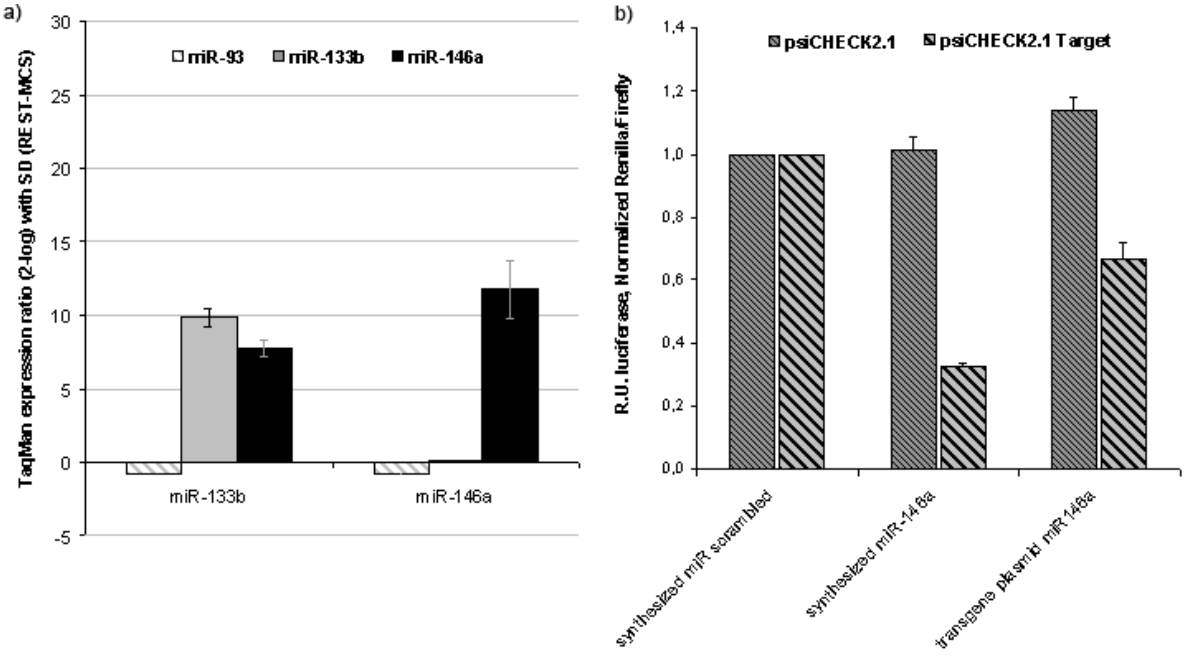

Figure 4.21: MicroRNA overexpression and knockdown effects in HeLa cells. a) Analysis of miRNA expression levels analyzed by TaqMan RT-PCR and REST-MCS [7], values normalized to RNU6B small non-coding RNA, one experiment each with triplicates. X-axis: effective miRNA from different origins: a) 48 hours post transgene plasmid transfection (Lipofectamine2000), control plasmid expressed no artificial miRNA, b) Knockdown effect comparison between synthesized mature and transgene plasmid expressed human miRNA-146a co-transfected with luciferase expressing psiCHECK2.1. Firefly luciferase expression (normalized to renilla luciferase expression) 48 hours post transfection (Lipofectamine2000). psiCHECK2.1 Target: 3' UTR of miR-146a target cloned behind firefly luciferase CDS, scrambled miRNA is premiRNA control 1 (Ambion), one experiment.

The knockdown effects detected with transfected transgene vector were lower than the effects of the pre-synthesized mature miRNA (fig. 4.21 b).

Overexpression of miR-133b, miR-146a and luciferase (control) was performed in THP-1 cells too by transduction with the designed lentiviral platform. First transduction was performed with lentiviral particles based on pHIV8 (GFP as selection marker). FACS sorting for GFP and constant cell line screening for GFP expression was performed indicating high expression of the transgene cassette but no elevated levels of miR-146a could be measured (data not shown). The same transgene plasmid batch led to strong GFP ex-

pression with clearly increased miR-146a levels in HeLa cells (fig. 4.21 a). Re-Sequencing of the pre-miRNA in the transgene plasmid confirmed an unmutated sequence. The miR-133b construct performed well on the pHIV8-GFP backbone in THP-1 as well as HeLa cells. Re-cloning of the pre-miRNA cassette into the pHIV9 backbone (Puromycin resistance cassette) showed good miRNA overexpression in THP-1 cells for miR-133b and miR-146a but with different patterns compared to HeLa cells (fig. 4.22).

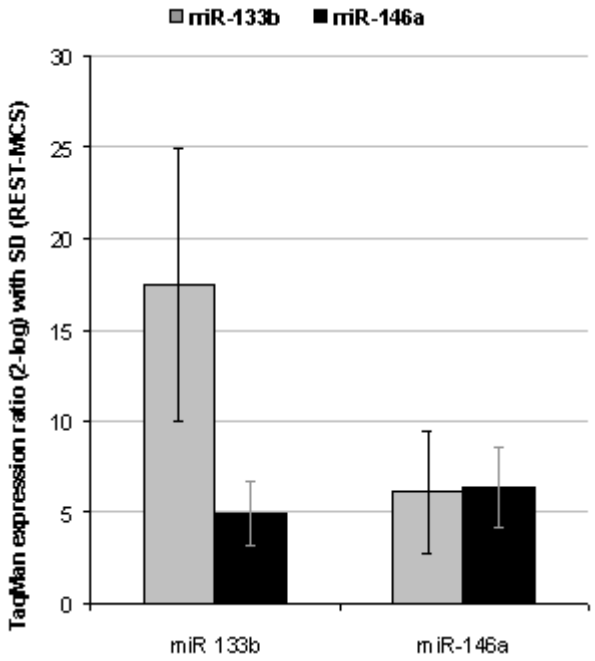

Figure 4.22: MicroRNA overexpression in THP-1 cells. Overexpression if miRNAs in THP-1 cells after lentiviral transduction and puromycin selection for transgene expression, expression compared to control expressing amiRNA against luciferase (pHIV9-luc). Analysis of miRNA expression levels analyzed by TaqMan RT-PCR and REST-MCS [7], values normalized to RNU6B small non-coding RNA, one experiment each with triplicates. X-axis: effective miRNA.

4.4 Summary of results on establishment of an RNAi platform in eukaryotic cells

We could show that for expression of transgenes in both THP-1 cells as primary human monocytes purified from peripheral blood promoter selection is crucial. In our experiments only CMVie promoter yielded a detectable expression. Nevertheless the expression declined rapidly over a time frame of 49 hours thus temporary plasmid based mediation RNAi platforms are not feasible for long term *M. tuberculosis* infection models. Transfection of siRNAs into THP-1 cells proved extremely low for several methods. Only Nucleofection let to useful transfection efficiencies as measured by plasmid transfection (40 % after 24 hours with DN100 protocol). As Nucleofection caused high cell toxicity (70 % after 24 hours with DN100 protocol) we tested lentiviral delivery as well. Lentiviral transduction of THP-1 cells with pHIV7-GFP was consistently 63 % and higher with an MOI of 1:10 and was confirmed with constructs based on pHIV8-GFP and pHIV9-Pur. Cell sorting for GFP or Puromycin resistance selection showed a stable transgene expression over several month. Transduction of primary human dendritic cells derived from peripheral blood caused high toxicity (100 % cell death for two out of three batches after 2-3 days post transduction) or low transduction efficiency (12 % of cells transduced) with the protocol used for THP-1 cells. Transduction of human primary dendritic cells derived from $CD34^+$progenitor cells (cord blood) showed no toxicity but low efficiency as well (around 10 %). Transduction of primary human macrophages derived from peripheral blood led to low transduction efficiency (3.8 % of cells transduced) with the protocol used for THP-1 cells.

Processing of amiRNA from pre-miR30 structure inside the transgene intron was confirmed with Northern Blot for three TLR2 targeting constructs (one mismatch control, two fully complementary amiRNA) with no unprocessed pre-miR30 detectable. The impact of amiRNA sequences on the knockdown efficiency was clearly detectable. None of the tested TLR2 constructs showed any knockdown in THP-1 cells on pHIV9-Pur background. We tested two pHIV8-GFP based constructs expressing amiRNA targeting Raf-1 in human primary dendritic cells derived from $CD34^+$progenitor cells. Only one amiRNA construct lead to reliable knockdown in infection experiments and on protein level once Raf-1 was induced. Downstream effect of proposed regulated genes could be detected as well. From four constructs used in THP-1 cells (two against CARD9-1, two against NALP1) on pHIV9-Pur background three performed well (knockdown of 80 % and more on mRNA level), while one did not yield any useful knockdown effects.

