DEVELOPING A CELL-BASED FLUORESCENT ASSAY FOR SCREENING DICER-ACTIVATING COMPOUNDS

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Tiivistelmä/Referat – Abstract

MicroRNAs are ~22 nucleotide long RNA strands which regulate gene expression by binding to the 3'UTRs of messenger RNAs. MicroRNAs are predicted to regulate about a half of all protein-coding genes in the human genome thus affecting many cellular processes. One crucial part of microRNA biogenesis is the cleaving of pre-miRNA strands into mature microRNAs by the type III RNase enzyme, Dicer.

Dicer has been shown to be downregulated due to aging and in many disease states. Particularly central nervous system disorders are linked to dysregulated microRNA processing. According to the latest studies, Dicer is crucial to the survival of dopaminergic neurons and conditional Dicer knockout mice show severe nigrostriatal dopaminergic cell loss, which is a hallmark of Parkinson's disease.

By activating Dicer with a small-molecule drug, enoxacin, the survival of dopaminergic cells exposed to stress is significantly improved. However, enoxacin, which is a fluoroquinolone antibiotic, activates Dicer only at high concentrations (10-100 µM) and is polypharmacological, which may cause detrimental side effects. Therefore, enoxacin is not a suitable drug candidate for Dicer deficiencies and better Dicer-activating drug candidates are needed. The aim of this work was to develop a cell-based fluorescent assay to screen for Dicer-activating compounds.

Assays which measure Dicer activity have already been developed, but they have some pitfalls which don't make them optimal to use for high-throughput screening of Dicer-activating compounds. Some are cell-free enzyme-based assays and thus neglect Dicer in its native context. The RNA to be processed by Dicer does not represent a common mammalian RNA type. Most assays do not have internal normalizing factors, such as a second reporter protein to account for e.g. cell death, or the analysis method is not feasible for high-throughput screening data. Considering these disadvantages, the study started by designing a reporter plasmid *in silico.* The plasmid expresses two fluorescent proteins, mCherry (red) and EGFP (green), and a mCherry transcript-targeting siRNA implemented into a pre-miR155 backbone which is processed by Dicer. Thus, measuring the ratios of red and green fluorescence intensities will give an indication on Dicer activity. The plasmid also has additional regulatory elements for stabilizing expression levels. The plasmid was then produced by molecular cloning methods and its functionality was tested with Dicer-modulating compounds. The assay was optimised by testing it in different cell lines and varying assay parameters, and stable cell lines were created to make large-scale screening more convenient. Finally, a small-scale screen was done with ten pharmacologically active compounds.

Transiently transfected, in Chinese hamster ovarian cells, mCherry silencing was too efficient for reliable detection of improvement in silencing efficiency due to floor effect. With an inducible, Tet-On, system in FLP-IN 293 T-Rex cells, the expression could be controlled by administering doxycycline and the improvement in silencing was quantifiable. The assay seemed to be functional after 72 hours and 120 hours of incubation using enoxacin (100 µM) as a positive control. However, the screening found no compounds to significantly reduce mCherry/EGFP fluorescence ratio and, additionally, the effect of enoxacin was abolished. Therefore, a more thorough analysis on the effects of enoxacin was done and, although statistically significant, enoxacin was only marginally effective in reducing mCherry/EGFP fluorescence ratio after 72 hours of treatment. It should be noted from the small-scale screening that metformin and BDNF, compounds previously shown to elevate Dicer levels, showed similar effects to enoxacin. The quality of the assay in terms of high-throughput screening was determined by calculating Z-factors and coefficients of variations for the experiments, which showed that the variability of the assay was acceptable, but the differences between controls was not large enough for reliable screening. In conclusion, the effects of metformin and BDNF should be further studied and regarding the assay, more optimisation is needed for large-scale, high-throughput, screening to be done with minimal resources.

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Dicer, RNA interference, microRNA, cell-based screening assay, fluorescent reporter, enoxacin, Parkinson's disease

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Tiivistelmä/Referat – Abstract

MikroRNA:t ovat noin 22 nukleotidiä pitkiä RNA-juosteita, jotka estävät geenien ilmentymistä sitoutumalla lähetti-RNA:n 3'UTR-alueille. MikroRNA:t osallistuvat laajalti moneen soluprosessiin säätelemällä noin puolta kaikista proteiineja koodaavista geeneistä. MikroRNA:n ilmentymisessä, eräs tärkeä vaihe on III tyypin RNaasin, Dicerin, suorittama pre-miRNA:n prosessointi valmiiksi mikroRNA-juosteeksi.