Thus from eight amiRNA constructs predicted to yield knockdown for four genes only

four sequences were active and three genes could successfully be knocked down. The knockdown controls (no amiRNA expressed, luciferase amiRNA expressed) performed as expected.

Overexpression of endogenous miRNA was tested as well. Two constructs based on pHIV8-GFP performed well in HeLa (human epithelial cell line) and showed the same effects as transfected synthesized miRNA even though the knockdown effect of the transgene expressed miRNA was lower. Only one construct (miR-133b) led to miRNA overexpression in THP-1 cells (human pro-monocytotic cell line) even though both constructs were not mutated and the selection marker was clearly expressed. Repetition of the experiment with pHIV9-GFP based constructs in THP-1 cells led to a overexpression of both miRNA but once more miR-146a overexpression was only low and - contrary to HeLa cells - miR-133 was upregulated too. Thus overexpression of endogenous miRNA caused different expression patterns in both cell types.

5 Discussion

The goal of this thesis was to research potential ways of generating gene knockouts or knockdowns in *M. tuberculosis* infection models by RNAi in prokaryotes and eukaryotic cells.

To allow sensitive and reliable detection of snRNA delivery and expression the hairpin primer based TaqMan RT-PCR method published previously was further dissected and modified. The measured sensitivity of both designs in comparison with the published results will be discussed including its impact in miRNA research and an outlook given for potential further analysis.

A proposed method for gene knockdown in prokaryotic cells by complementation with eukaryotic RNAi components was tested thoroughly. The potential underlying mechanism for temporary as well as persistent effects are discussed as well as its potential as research tools. Furthermore the effects and potential mechanisms of another protocol developed to cause target specific gene expression regulation in prokaryotes is debated.

For regulation of gene expression in eukaryotes a newly established lentiviral RNAi platform characterized by an intron-exon combination for expression of amiRNA has been developed. It is compared to existing lentiviral platforms and potential further improvements are discussed. Additionally possible applications in research on tuberculosis and endogenous miRNA are debated.

5.1 Hairpin primer based TaqMan RT-PCR

To detect RNA's a wide range of methods has been developed including Northern Blot, RACE protocols and RT-PCR. Each of those has advantages and disadvantages. While Northern Blot for example allows detection of specific RNA's and definition of the length of the detected RNA fragment the method has a low sensitivity even with the use of radioactive labeling. Furthermore screen development can be time consuming. 3' and 5' RACE protocols allows the detection of all RNAs in a mRNA purification including length definition and cloning but the protocol is time consuming, complicated and expensive. RT-PCR is the most sensitive detection method known today especially when using TaqMan probes. But it is cost intensive with each gene needing a specific probe and primer sets and allows no definition about the length of an RNA or how the 5' and 3' ends look like. Recently a protocol was published by Applied Biosystems (AB) which allows detection of even extremely short RNAs including miRNAs with hairpin primers and employs the TaqMan RT-PCR system [189]. Unfortunately further information such as sequences of

primer included in the kits are under non-disclosure even in case of purchase. The setup from AB makes it necessary to buy individual snRNA specific kits for reverse transcription and RT-PCR which are expensive and delivery often needs a long time even for miRNA predefined kits. This is even worse in case of custom kits. Especially when only a few samples per snRNA need to be tested this is inefficient. Because the expression of snRNAs in lentiviral systems should be confirmed and Northern Blots are not feasible in more high-throughput oriented approaches we tried to understand the hairpin design to come up with a less costly design.

In our experiments a 5-6 basepair overhang of the hairpin primer performed best and could be shown to have the same sensitivity as the custom kit supplied by AB. Since our design needs only specific reverse transcription hairpin and forward primer for each shRNA while conserving the remaining kit content the delivery time and price for small-size samples is significantly reduced.

Surprisingly our design as well as the one from AB had a 100 fold lower sensitivity than reported in the original AB publication for let-7 [189]. It is possible that the sequence of the snRNA which pairs with the reverse hairpin primer strongly influences the binding sensitivity due to its melting temperature. Tests with other siRNAs (for CD4-directed siRNA see [190], data not shown for a third one) with our design seem to support that there is a certain dependency between siRNA sequence and complementary hairpin overhang but all three tested siRNAs and hairpin primer showed a sensitivity threshold of 10^3 to10^4 molecules per reverse transcription. In common RT-PCR the primer design is based on a fixed melting temperature and the length of RNA sequence allows a comfortable buffer for primer design. This is not feasible for siRNA or miRNA quantification because of the length of the template that needs to be reverse transcribed. Which makes the method much more susceptible to systemic errors. Recent research in diagnosis of diseases is increasingly focusing on miRNA expression patterns. Differences in quantification between different kits in log fold dimensions due to bias of the method would impact findings in that research and lead to false conclusions about miRNA expression patterns if not confirmed by Northern Blot. Thus it would be interesting to test the sensitivity of different kits for individual miRNA further to understand the quantification reliability. Additionally confirmation of 3' end specificity would be important to understand if only mature miRNA are detected and no pre-miRNA or even whole transcripts influence the detection rate.

5.2 Regulation of gene expression in prokaryotes

Despite its availability, genetic manipulation of Mycobacteria is still time consuming and often very inefficient. It takes at least several month for homologous recombination in *M. tuberculosis* or BCG strains to yield genetically modified clones. In case of some genes this has proven with many methods to be impossible - probably due to polar or cistronic effects of the target gene or the cassette to be used as replacement, essential genes or in case of vaccine design the exclusion of antibiotic resistance selection marker. For research a method for fast and easy temporary gene knockdown as well as for vaccine generation a method to cause target specific mutations leading to reliable knockout strains is very much in demand.

5.2.1 Original method and deletions

The originally proposed method to complement prokaryotes with eukaryotic components to artificially built an RNAi machinery in those cells and have gene knockdown with siRNA proved very unreliable. While proof of principle results previous to this thesis were promising, the parameters that would lead to the successful and reliable application of this method could not be determined. The main impact seemed to be the cell extract quality with best (albeit low and unreliable) effects seen with the extract protocol published previously [184]. Furthermore success seemed to depend strongly on parameters intrinsic to the batch of competent bacteria and could not be standardized. Even most careful parameter variation in cell extract production like different concentrations and mixtures of protease inhibitors or cell numbers failed to increase the methods efficiency to values that would provide a reliable research tool when using different batches.

The observed unreliability of effects can not originate from lack of siRNA delivery or siRNA degradation of siRNAs when incubated with cell extracts. We could show that siRNAs are delivered inside the cell by electroporation. The siRNA which was detected in unpulsed samples after RNA purification from *L. monocytogenes* likely originates from siRNA attached to the outer cell wall or could be incorporated by diffusion or active transport into the bacterium as seems to be possible for asRNA [206]. Since siRNA buffer or control siRNA treated bacteria did not show any detectable signal those signals are most likely not false positive.

In experiments with *E. coli* RNaseIII knockout and wildtype strains single stranded antisense RNA and DNA oligomers caused stronger effects on the target gene expression than siRNA in wildtype strains and no effect in the RNaseIII knockout strain. The lack of effect in the RNaseIII knockout strain suggests already a purely antisense based effector mech-

anism. Furthermore antisense effects are based on kinetically controlled hybridization of the antisense nucleic acid to the complementary strand [207]. Reports about comparison of asRNA and siRNA in terms of kinetics differ. One study reports similar capacities of asRNA and siRNA mediated gene regulation [208] while another study found higher efficiency of siRNAs (up to 1000 fold higher in some cases [209]). Determining factor if siRNAs outcompete asRNAs in knockdown efficiency is considered to depend on the siRNA design and activity. It is unclear how active the in-house designed *ftsl* targeting siRNA is. Assuming that it is not of optimal design would lead to the expectation of asRNA and siRNA be comparable in activity. If in the wildtype strain any ftsl asRNA was incorporated into cell extract RISC at least an effect similar to the asRNA based antisense effect would have been expected - if not synergy from antisense and RNAi based mRNA degradation in parallel. Not only was there no significant difference between asRNA and siRNA visible, the variation in effect was so strong that no significant decrease in cfu at all could be detected. Thus our results in *E. coli* RNaseIII knockout and wildtype strains indicated that the siRNA is either not or only rarely incorporated into a RISC. Alternatively this RISC is not effective in *E. coli* cells.

Antisense RNA (just the guide strand of the siRNA) can already be incorporated into Ago2 ("minimal RISC") causing slicing effects on complementary mRNA [196]. Antisense DNA oligomers can not be incorporated into full or minimal RISC but yielded in our experiments the same effect as the asRNA. That indicates that the presence of Ago2 in *E. coli* strains seems to play no role on the effect or even hinder it. As this is contrary to RNAi in mammalian cells [210] it would rule out any RNAi based mRNA slicing pathway. Electroporation of *E. coli* overexpressing functional human Ago2 or an Ago2 mutant with defective PIWI slicing domain with siRNA or asRNA caused no significant knockdown with or without cell extract. Thus our results indicate that the environment inside the bacteria cell inhibits either the assembly or the function of even minimal Ago2 RISC.

In conclusion it seems to be impossible at least in *E. coli* to rebuild the RNAi machinery with the technologies and tool applied here.

In case the lack of target specific effects originated from the toxicity of human Ago2 in *E. coli* interfering with the readout (cfu) this can not be avoided. It might be possible that Argonautes with slicing activity from another organism might be less toxic but since this protein family is highly conserved that is unlikely. A gene regulation method that makes cultivation of cells at room temperature necessary to keep the bacterial cells alive is only in rare cases usable as research tool. Furthermore a RISC able to mediate siRNA based RNAi and not only asRNA triggered slicing seems to need at least two more components: Dicer and TRBP (TAR RNA binding protein, [36]). So far the overexpression

of human Ago2 in *E. coli* without codon optimization makes it necessary to express two plasmids for tRNAs that are common in eukaryotes but rare in prokaryotes. Furthermore the plasmid for Ago2 needs to be introduced as well plus a fourth plasmid which carries the eukaryotic heat shock protein 90 too and is necessary for successful folding of Ago2 (personal communication N. Tolia, CSHL). The latter two plasmids need to be under constant antibiotics selection or either become instable (data not shown, unpublished observations I. Dietrich & U. Jung, MPIIB). The overexpression of two more high molecular proteins - Dicer (220kDa [36]) and TRBP (30-50kDa depending on isoform [211]) - would add further constraint on the bacterial cell.

So the overexpression of all three eukaryotic components in *E. coli* to achieve full RNAi functionality seems not feasible.

5.2.2 A hypothesis based on the appearance of mutations

One experiment in the experiments trying to complement Prokaryotes with eukaryotic RNAi components caused in a single experiment in *M. smegmatis* frequent mutations in the target gene. Cell extracts from the same batch caused none of those effects in the same batch of electrocompetent *M. smegmatis* in repetition of the experiment. Another batch caused basepair exchanges in *L. monocytogenes* in one experiment but showed no effect in a repeated experiment or in *M. smegmatis*. Thus there seems to be a mechanism intrinsic to the bacteria cell that is not yet understood and can lead to permanent target gene specific mutations. No consensus motif in the sequences adjacent to the deleted sequences could be found and no peculiar 3D structures were found indicating that restriction enzyme activity could be excluded as reason for the mutations. Two potential notions were developed how the mutations could have been caused.

The complementary siRNA strand could have hybridized with the target gene DNA and prime Polymerase II activation. This could cause either activation of exonuclease activity of bacterial Polymerase II [188] or activation of Flap endonuclease 1 (FEN-1) [198]. FEN-1 is a highly conserved enzyme in bacteria and eukaryotic cells which cuts DNA single strands in case of Okazaki fragment generation during replication and allows thus the ligation of the lagging strand fragments. Additionally FEN is activated in case of single base exchanges and UV radiation caused abasic site generation in the DNA. Both mechanisms could cause double strand breaks (DSB) when happening on both strands in parallel and lead to erroneous DSB repair.

As proof-of-principle we have performed four experiments with two different DNA-oligomer types (chemically modified and unmodified) targeting the hygromycin selection marker of

a rBCG:ΔureC:hly vaccine candidate [160]. This BCG strain is in a phase I clinical trial but still contains a hygromycin resistance that needs to be removed permanently but so far this couldn't be achieved with common protocols despite great and long term efforts. The oligomer design based on the first hypothesis did not yield any detectable effect on the hygromycin resistance.

The design aiming to activate FEN-1 with a single abasic site and phosphorothioate stabilization showed strong and reproducible effects in four experiments: up to 98% decrease of cfu on plates with hygromycin compared to plates without hygromycin. This effect was reached with a very small dosage of less than 15 pmol per oligomer.

Generated clones were analyzed for erratic DNA DSB repair in the target genes by PCR and no deletions were detectable. Six weeks after electroporation the effect was not detectable anymore. Since the electroporation of sense oligomers has caused 48-80% decrease in cfu (respective to oligomer location) RNaseIII effects can be excluded as sole underlying mechanism. Currently, we cannot exclude that the temporary effect is due to general vitality decreases caused by the oligomer design. But several observations make that unlikely. The electroporation of DNA-oligomers with the same hygromycin binding sequence but no chemical modification (design one) decreased cfu numbers on non-selective agar plates to the same degree as electroporation of FEN-1 modified oligomers but did not show strong cfu differences between hygromycin and hygromycin-free agar (maximum cfu difference 10% in one experiment, data not shown). Thus the electroporation of DNA-oligomers compared to plasmids seems to be toxic to the cell in general while not having a strong impact on the hygromycin resistance. Additionally the colonies grown on hygromycin and hygromycin-free agar plates show the same phenotype and growth speed compared to untreated colonies. Phosphorothioate stabilized DNA-oligomers are used quite frequent for antisense effects in eukaryotic [208] and even prokaryotic cells including *M. tuberculosis* in much higher concentrations than used in our experiments without showing a general toxic effects on the cell [206]. UTP-modified DNA-Oligomers or abasic DNA-Oligomers up to 33 basepairs apart have been shown to cause in *E. coli* targeted deletions in plasmids without a general decrease in cell vitality [212]. This goes along with the observed effects that closely adjacent oligomer pairs have stronger effects than far distanced oligomer binding locations. These results make it likely that the decreased cfu number we see on hygromycin-plates is based on targeted DSB in the chromosome but not general vitality decreases from the DNA-oligomer design.

We aim to elucidate the underlying mechanisms of gene expression regulation using DNA-oligomers further and plan to perform time response analysis with a wider range of gene targets including markers like GFP. The use of such markers will help us understand

whether the efficacy depends on chromosomal and/or extrachromosomal targets. If DSBs are the reason for the observed effects the most crucial parameter most likely leading to a permanent deletion in the targeted region would be further decrease of the DNA-Oligomer abundance. To have only single DSB events per cell could improve the chance for re-ligation without causing an immediate new DSB. Maybe the overexpression of a ligase via plasmid would help to overcome the generation of too many DSBs that are not repaired in many cases and thus lethal. Since it was shown that DNA-oligomers can even be added directly to the medium [206] this could improve the ease of the method further.

Research on DNA damage, its induction and repair in eukaryotic and prokaryotic cells is quite advanced [212]. Still none of the mechanisms have been used yet to develop a method for permanent targeted genetic manipulation. In case it can be proven that the observed effects are target gene specific and leading to DSBs this would - to our knowledge - be the first methodical application of targeted erratic DNA repair causing gene knockouts. Until the underlying mechanism for the observed effects has not been dissected it is unclear if the hypothesis of what led to the earlier detected mutations in *M. smegmatis* and *L. monocytogenes* is correct and can reproduce these results. If this protocol does not cause any DSB there is still no explanation for the mutations found previously. In case only temporary effects can be obtained with the method referred to as "FEN-1 method" those might be based on epigenetic expression regulation on the genomic level (e.g. by methylation [213]) or an yet unknown mechanism.

Nevertheless the method can be quite useful in research even if only temporary effects are achieved. The protocol is much faster than common methods for genetic manipulation of *Mycobacteria* and the efficacy is very promising already at very low oligomer concentration compared to what would be expected from antisense based systems.

5.3 Gene expression regulation on host side

5.3.1 Reasons for the use of a lentiviral system and the construction of a new platform

RNAi has become a widely used tool in research. The understanding of the many different pathways and details of endogenous RNAi as well as artificially triggered RNAi is improving with a tremendous speed and uncovers an increasingly complex number of underlying mechanisms. There are a lot of commercially available platforms meanwhile for siRNA and shRNA based systems. But the application of RNAi as research tool lags behind in translation of the new knowledge into advanced RNAi platforms.