Dicerin toiminnan ja ilmentymisen on mitattu heikentyvän ikääntymisen johdosta, sekä useissa eri taudeissa. Erityisesti keskushermostotautien ja mikroRNA prosessointiin liittyvien ongelmien välillä on löydetty yhteys. Tuoreimpien tutkimusten mukaan Dicerilla on tärkeä rooli myös dopaminergisten hermosolujen selviytymisen kannalta ja lisäksi Dicer muuntogeenisillä hiirillä mustatumakkeen dopamiinihermosolut kuolevat, joka on Parkinsonin taudin keskeisin patofysiologinen ilmiö.

Dicerin aktiivisuuden tehostamisella, käyttäen enoksasiinia, on suojaava vaikutus dopamiinihermosoluille. Enoksasiini, joka on fluorokinoloneihin kuuluva antimikrobinen yhdiste, tehostaa Diceria vain suurilla pitoisuuksilla (10-100 µM). Lisäksi se on polyfarmakologinen voiden aiheuttaa paljon vakavia haittavaikutuksia, joten se ei ole optimaalinen lääkeaine Dicer-puutoksiin liitettyjen tautien hoitamiseen. Tämän erikoistyön tavoitteena oli kehittää solupohjainen, fluoresenssiin perustuva menetelmä, jolla voisi seuloa parempia Diceria aktivoivia yhdisteitä.

Dicerin aktiivisuutta mittaavia menetelmiä on jo kehitetty aiemmin muutamia, mutta ne eivät ole optimaalisia Diceria-aktivoivien yhdisteiden seulomiseksi. Osa kokeista on entsyymipohjaisia eivätkä ne ota huomioon solunsisäistä endogeenistä Diceria. Kokeissa käytettävä RNA, jonka Dicer prosessoi, ei edusta yleisiä nisäkässolujen RNA-tyyppejä. Tietyissä kokeissa ei ole sisäistä suhteuttavaa tekijää (esimerkiksi toista fluoresoivaa proteiinia) tai niillä ei ole mahdollista suorittaa laajoja seulontoja. Työssä suunniteltiin ensiksi edellä mainitut puutteet huomioon ottaen reportteriplasmidi *in silico*. Plasmidi ilmentää kahta fluoresoivaa proteiinia, mCherry:ä (punainen) ja EGFP:tä,(vihreä) sekä mCherry:n ilmentymistä estävää siRNA-juostetta pre-miR155:n runkoon liitettynä, jonka Dicer prosessoi. Näin ollen, mittaamalla punaisen ja vihreän fluoresenssi-intensiteettien suhdetta, voidaan tutkia Dicerin aktiivisuutta. Plasmidissa on myös useita säätelyelementtejä ilmentymisen tasaamiseksi. Plasmidi valmistettiin molekyylikloonausmenetelmin ja sen toiminnollisuutta testattiin Dicerin aktiivisuuteen vaikuttavilla yhdisteillä. Seulontakoetta optimoitiin eri solulinjoilla ja olosuhdemuutoksilla, ja lisäksi valmistettiin stabiileja solulinjoja laajamittaisen seulonnan helpottamiseksi. Lopuksi suoritettiin pienen mittakaavan seulonta kymmenelle farmakologisesti aktiiviselle yhdisteelle.