We needed a system that could trigger RNAi in the THP-1 cell line as well as primary human monocytes and monocyte derived DC and macrophages while avoiding activation of these cells. The focus of commercially available products is often on the delivery of siRNAs into hard to transfect cell lines without applicability to primary cells or *in vivo* systems due to toxicity, cell activation or missing possibilities to sort for successfully transfected cells. Naked or even lipo-complex packaged siRNAs can activate Toll-like receptors by immunostimulatory sequences (activation of TLR3,7,8 have been reported) [214]. Chemical modifications like 2-O-methylations of the sense strand of the siRNA strongly diminish this immune activation or even decrease potential off target effects caused by sense strand incorporation into RISC [215] [216]. But at least in THP-1 cells and primary human monocytes we could never achieve any transfection efficacy above 5% with any transfection reagent including lipo-complex based systems. While the transfection efficiency by Nucleofection was better, the cell mortality was very high in THP-1 cells and primary human DC. A single group claims to have 100% transfection efficiency with the DF4 transfection reagent (a lipo-complex system, Dharmacon) in primary human DC [217]. This group used CD14 positively selected monocytes from peripheral blood which have been differentiated into DC before transfection with 100nM to 50μM siRNA. Unfortunately they do not reveal the cell number that concentration is used at. Additionally there are no activation markers for those DC shown. For our experiments we avoid positive selection when purifying monocytes to not activate these cells. It might be possible that the transfection of differentiated (and maybe because of the purification process even activated) DC yields higher efficacies with lipo-complex based system than we observed in monocytes.

Another problem with siRNA transfections is the interference with endogenous RNAi pathways due to the sheer amount of siRNAs which can be (and often is) transfected [218]. The short half life of knockdown (peak is mostly after two to three days) because of the instability of siRNA is another disadvantage as well as the impossibility to sort for successful transfection. Because of those reasons we decided for a lentiviral platform. Still it might be useful to test siRNA delivery and knockdown by DF4 as a potential supporting method for findings by the lentiviral method in case the cells are not activated by the transfection and there is no artifact created by interference with the endogenous miRNA pathways.

5.3.2 Reasons for the selection of an intron derived artificial miRNA and the lentiviral system

One important factor in the design of shRNAs and artificial miRNA is the hairpin loop design. More recent research has shown that the processing efficacy of shRNA and artificial miRNA strongly depends on the loop to stem sequence combination of a hairpin. A very effectively processed stem-loop combination can be totally inert to processing when a single base pair in the stem is exchanged (personal communication C. Köberle, MPIIB). It seems at the same time that the loop of endogenous human miR-30 is very independent of the stem sequence leading to well processed shRNAs (personal communication Dr. G. Hannon, CSHL, confirmed by own results fig. 4.17 b). Thus we decided for miR30 based loop design that was further optimized by work in G. Hannons lab (loop comparison study unpublished, final loop sequence commercially available via OpenBiosystems).

Most RNAi platforms that are commercially available are U6/H1 based shRNA expression systems while the knowledge about advantages of artificial miRNA based RNAi systems has not made it into wider RNAi application yet. The Hannon-Elledge database for artificial miR30 based constructs is available commercially (OpenBiosystems). The original system available at the start of this thesis was driven by U6. The current system uses a Polymerase II system too now. It consists out of the CMV immediate early (CMVie) driven transcript that has a GFP CDS followed by an IRES sequence, a puromycin selection marker, the pre-miRNA and a PolyA sequence. That construct design might cause several problems though. Drosha is known to process pre-miRNAs from constructs co-transcriptionally [15] which causes free 3' and 5' ends in the mRNA. The 5'-3' exonuclease Xrn2 (5'-3' Exoribonuclease 2) binds to those free 5' and 3' RNA ends which can lead to transcriptional termination or degradation of downstream mRNA [219]. The recent GZIP design by Open Biosystems places the pre-miRNA after the IRES translated selection marker but before the polyA signal. Most likely the reasoning behind this design is to counter Xrn2 based 5'-3' degradation while loosing only the polyA tail. The upstream placed IRES are sequences which are commonly found in viral mRNA [18]. Binding of RNPs to the upstream located IRES allows the nuclear export without a polyA tail and even translation of cap-free mRNA. [222]. But at least IRES containing Hepatitis B mRNA tends to be exported into the cytosol extremely fast. Faster even than splicing - which happens co-transcriptionally too - thus the export could take place before the mRNA is completely transcribed [220]. And since the IRES sequence is far upstream of the pre-miRNA that could prevent miRNA expression. So far it hasn't been shown that PTB based export depends on the full transcription of the mRNA but only that

binding of PTB to mRNA decreases its nuclear-cytosol cycling speed [221]. Because of this the pre-miRNA transcription/processing and nuclear export (while still leading to translation of the selection marker) can compete with each other. If the nuclear export happens prematurely this would lead to a decrease in the knockdown efficiency. Even if the pre-miRNA structure is fully transcribed before the nuclear export but previously to Drosha based processing there is no knockdown to be expected. That export of the mRNA without processing of the pre-miRNA by Drosha seems to happen is supported by their choice of placing a GFP CDS without IRES upstream of the IRES translated puromycin resistance cassette. The GFP selection marker can only be translated (as it has no IRES) if the PolyA - cap structure is formed (see chapter "Translation" 1.1.4). Thus the PolyA must still remain at the end of the mRNA without Drosha being able to process the pre-miRNA first (and thus removing the polyA). Belated processing in the cytosol (as it happens with shRNA structures) is not possible for pre-miRNAs or the whole mRNA as Dicer can not bind to double stranded RNAs without two bp 3' overhang. This can be a problem in case of a single copy systems as the lentiviral vectors tend to be. Additionally especially in primary cells or monocytes which are more prone to promoter silencing the shRNA expression can be further reduced.

In conclusion there seems no mechanism involved in case of the GZIP design to make sure the transcription is completed or the pre-miRNA is retained in the nucleus until complete processing occurred before the export takes place. This is not an issue with an intron based system which lacks the described potential competing mechanisms. The generation of free 5' and 3' ends by splicing and Drosha processing happens in an intron-exon construct too. But it is countered in those systems by exon tethering by the Polymerase II thus the single exons are afterwards re-ligated efficiently [16]. Since the nuclear export signal in this case is only at the 3' end of the construct no pre-mature export is to be expected.

It would be interesting to quantify the individual expression cassette RNA components for both the GZIP and pHIV system in general and depending on their location in nucleus and cytosol to evaluate the efficacy of both construct types. Unfortunately there seem to be no publications available using the OpenBiosystems construct so far.

Additionally Open Biosystems only guarantees a knockdown efficacy of at least 70% if three different constructs for one target gene are obtained. While the success rate of shRNA prediction algorithms is still not very good prediction tools like DSir seem to be better than the original prediction algorithm used for the Hannon-Elledge database (personal communication Dr. G. Hannon, CSHL). The Qiagen shRNA database seems to be relatively effective compared to RNAScout [182] predictions (personal communication Alexander Kardas, MPIIB). Translation of validated siRNAs into shRNAs or amiRNAs

can in some cases lead to inactive constructs for reasons not yet understood but at least in our construct the knockdown efficacy of a construct derived from a published siRNA for Raf-1 (shRaf-1) performed well while the amiRNA directly taken from the Hannon-Elledge database (Open Biosystems, shRaf-2) did not yield any knockdown. Under those conditions in-house cloning to obtain active amiRNA expression constructs was necessary in any case.

Thus I decided to choose an expression cassette published recently [186] because it expresses selection marker and pre-miRNA from the same promoter so promoter silencing would be detected and those cells removed by selection. Furthermore the intron structure aims to retain the pre-miRNA in the nucleus for maximal pre-miRNA processing efficacy while not decreasing the translation of the selection marker due to Xrn2 based downstream degradation. The chosen intron-exon combination hasn't been used in gene validation studies or in combination with a viral delivery platforms yet [224]. Moreover it was shown that the lentiviral system we used does not activate interferon responses and has been cleared by FDA for clinical trials in human in RNAi application ([223] and unpublished data, personal communication Dr. John J. Rossi, Beckman Research Inst. CoH). This hasn't been shown for the OpenBiosystems platform yet. Thus with our system we aim to combine the advantages of good prediction databases or validated siRNA sequences with an *in vivo* proven lentiviral expression system and minimal interference with endogenous miRNA processing and function.

5.3.3 Influence of promoter-, intron-, artificial miRNA- and artificial miRNA target site sequence as well as infection model on the knockdown effect

Expression of amiRNA driven by a CMVie promoter leads to efficient RNAi knockdown in HEK 293T and HeLa even if the construct is only integrated in the genome as a single copy [121]. Promoter comparisons indicate that CMVie is most suitable for primary human DC [225]. In case of experiments that include infection with *M. tuberculosis* or BCG that promoter choice might be even more favorable: those infections induce NF-κB ([227]) which trans-activates the CMVie promoter [228]. This would increase the amiRNA expression and overcome silencing which can happen in case of CMVie in primary cells and *in vivo* [228]. Nevertheless replacing the promoter in case of low knockdown efficacies with a promoter less prone to silencing could be useful for targets that might be difficult to knock down by more than 70% otherwise (e.g. Raf-1). The human constitutionally active promoter E1Fa might be a reasonable choice and has been used in lentiviral protein overexpression platforms already (personal communication Dr. T. Joeris, MPIIB). In this

case it remains to be tested if the increase in amiRNA amount is not causing similar side effects as has been described for U6 or H1 driven expression of shRNAs. Furthermore our results for CARD9 and NALP1 confirm the strong impact of the specific amiRNA sequence on the knockdown efficiency when expressed under the same promoter. Thus it might be a better option to screen for more effective miRNA sequences first (because the sequence itself is more effective or because the target site has a higher accessibility [229]). In case a cell type that allows transfection expresses the target gene no cloned transgene plasmids are necessary. Instead the screen can be done easily by modifications of a recently published protocol [96] by addition of SV40 minimal promoter and terminator via PCR.

Finally it has been shown that intron-containing expression cassettes show higher translation of the exons increasing the copy number of the artificial miRNA compared to linear constructs [231] [232]. Because of this the generation of expression systems with the rabbit beta-globin intron being placed between promoter and the translated regions has become quite common and is applied in the packaging system of the lentiviral platform too.

5.3.4 Gene knockdown with a lentiviral RNAi platform to research the DC-SIGN - Raf1 - PD-L1 signaling cascade

For a test of our lentiviral platform in an infection model we did chose Raf1 as we have indications that Raf-1 plays a role in the DC-SIGN mediated signaling cascade to regulate PD-L1 expression on DCs. Using our lentiviral system we could show a knockdown of Raf-1 in primary dendritic cells derived from cord blood CD34+ progenitor cells by 45% after *M. tuberculosis* infection and 73% after BCG infection.

The different efficiencies might be related to differential gene expression in both cord blood donors. The CD34+ progenitor cell batch we had obtained for the infection with *M. tuberculosis* showed lower cell division rates than the one used for the BCG infection. As the cell number necessary for each transgene construct with and without infection is high, it is not possible to perform several infection experiments with one batch. Cell sorting for GFP expression after transduction yields a very low number of cells as it is very difficult in FACS to detect low GFP expression and a stringent sorting protocol discards those cells. Since the expansion of those progenitor cells is complicated and spontaneous differentiation can happen in case of too long expansion times those need to be limited. The variety in batch vitality and the laborious process of producing the necessary high numbers of viral particles is currently the largest bottle neck for RNAi application in human primary DCs.

Another reason for the variety in knockdown results could be differential Raf-1 induction by *M. tuberculosis* and BCG. In studies with antisense oligonucleotides directed against Raf-1 in ovarian cancer cells those cell lines with higher Raf-1 mRNA levels compared to constitutively expressed A-Raf and B-Raf were more sensitive to Raf-1 antisense effects too. In those studies degradation of Raf-1 mRNA by antisense effects induced apoptosis [226]. If apoptosis is induced by RNAi mediated Raf-1 knockdown as well a knockdown should not undercut a necessary basal level of Raf-1. In this case a smaller Raf-1 knockdown - while still impacting the regulation cascades Raf-1 is involved in - would be favorable to validate its cellular function. Knockdown effects are measured as relative expression levels by RT-PCR. Thus an increase in the absolute Raf-1 mRNA level (e.g. by infection) would be measured as a stronger knockdown while not being detectable without previous expression induction. We can observe this in both BCG and *M. tuberculosis* infections on mRNA level. Our results in HEK 293T in primary human DC's (fig. 4.18) on protein level support the idea that Raf-1 knockdown is favored if the Raf-1 expression is increased.

The third reason for the strong knockdown in infected cells compared to uninfected cells could be due to the CMVie trans-activation by NFkβ which would increase the amount of artificial miRNA and thus the knockdown effect [228].

There exists no human DC cell line which could be used to overcome individual variations among donors. Buffy coat can be used as relatively low cost source of high numbers of primary human monocytes which can be differentiated into DCs as well [230]. But our experiments prove a strong individual difference variability in cell viability for those cells after transduction too. In tests with three different buffy coats transduction only in one case DCs remained viable while the cells of the other two donors died two to three days after transduction. It would be useful to research if optimized transduction protocols on buffy coat derived DCs can overcome the vitality problem as this could decrease costs on experiments with primary human DC's. Waiting two days after purification of monocytes before transduction while already starting the differentiation might be helpful to increase vitality. Furthermore a new second generation of the lentiviral platform was designed (pHIV9) which is based on antibiotic selection (the exon codes for puromycin resistance instead of GFP) to improve the selection sensitivity for detection of low transgene expressing cells. At least in case of infection with *M. tuberculosis* and BCG the NF-kβ based trans-activation of the transgene should increase knockdown effects even in those cells. It is not clear how strong the transgene needs to be expressed to yield reasonable knockdown effects, the amiRNA sequence seems to have the major impact at the moment on knockdown efficiencies.

Another basic problem for this specific pathway in DCs and the use of lentiviral vectors could be an unspecific decrease of the DC-SIGN expression by lentiviral platforms. The reason might be related to viral proteins rather than receptor binding or transgene design as receptors differ in both cases and MULV based integrating vectors with the same transgene caused no DC-SIGN knockdown [233]. We can confirm that DC-Sign induction in DCs transduced with lentiviral vectors expressing no amiRNA or a luciferase targeting amiRNA is significantly lower after infection with BCG or *M. tuberculosis* than DC-Sign induction in untreated DCs (unpublished data, personal communication K. Fae, MPIIB). This decrease in DC-SIGN induction is detectable in lower PD-L1 expression levels too. Nevertheless the platform is still effective to provide evidence on the regulation of PD-L1 by DC-SIGN via Raf-1 as transduced cells with an amiRNA targeting Raf1 show further PD-L1 decrease compared to cells transduced with an empty or luciferase amiRNA expressing construct.

In conclusion we did not see a knockdown for Raf-1 that was as strong as has been reported with siRNA transfected cells. But the phenotype was clearly visible with our lentiviral platform while our approach should minimize the risk of artifacts caused by a general perturbation of endogenous miRNA gene regulation.

5.3.5 Overexpression of endogenous miRNA in different cell lines

Overexpression of endogenous miRNAs was performed in collaboration with J. P. Patron (MPIIB) to research the effects of candidate miRNAs (hsa-miR-133b and hsa-miR146a) on THP-1 cells in infection with BCG and *M. tuberculosis*. First constructs were based on pHIV8 transgene plasmid with GFP selection. The overexpression of the miRNA could be detected in HeLa cells transfected with the transgene plasmid or transduced with the packaged vector for both miRNAs. But surprisingly only miR-133b overexpression could be detected in THP-1 cells - even though strong GFP-expression was detectable in those cells and the transgene plasmid was confirmed to be unmutated. Repeating the experiments in THP-1 cells with transgene plasmids based on pHIV9 (puromycin resistance as transgene expression marker) yielded the expected miRNA overexpression for both constructs. It remains unclear what caused the undetectable miRNA over-expression in the GFP transgene construct. Since both RNAs (GFP mRNA and miRNA) are transcribed from the same construct and the GFP encoding exon lies behind the miRNA encoding intron the miRNA must have been transcribed as its pre-miRNA and should have been processed properly. As the same transgene plasmid batch performed well in HeLa and was proven not to be mutated it is unlikely that a mutation of the packaged virus sequence

would play a role. If over-expression of miR-146a in THP-1 cells is toxic it is possible that over the long-term selection for GFP expression and cell line expansion a clone which was mutated after reverse transcription of the virus particle transgene was favored and thus displacing clones with the correct sequence. Genomic sequencing of the cell line would confirm this. Nevertheless a strong toxicity of the construct is not likely as in the cell lines generated on the pHIV9 background (puromycin resistance) a miRNA 146a overexpression could be detected - albeit lower compared to the results in HeLa supporting the idea of a certain toxicity and with upregulation of miR-133b.