Ohimenevästi transfektoituna, Kiinanhamsterin munasarjasoluissa, mCherryn hiljentäminen oli niin tehokasta, että hiljentämisen tehostamista ei voitu luotettavasti mitata. Hallitsemalla ilmentymistä doksisykliinin avulla, Tet-On-systeemillä FLP-IN 293 T-Rex soluilla, saatiin ilmentymistä kontrolloitua ja mittaukset luotettaviksi. Seulontakoe saatiin toimimaan 72 tunnin ja 120 tunnin aikapisteillä käyttäen enoksasiinia positiivisena kontrollina. Seulonnasta ei löydetty mCherry/EGFP fluoresenssien suhdetta merkitsevästi vähentäviä yhdisteitä ja lisäksi enoksasiinin vaikutus ei ollut enää tilastollisesti merkitsevä. Tämän perusteella suoritettiin laajempi analyysi enoksasiinin vaikutuksista, missä havaittiin, että sen vaikutus mCherry/EGFP fluoresenssien suhteen vähentämisessä oli, vaikkakin tilastollisesti merkitsevä, hyvin vähäinen 72 tunnin kokeessa. Huomioitavaa pienen mittakaavan seulonnasta on, että metformiinin ja BDNF:n, joiden on aiemmin osoitettu lisäävän Dicerin ilmentymistä, vaikutukset olivat vastaavia enoksasiinin vaikutukseen. Seulontakokeen laatu laajamittaisen seulontakokeen suhteen määritettiin laskemalla kokeille Z-tekijän sekä hajonnan koeffisienttien arvot. Nämä osoittivat, että kokeen hajonta oli hyväksyttävä, mutta ero kontrollien välillä oli liian pieni, jotta koetta voisi käyttää luotettavasti seulomiseen. Tärkeimpinä johtopäätöksinä, metformiinin ja BDNF:n vaikutuksia Diceriin tulisi tutkia tarkemmin, ja koetta on optimoitava lisää, jotta laajamittaisia seulontoja voidaan suorittaa mahdollisimman vähillä resursseilla.

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Dicer, RNA interferenssi, mikroRNA, solupohjainen seulontakoe, fluoresenssireportteri, enoksasiini, Parkinsonin tauti

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ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
bp	base pair
CFP/RFP	Cyan/Red fluorescent protein
CHO	Chinese hamster ovarian
CKO	conditional knockout
CMV	cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
DGCR8	DiGeorge syndrome critical region 8
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's modified eagle medium
ds/ss	double-stranded/single-stranded
DUF283	domain of unknown function
EGFP/ EYFP	Enhanced Green/Yellow fluorescent protein
ER	endoplasmic reticulum
Exp5	exportin 5
FLP	Flippase (Flp recombinase enzyme)
HEK293T	human embryonic kidney cells
HSV-TK	herpes simplex virus thymidine kinase
HTS	high-throughput screening
JNK	c-Jun-N-terminal kinase
kDa	kiloDalton
mCherry	modified Cherry (red fluorescent protein variant)
mRNA	messenger RNA
miRNA	microRNA
NLS	nuclear localization sequence

nt	nucleotide
pA	polyadenylation
PACT	protein activator of PKR
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PEI	polyethylenimine
piRNA	piwi-interacting RNA
PIWI	P-element-induced wimpy testis
pol II/III	RNA polymerase II/III
pri-/pre-miRNA	primary/precursor microRNA
RBP	RNA-binding protein
RLC	RISC loading complex
RNAi	RNA interference
RT	room temperature
sncRNA	short non-coding RNA
shRNA	short hairpin RNA
siRNA	short interfering RNA
SV40	simian virus 40
tdTomato	tandem dimer Tomato (red fluorescent protein)
T-Rex	FLP-IN 293 T-Rex cells
TRBP	human immunodeficiency virus transactivating response RNA-binding protein
WPRE	Woodchuck virus post-transcriptional regulatory element

1 INTRODUCTION

MicroRNAs (miRNAs) are 22 nucleotide (nt) long strands of RNA which post-transcriptionally regulate gene expression (Bartel 2004). They are crucial to the development of plants and animals and are expressed in response to cellular stressors. One important part of miRNA biogenesis is the cleaving of precursor (pre-)miRNAs into mature miRNAs by the type III RNase Dicer (Bernstein et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Carmell and Hannon 2004; Cullen 2004).

Dicer dysfunctions and abnormal miRNA processing have been linked to various neurodegenerative diseases (Shin et al. 2009; Emde et al. 2015; Chmielarz et al. 2017; Varol et al. 2017). In particular, Dicer levels are downregulated in patients suffering from aging-related disorders, such as Parkinson' disease (PD) and amyotrophic lateral sclerosis (ALS). Neurodegenerative diseases are an increasing burden not only on patients and their families, but also on society since considerable resources is spent on the healthcare of patients suffering from neurodegeneration (Rodriguez-Blazquez et al. 2015; McDade and Bateman 2017).