The knockdown effects caused by miRNAs after transfection of the transgene vector were lower than the effects of transfection of the pre-synthesized mature miRNA. But the amount of transfected synthesized miRNA (15 pmol) was more than 200 times higher than the amount of transgene vector (300 ng of transgene vector equals 60 fmol) while there was only a ten to twelve fold increase of miRNA expression from the transgene vector transfection (fig. 4.21 a) compared to endogenous levels. Thus the effect could be due to quantitative difference and might not indicate a generally lower knockdown efficacy of the transgene vector.

In general over-expression of miRNA 133b and miRNA 146a had in both cells lines different effects on the levels of miRNAs which were not overexpressed. It remains to be tested if those effects are based on temporary transfection versus stable genomic integration or if endogenous miRNAs in general have regulatory functions on other miRNAs. In that case those regulatory networks could differ in different cell lines and cell types. So far miRNA expression has been only studied in certain conditions and certain cell lines. Comparisons of potentially differential effects on the miRNA expression pattern after miRNA over-expression in different cell types have not been researched yet.

5.3.6 Outlook on the application of the established lentiviral RNAi platform

With the development of the lentiviral platform for expression of amiRNA from an exon-intron combination for gene validation in *M. tuberculosis* infection models several requirements could be fulfilled. Those are the effective transduction of THP-1 cells without causing high cell mortality providing stable RNAi mediation with minimal interruption of the endogenous miRNA pathway to be expected. Furthermore primary human DCs derived from $CD34^+$progenitor cells could be transduced and reproducible knockdown be shown without making a cell activation necessary as reported by Geijtenbeck for the Raf-1 target gene so far. The transduction efficiency of primary DC and macrophages - especially when purified from peripheral blood - is still too low or the original transduction

protocol too toxic thus needing optimization.

All the experiments performed so far were done *in vitro*. Mouse *in vivo* models for TB research would be the next step of RNAi application. Collaboration with Dr. S. Reece (MPIIB) has been started to apply the lentiviral system in mice *in vitro* and *in vivo*. It needs to be tested if the VSV-G pseudotyping of the lentiviral particles works efficiently for mouse cells. But since the lentiviral system is modular more effective pseudotypes - if necessary - can be used. For mice lung e.g. epithelia baculovirus gp64 seems a useful ligand in lentiviral transduction [234]. Previous studies indicate that the stability of transgene expression *in vivo* is strongly dependent on the expression rate in APC [235]. The reason is most likely $CD8^+$ T cell priming by the transgene expression when presented on MHC I. In case of TB research transgene expression in APC by miRNA target site regulation is possible too. Unfortunately this would abrogate the advantage of the intron-exon system for amiRNA expression control. One way to overcome this problem would be incorporation of sequences inhibiting the proteasomal processing [236]. But this area of gene therapy focus is still in its infancy.

Another topic is the expression of multi-meric constructs - several amiRNA in one construct - to either increase knockdown efficiency or regulate multiple targets with one construct. It has been reported in a previous study that this is possible with pre-miRNA-30 based approaches [102]. Collaboration with Dr. U. Steinhoff (MPIIB) on this is topic to verify those results is ongoing.

6 Summary

Aim of this thesis was to develop a platform to research the impact of gene knockouts or knockdowns in *M. tuberculosis* infection models by RNAi.

The proposed method about complementation of prokaryotic cells with eukaryotic RNAi components to mimic an RNAi like pathway of gene regulation had to be suspended. Previous results of significant and reliable generation of gene knockdown/knockout could not be reproduced. Results indicate that at least in *E. coli* Argonaute 2 based RNAi is not possible.

It remains unclear what caused the mutations observed in *M. smegmatis* and *L. monocytogenes* in one experiment each. Verification of the hypothesis that an activation of FEN-1 with subsequent erratic DSB repair could be the underlying mechanism did not find similar changes on the genetic level with the current protocol. Nevertheless this hypothesis led to a new protocol which yielded promising transient phenotypic effects in several experiments. The underlying mechanism is so far not understood. While RNaseH effects could be ruled out as sole mechanism, inhibited transcriptional activity of the targeted gene could play a role. Further research on this protocol to understand the underlying mechanism and applicability in research is considered.

The application of RNAi in eukaryotic cells could be achieved with the combination of two systems. The first is a lentiviral system which has shown its *in vitro* and *in vivo* safety. The second component is an intron-exon expression cassette for artificial miRNAs. Since the superior properties of artificial miRNAs compared to shRNAs in terms of target specificity of effects, safety *in vivo* and avoidance of IFN I response activation have been shown, the resulting platform is state of the art and should outperform commercially available systems.

The efficacy of the system *in vitro* in infection models has been shown while analyzing the Raf-1 mediated regulation of PD-L1 by DC-SIGN in human primary DCs that were infected with BCG or *M. tuberculosis*. Knockdown of expression of two other genes in THP-1 cells below 20% could be achieved as well. Control constructs without miRNA or luciferase targeting miRNA performed as expected.

The impact of target gene and amiRNA sequence on the knockdown level could be demonstrated for several genes and constructs. Comparison of two Raf-1 targeting artificial miRNAs with one derived from a validated siRNA and one predicted by the Hannon-Elledge

database showed reliable knockdown on mRNA and protein level only for the first one. Since our results confirm published data about sequence independent processing efficacy of the pre-miRNA the reason for this is to search in the activity of the artificial mature miRNA. Further research needs to explore the optimal design rules for artificial miRNA as a critical parameter for the successful application of this platform. Additional tests with different promoters driving the expression of the artificial miRNA could improve the knockdown efficiency further and abrogate potential silencing of the CMVie promoter.

The feasibility to overexpress endogenous miRNA with the developed platform could be shown as well. Overexpression of the same miRNA in different cell types seems to have different effects on the miRNA expression patterns. Predicted mRNA targets could be validated with similar results to transfection of synthesized mature miRNA.

In conclusion the developed platform seems well usable for both applications.

7 References

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8 Online Resources

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[3] http://www.origene.com/shRNA/

[4] http://www.who.int/mediacentre/news/notes/2006/np29/en/index.html

[5] http://www.rnainterference.org/HumanSequences.html

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[8] http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi

[9] http://www.premierbiosoft.com/netprimer/

[10] http://bioinformatics.weizmann.ac.il/blocks-bin/oligo_melt.pl

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9 Appendix

9.1 Zusammenfassung

Ziel dieser Arbeit war es, eine Plattform zu entwicklen, mit deren Hilfe die Auswirkungen von Gen-Knockouts oder Gen-Knockdowns in *M. tuberculosis* Infektionsmodellen durch RNAi untersucht werden können.

Die ursprünglich vorgeschlagene Methode, prokaryotische Zellen mit eukaryotischen Komponenten des RNAi Regulationsweges auszustatten und diesen so nachzubauen, mußte aufgegeben werden. Vorherige Ergebnisse über signifikante und zuverlässige Generierung von Gen-Knockdown und -Knockout ließen sich nicht reproduzieren. Stattdessen legen die im Rahmen dieser Arbeit gewonnenen Ergebnisse nahe, daß zumindest in *E. coli* kein Argonaute 2 basierter RNAi Regulationsweg nachgebaut werden kann.

Es bleibt weiter unklar, welcher Mechanismus zu den beobachteten Mutationen in *M. smegmatis* und *L. monocytogenes* in je einem Experiment geführt hat. Die Untersuchung des Hypothese, daß der zugrundeliegende Mechanismus die Aktivierung von FEN-1 wäre und nachfolgender fehlerhafter Doppelstrangbruch-Reparatur, konnte mit dem entwickelten Protokoll keine vergleichbaren Veränderungen auf genetischer Ebene erzeugen. Nichts desto trotz wurde aufgrund dieser Hypothese ein neues Protokoll entwickelt, welches in mehreren Experimenten zu erfolgversprechenden (wenn auch vorübergehenden) phänotypischen Effekten führte. Der diesen zugrundeliegende Mechanismus ist noch nicht aufgeklärt. RNaseH Effekte konnten als einziger Wirkungsmechanismus ausgeschlossen werden, so daß transkriptionelle Inhibition des Zielgenes eine Rolle spielen könnte. Eine weitere Untersuchung dieses Protokoll, um den Wirkungsmechanismus zu verstehen, ist geplant.

Die Anwendung von RNAi in eukaryotischen Zellen konnte durch die Kombination von zwei Komponenten erreicht werden. Die erste ist ein lentivirales Verpackungssystem, dessen seine Sicherheit *in vitro* und *in vivo* demonstriert worden ist. Die zweite Komponente ist eine Intron-Exon Expressionskassette für künstliche miRNAs. Da die überlegenen Eigenschaften von künstlicher miRNAs im Vergleich zu shRNAs bezüglich Zielspezifität der Effekte, Sicherheit *in vivo* und Vermeidung von Interferon I Antworten erwiesen sind, entspricht die entwickelte Plattform dem aktuellsten Forschungsstand und sollte kommerziell verfügbare Systeme übertreffen.

Die Wirksamkeit des Systems *in vitro* in Infektionsmodellen wurde nachgewiesen, indem die Raf-1 vermittelte Expressionsregulation von PD-L1 über DC-SIGN in menschlichen primären DC nach Infektion mit BCG oder *M. tuberculosis* analysiert wurde. Der Knockdown der Expression zweier weiterer Gene in THP-1 Zellen auf unter 20% des Ursprungsniveaus konnte ebenfalls gezeigt werden. Die Kontrollkonstrukte ohne amiRNA oder mit einer amiRNA, welche gegen Luziferase gerichtet ist, funktionierten wie erwartet.

Der Einfluß von Zielgen und amiRNA Sequenz auf die Knockdownstärke konnte für mehrere Gene und Konstrukte demonstriert werden. Der Vergleich zweier Raf-1 amiRNA Konstrukte (eins abgeleitet von einer funktionalen siRNA, eins aus Vorhersagen der Hannon-Elledge Datenbank) führte nur für das erste Konstrukt zu verlässlichem Knock-

down auf mRNA und Protein-Ebene. Da unsere Ergebnisse eine effiziente Prozessierung der pre-miRNA unabhängig von deren doppelsträngigen Sequenz bestätigen, muß die Ursache dafür in der Aktivität der reifen amiRNA zu suchen sein. Zukünftige Experimente sollte daher die Regeln für optimale amiRNA Auswahl zum Thema haben, da dies der kritische Parameter einer erfolgreichen Anwendung ist. Zusätzliche Tests mit verschiedenen Promotoren, welche die Expression der amiRNA steuern, könnte die Knockdown Effizienz weiter erhöhen, wenn mögliche Abschalt-Effekte ("silencing") des CMVie Promoters so vermieden werden würden.

Die Möglichkeit, endogene miRNAs mit Hilfe der entwickelten Plattform zu überexprimieren, wurde ebenfalls erfolgreich genutzt. Die Überexpression in verschiedenen Zelltypen scheint unterschiedliche Wirkungen auf das miRNA Expressionsmuster zu haben. Die Validierung vorhergesagter miRNA Zielgene war vergleichbar gut möglich wie nach Transfektion synthetisierter reifer miRNAs.

Zusammenfassend scheint die entwickelte Plattform für beide Anwendungsgebiete gut geeignet zu sein.

9.2 Danksagung

Zuerst möchte ich Herrn Prof. Stefan H. E. Kaufmann dafür danken, meine Arbeit in seiner Abteilung anfertigen zu dürfen. Im Vorstellungsgespräch wurde ich von ihm gefragt, warum er mich einstellen sollte. Meine Antwort, daß ich wieder ein Projekt bräuchte, um mich daran festzubeißen, eine Herausforderung, war möglicherweise nicht die, welche ein Bewerbungstrainer empfehlen würde, aber spontan und ehrlich. Mein Wunsch wurde erfüllt - in einer Umgebung, die charakterisiert war von großer wissenschaftlicher Freiheit, einer hervorragenden Ausstattung, Vertrauen und vielen befruchtenden Diskussionen. Dafür, sowie die Unterstützung, Betreuung und das Korrekturlesen der Arbeit gilt mein großer Dank.

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9.3 Lebenslauf

geb. 23.11.1979 in Dresden

Bildungsweg und beruflicher Werdegang

1986 - 1992 Oberschule "Fritz Schultze", Dresden

1992 – 1998 Gymnasium Dresden Plauen, Dresden

1998 Abitur, Note 1,8

1999 - 2005 Studium Humanbiologie, Ernst Moritz Arndt Universität, Greifswald

2001 Vordiplom – Note 1,4 (Prüfungsfächer Physikalische Chemie, Mathematik, Morphologie, Mikrobiologie, Biochemie)

2005 Diplom – Note 2,1 (Hauptfach Immunologie, 1. Nebenfach Virologie, 2. Nebenfach Biochemie/Molekularbiologie, Diplomarbeit "Rolle von LBP in der Darmschleimhaut")

2005 – heute Doktorarbeit am Max-Planck-Institut für Infektionsbiologie, Berlin, Abteilung Immunologie, Prof. Kaufmann

Praktika

1994 & 1996 einwöchige Praktikum am Max-Planck-Arbeitsgruppe "Mechanik heterogener Festkörper",Technischen Universität Dresden, Prof. Wolfgang Pompe

1995 dreiwöchiges Praktikum am Institut für Festkörper- und Werkstoffwissenschaft e. V. Dresden, Dr. Ingolf Mönch

1999 vierwöchiges Praktikum am Institut für Immunologie und Transfusionsmedizin TU Dresden, Prof. Ernst P. Rieber

2002 viermonatiges Praktikum, University of Massachusetts Medical School, Department of Pathology, Prof. Kenneth Rock

2003 - 2004 Studentische Hilfskraft, Institut für Immunologie und Transfusionsmedizin Ernst Moritz Arndt Universität Greifswald, Frau Prof. Christine Schütt

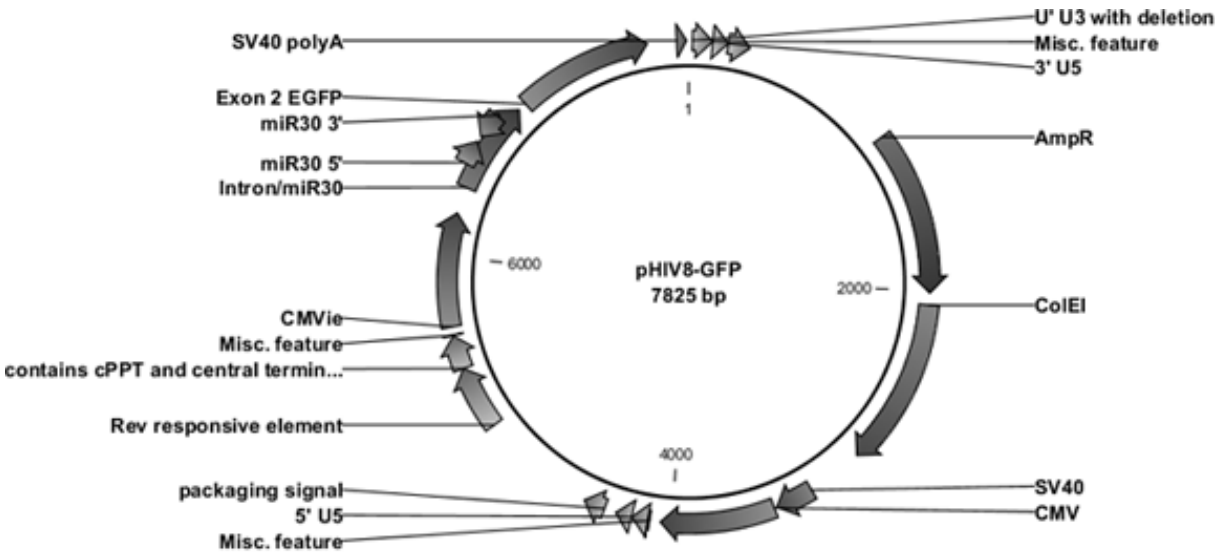
2008 zweimonatiges Praktikum, Beckman Research Institute, City of Hope, Prof. John J. Rossi