Activating Dicer with a small-molecule compound, enoxacin, has neuroprotective effects on midbrain dopaminergic neurons (Chmielarz et al. 2017). Enoxacin is not an optimal drug for enhancing RNA interference (RNAi) *in vivo* since it only activates Dicer in high cellular concentrations and, thus, may lead to a plethora of detrimental side effects e.g. convulsions (Delon et al. 1999).

Therefore, there is a need for better compounds to be developed as drugs which could activate Dicer and possibly slow down or cure neurodegeneration reducing the cumulating burden on society. Current assays for measuring Dicer activity are not optimal for screening compounds in high-throughput (Chiu et al. 2005; Davies and Arenz 2006; Shan et al. 2008; Li et al. 2012; Podolska et al. 2014). The disadvantages are that some of them are biochemical disregarding Dicer in its native context, thus, not taking its interactome into account. Those that are cell-based don't have an additional reporter to account for other factors, which may affect the reporter protein e.g. cell death, to rule out false positives. The assays measure double-stranded (ds)RNA or short hairpin (sh)RNA processing instead of primary (pri-)miRNA which are more relevant to human diseases. Moreover, most assays

use transiently transfected reporters which is more problematic compared to stably expressing cell lines when screening thousands of compounds and may cause variation in results due to variation in transfection efficiency.

The purpose of this M.Sc. thesis was to discuss Dicer as a drug target and to develop a sensitive and scalable, high-throughput enabling, cell-based fluorescent assay for measuring Dicer activity and use it to screen for compounds which could stimulate Dicer activity.

2 REVIEW OF THE LITERATURE

2.1 RNA interference

The first miRNAs were discovered in the early 1990s but their function remained unclear for many years (Lee et al. 1993; Wightman et al. 1993). A midst the turn of the millennia, RNAi, by dsRNA, was elucidated and subsequently many classes of short non-coding (snc)RNAs, such as miRNAs, were discovered to be mediated by the same RNAi processing machinery (Fire et al. 1998; Reinhart et al. 2000; Elbashir et al. 2001; Lee and Ambros 2001). This evolutionarily conserved mechanism has been found in many eukaryotes, and sncRNAs in all their endogenous forms have been shown to be very important in the development and homeostasis of plants and animals, regulating many cellular functions such as differentiation and maintenance of stem cells (Bartel 2004; Wilson and Doudna 2013; Wang et al. 2017).

RNAi has also been harnessed to research and medical applications (Rossbach 2010). RNAi can be achieved with many types of sncRNAs e.g. artificial short interfering (si)RNAs to control expression of any gene despite of whether it is regulated by endogenous sncRNAs or not. For example, many siRNAs are being studied as therapeutics for central nervous system disorders, although are still in early stages of development (Boudreau and Davidson 2010). However, a siRNA-based therapeutic, patisiran, targeting transthyretin (TTR) for the treatment of hereditary ATTR amyloidosis has already undergone Phase III trials and may be the first siRNA-based therapeutic to reach the market (Adams et al. 2017). In

addition, siRNA-mediated RNAi screens are a useful tool to screen for drugs, find out functions of genes and to figure out signaling pathways (Ghosh et al. 2017).

2.1.1 RNAi mechanism

RNAi functions by sncRNAs which interfere with messenger (m)RNAs. The biogenesis of endogenous miRNAs involves many steps before interfering can occur (Figure 1). First, the miRNA is transcribed, either mono- or polycistronically, from the genome, by polymerase (pol) II, or in some cases by pol III, as a capped and polyadenylated (pA) pri-miRNA form (Cai et al. 2004; Lee et al. 2004; Borchert et al. 2006). The pri-miRNA is processed in the nucleus by the Drosha/DGCR8 (DiGeorge syndrome critical region 8) microprocessor complex to a 70 nt pre-miRNA hairpin structure (Lee et al. 2003; Gregory et al. 2004). Subsequently, the pre-miRNA is transported by the exportin-5/RanGTP (exp5) pore complex to the cytoplasm where it is processed by Dicer to produce a mature miRNA consisting of a passenger strand and a guide strand (Bernstein et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Zhang et al. 2002; Yi et al. 2003; Lund et al. 2004). The passenger strand is, in most cases, degraded and the guide strand is required for the actual interfering. Next, the guide strand is incorporated into a RNA-induced silencing complex (RISC) composed of argonaute proteins capable of cleaving RNA (Hammond et al. 2000; Liu et al. 2004; Gregory et al. 2005; Haase et al. 2005; Fabian et al. 2009). Loading of the guide strand is mediated by the RISC loading complex (RLC) which is composed of Dicer, argonaute 2 (Ago2) and the human immunodeficiency virus transactivating response RNA-binding protein (TRBP). RISC, with the loaded miRNA (miRISC), then proceeds to deadenylate, repress the translation of, or degrade mRNAs, by targeting mainly the complementary 3'UTRs of mRNAs. Blocking translation can be achieved similarly through various sncRNAs or miRNA mimicking intermediate RNAs, *i.e.* artificial shRNAs and siRNAs, which will be covered in section 2.2 of this thesis.