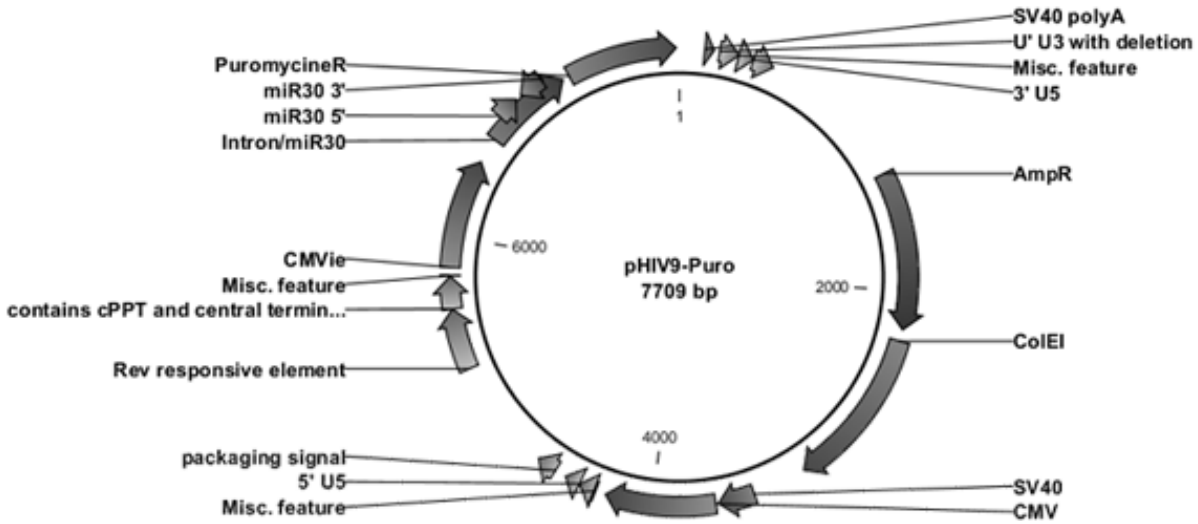
Auszeichnungen

1996 Deutsche SchülerAkademie 1996 für Hochbegabte, Braunschweig, Kurs "ATP – Energiehaushalt der Zelle"

1998 Zwölfter Platz IX. Internationale Biologieolympiade Runde 3 (beste 42 Deutsche)

9.4 Plasmidkarten





9.5 Supplier

abcam (Cambridge, UK, www.abcam.com)

Active Biosciences (Hamburg, Germany, www.activebioscience.de)

Agilent Technologies (La Jolla, CA, USA, http://www.stratagene.com) comprising products from Stratagene

Ambion (Austin, TX, USA, www.ambion.com)

Applied Biosystems (Carlsbad, California USA, www.appliedbiosystems.com)

ATCC (Manassas, VA, USA, www.atcc.org)

Bacto Laboratories Pty Ltd (Liverpool, NSW, Australia, http://www.bacto.com.au)-comprising products from Difco

Beckman Coulter GmbH (Krefeld, Germany, www.beckmancoulter.de/)

Becton Dickinson GmbH (Heidelberg, Germany www.bdbiosciences.com)

Biochrom AG (Berlin, Germany, www.biochrom.com)

Biomol (Hamburg, Germany, www.biomol.de)

Biontex Laboratories GmbH (Martinsried/Planegg, Germany, www.biontex.com)

Biozym (Hess. Oldendorf, Germany, www.biozym.com)

Carl Roth (Karlsruhe, Germany, www.carl-roth.de)

Cell Signaling (Danvers, MA, USA, www.cellsignal.com)

Corning Life Scienes (Amsterdam, The Netherlands, www.corning.com/lifesciences/)

Dharmacon Inc. (Lafayette, CO, USA, www.dharmacon.com/)

Fermentas (St. Leon-Rot, Germany, www.fermentas.de)

GE Healthcare Life Sciences (Little Chalfont, UK, www.gelifesciences.com) - comprising products from Amersham Pharmacia

Gibco (Karlsruhe, Germany, www.Invitrogen.com)

Invitrogen (Carlsbad, CA, USA, www.invitrogen.com)

Lonza (Koeln, Germany, www.lonzabio.com) - comprises products from amaxa

Macherey-Nagel (Dueren, Germany, www.mn-net.com/)

Merck Chemicals LTD (Nottingham, UK, www.merck-chemicals.de) - comprising products from Calbiochem

Millipore GmbH (Schwalbach, Germany, www.millipore.de)

Miltenyi Biotech (Bergisch Gladbach, Germany www.miltenyibiotec.com)

New England Biolabs NEB (Ipswich, MA, USA, www.neb.com)

Nunc (Wiesbaden, Germany, www.nuncbrand.com)

PAA Laboratories (Pasching, Austria, www.paa.com)

Perkin Elmer (Waltham, MA, USA, www.perkinelmer.de)

Promega (Mannheim, GERMANY, www.promega.com)

Qbiogene/MP biomedicals (Solon, OH, USA, www.mpbio.com)

Qiagen (Hilden, Germany, www.qiagen.com)

Roche Diagnostics (Mannheim, Germany www.roche-appliedscience.com)

Sarstedt (Nuembrecht, Germany www.sarstedt.com)

Serva Electrophoresis (Heidelberg, Germany, www.serva.de)

Sigma-Aldrich (München, Germany www.sigmaaldrich.com) - comprises products from Fluka

Strathmann GmbH & Co. KG (Hamburg, Germany, www.strathmann.de) - via eubio.at

Takara Bio (Madison, WI, USA, www.takara-bio.us) - comprising products from Clontech Laboratories, Inc.

Thermo Fischer/Electron GmbH (Dreieich, Germany www.thermo.com)

9.6 Abbreviations

°C (degree celsius)
AAV (adenoassociated virus)
A (Ampere)
A. dest. (Aqua destillata)
Ago (Argonaute)
amiRNA (artificial microRNA)
ATP (adenosine triphosphate)
AV (adenovirus)
BCG (bacillus Calmette Guerin)
bp (basepair)
BSL (bio-safety level)
CARD (caspase recruitment domains
CARD-9 (caspase recruitment domain family, member 9)
CDS (coding sequence)
cfu (colony forming units)
cm (centi meter)
CMVie promoter (Cytomegalie virus immediate early promoter)
DNA (deoxyribose nucleic acid)
DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non- integrin)
DdR Pol (DNA dependent RNA polymerase)
DGCR8 (DiGeorge syndrome critical region gene 8)
dsRNA (double stranded RNA)
FEN-1 (flap endonuclease 1)
g (gramm)
HIV (human immunodeficiency virus)
IFN (interferon)
INH (isoniazid)
IRES (internal ribosome binding sites)
kV (kilo volt)
LGP-2 (laboratory of genetics and physiology 2)
μFD (micro faraday)
μl (micro liter)
M (molar)
MDA-5 (melanoma differentiation-associated antigen 5)
MDR (multi drug resistant)

mg (milli gramm)
miRNA (microRNA)
mJ (milli Joule)
ml (milli liter)
mM (milli molar)
MMuLV (Molony murine leukemia virus)
mRNA (messenger RNA)
ms (milli seconds)
NALP1 (NACHT-LRR-PYD-containing protein-1)
ng (nano gramm)
nm (nano meter)
OAS-1 (2'-5'oligoadenylate synthetase)
PABP (PolyA tail binding protein)
PAZ (Piwi Argonaute Zwille)
PCR (polymerase chain reaction)
PD-L1 (Programmed Death Ligand-1)
PIWI (P-element induced wimpy testis)
pmol (pico mol)
PNK (polynucleotide kinase)
PolyA signal (Polyadenylation signal)
pre-miRNA (preliminary microRNA)
pri-miRNA (primary microRNA)
PTBP (Polypyrimidine tract binding protein)
Raf-1 (v-raf-1 murine leukemia viral oncogene homolog 1)
RdRP (RNA dependent RNA polymerases)
RIG-I (retinoic acid-inducible gene-I)
RISC (RNA induced silencing complex)
RITS (RNA induced transcriptional silencing complex)
RMP (rifampicin)
RNA (ribose nucleic acid)
RNAi (RNA interference)
RNP (ribo nucleo protein)
rpm (rounds per minute)
shRNA (small hairpin RNA)
siRNA (small interfering RNA)
SIV (simian immunodeficiency virus)
SV40 promoter (simian virus 40 promoter)

TB (tuberculosis)
TDM (trehalose 6,6 dimycolate)
TK promoter (herpes simplex virus thymidine kinase promoter)
TLR (toll like receptor)
xg (fold centrifugal force)
Xrn2 (5'-3' Exoribonuclease 2)