Despite that the biogenesis and production of miRNAs is very well characterized, elimination and disposal of miRNAs is not as thoroughly studied. MiRNA turnover is regulated, in part, by the Translin/Trax complex which competes with pre-miRNA processing, and degrades pre-miRNA instead of producing mature miRNAs (Taira et al.

1998; Chennathukuzhi et al. 2001; Asada et al. 2014). MiRNA repression can also be regulated by other RNA-binding proteins (RBP), such as Lin28, which uridylates let-7 family pre-miRNAs and certain other miRNAs, such as the brain-specific miR-9, preventing their processing by Dicer. In contrast, other RBPs may assist with processing, such as protein activator of PKR (PACT), which assists Dicer similarly to TRBP (Lee et al. 2006). Additionally, there is some evidence that PCBP2, a poly(C)-binding RBP, may promote pre-miRNA processing by catalysing their interaction with Dicer (Li et al. 2012)

Even though miRNA-mediated RNAi is one of the most studied RNAi pathways, it is important to note that not all RNAi are processed in this manner. For example, there are also non-canonical pathways of RNAi which bypass Dicer cleavage, e.g. using Ago2 to execute silencing by AgoshRNA complexes (Harwig et al. 2017).

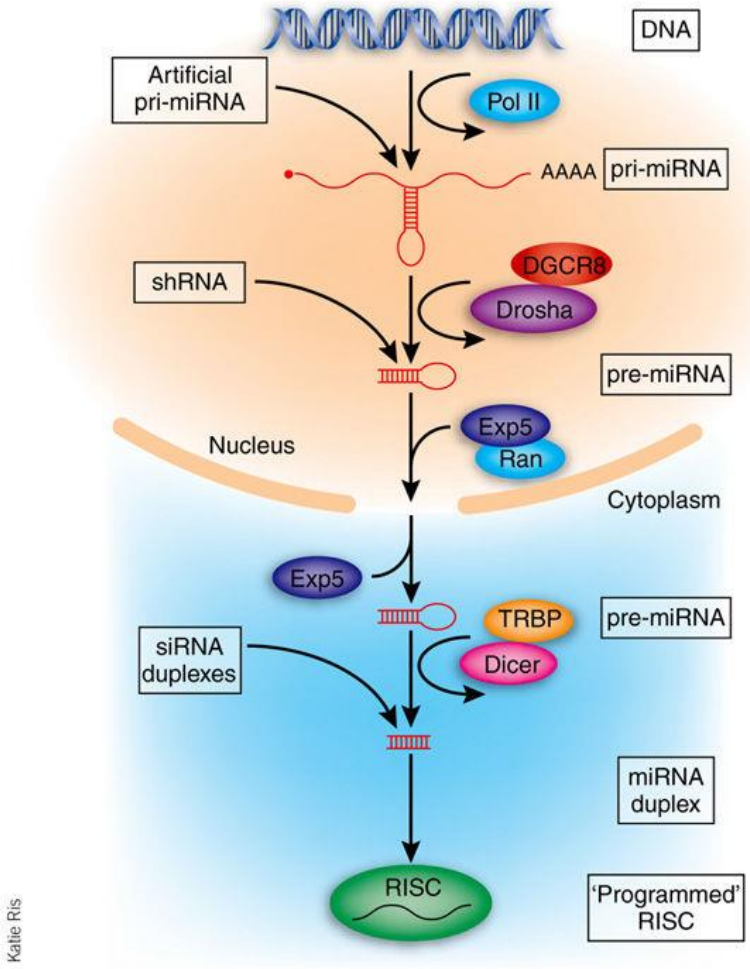

Figure 1. microRNA and short interfering RNA biogenesis from the genome or an artificial pri-miRNA, or their intermediates, respectively. Pri-miRNA, artificial or the natural, which

is transcribed from the genome, gets processed by Drosha/DGCR8 complex into a pre-miRNA/shRNA which is transported by the exp5/Ran pore complex into the cytosol where it is processed by Dicer into a mature miRNA/sirRNA that gets loaded into RISC to perform complementary mediated silencing. (Cullen 2006). DGCR8 = DiGeorge syndrome critical region 8 Exp5 = exportin 5, miRNA = microRNA, pre = precursor, pri = primary, Ran = Ras-related nuclear protein, RISC = RNA-induced silencing complex, shRNA = short hairpin RNA, siRNA = short interfering RNA, TRBP = human immunodeficiency virus transactivating response RNA-binding protein.

2.1.2 RNAi significance

In mammals, RNAi is involved in various cellular functions, mostly silencing genes through endogenous siRNA, miRNA and piwi-interacting-(pi)RNA pathways regulating many biological processes, such as stem cell maintenance and differentiation, embryogenesis and heterochromatin formation (Carmell and Hannon 2004; Cullen 2006; Wilson and Doudna 2013; Wang et al. 2017). Although its role in immune responses is clear in plants and invertebrates, it has not been very well characterized in mammals. However, recent studies suggest that in addition to the aforementioned processes, RNAi may have a role in mammalian immunity and is induced and suppressed by human viruses (Qiu et al. 2017).

2.1.3 Kinetics and stoichiometry of RNAi in mammals

Modelling RNAi kinetics mathematically and experimentally has been done to some extent. For example, a study published in 2011 by Cucatto et al. presents four different models to measure dsRNA produced siRNA-mediated RNAi kinetics in mammalian cells (Figure 2). One proposed model (model 3 on Figure 2) takes into account the rate of mRNA-siRNA complex formation (k_3), the cleavage and dissociation rate of the complex (c_3) and the number of siRNA target sites (h_3). The other proposed model (model 4 on Figure 2) considers the changes (δ) in mRNA concentration (X_m) and siRNA concentration (X_s) which factors the maximal degradation rate of mRNA due to RNAi, has a Michaelis-Menten like constant and the number of siRNA target sites (h_4). The authors also performed real-world experiments to see which one would be most precise in modelling

interference and found out that the fourth, a phenomenological, Hill-kinetic model was the most efficient way to model siRNA-mediated RNAi.

Regarding miRNA kinetics, in another study form the same year by Mukherji et al., and further adapted by Lemus-Diaz et al. in 2017, the proposed model considers targeted mRNA transcription rate, its decay rate, forming miRNA-mRNA complexes and free mRNA interaction with miRNAs. The model assumes the concentration of miRNA to be constant, and also takes into account the number of binding sites. These models show some of the differences, mainly in complex formation and in assumptions of concentration shifts, in modelling the kinetics of siRNA- and miRNA-based mRNA silencing. The best way to model RNAi quantitatively, regardless of the interference-mediating RNA type, is still very much under debate.

Furthermore, what complicates matters in terms of miRNA kinetics, is that the exact ratios of the outcomes miRNAs exert to mRNA — deadenylation, repression and degradation — are not known and depends on the complementarity of the sequences. However, it has been shown that translational repression outweighs the degradation in new mRNA synthesis, but at a steady state, deadenylation followed by degradation is more prominent (Bethune et al. 2012). This is important when considering situations, where endogenous gene expression is enhanced, and/or an exogenous gene is introduced to the cell.

In a study specifically focusing on Dicer kinetics, it was shown that mammalian Dicer cleaves pre-miRNA duplexes more than 100 times faster than long dsRNA under multiple turnover conditions (Chakravarthy et al. 2010).

	Model 3
$\delta(X_m, X_s) = \frac{c_3 k_3 h_3 X_s}{c_3 + k_3 h_3 X_m} X_m$	k_3: Rate of mRNA-siRNA* complex formation c_3: Cleavage and dissociation rate of mRNA-siRNA* h_3: Number of siRNA target sites
	Model 4
$\delta(X_m, X_s) = d_4 \frac{X_s^{h_4}}{\theta_4^{h_4} + X_s^{h_4}} X_m$	d_4: Maximal degradation rate of the mRNA due to RNAi θ_4: Michaelis-Menten like constant h_4: Number of siRNA target sites

Figure 2. Two different mathematical models of siRNA-mediated RNAi kinetics. Model 3 takes into account rate of mRNA-siRNA complex formation (k_3), the cleavage and dissociation rate of the complex (c_3) and the number of siRNA target sites (h_3). Model 4 takes into account the maximal degradation rate of mRNA due to RNAi, has a Michaelis-Menten like constant and the number of siRNA target sites (h_4). (Adapted from Cuccato et al. 2011). X_m = mRNA concentration, X_s = siRNA concentration.

2.2 Short non-coding RNAs

The human genome is regulated by many non-coding RNAs, such as long (>200 nt) non-coding RNAs e.g. lincRNAs and circular RNAs, and various sncRNAs (<30 nt) e.g. the aforementioned miRNAs and small neucleolar RNAs such as small Cajal body-specific RNAs (Wang et al. 2017). The landscape of non-coding RNAs is vast, and therefore this thesis covers only those which are processed or associated with Dicer, namely miRNAs and siRNAs, or are in some other way relevant to the work (piRNAs). Even so, Dicer can bind certain small neucleolar RNAs as substrates, for example the human small Cajal body-specific RNA ACA45, acting like miRNAs (Ender et al. 2008).

Generally, sncRNAs can be studied by using genome-wide bioinformatic screening and/or microarray analysis in combination with experimental data to validate predictions (Krutzfeldt et al. 2005; Lim et al. 2005; Minones-Moyano et al. 2011; Jvsinek Skok et al. 2013). Exeperimental data can be acquired, using for example sncRNA agonists (e.g. antagomiRs), or antisense morpholinos followed by quantitative polymerase chain reaction (PCR) for assessing effects of specific sncRNAs on levels of their predicted targets.

2.2.1 MicroRNAs and piwi-interacting RNAs

MiRNAs are abundant in mammals, and there are over 5000 miRNAs in the human genome, of which at least 50% are specific to humans (Londin et al. 2015). A single miRNA can regulate hundreds of genes and conversely a single gene can be regulated by hundreds of miRNAs (Krek et al. 2005; Lim et al. 2005). This is partly due to miRNAs being, in most cases, only partially complementary to mRNAs and therefore most miRNA's effects are subtle and don't completely knockdown the expression of a gene, but rather fine tune its expression (Lim et al. 2005; Mukherji et al. 2011). Expression can thus be further downregulated by compounding miRNAs. However, miRNAs may additionally regulate each other, making the fine tuning process even more sophisticated (Lai et al. 2004). Moreover, high-throughput screening (HTS) of miRNA function has revealed that many miRNAs are not active, and only abundant miRNAs repress their target(s) adding to the

complexity of their nature of regulators of the proteome (Mullokandov et al. 2012). Although a singular miRNA's effects may be weak, it is a *non-sequitur* to assume that it is only due to the binding of the miRNA itself, since it may also be dependent on their expression. Many experiments have shown that overexpression of individual miRNAs can have profound effects if they target the right genes in the right setting. For example, overexpression of miR-185 in and *in vitro* model of PD suppresses autophagy and apoptosis, showing that singular miRNAs may be useful therapeutics or research tools (Wen et al. 2018).

MiRNAs are predicted to affect the expression of more than half of all the protein-coding genes in the human genome (Lewis et al. 2005). Thus, one overarching function of miRNAs seems to be that they control protein expression noise (Schmiedel et al. 2015). Another, further adding to their multi-faceted regulatory roles, is the seemingly contradictory property that they do not just repress and degrade mRNAs regulating only translation, but may in fact (up)regulate translation, at least during cell cycle arrest (Vasudevan et al. 2007). This may be mediated by binding to the 5'UTRs, and thus, although main binding sites of miRNAs are 3'UTRs, they can also bind on the coding-region of transcript, possibly regulating splicing, adding yet another layer of regulation in the functionality of miRNAs (Lewis et al. 2005; Ellwanger et al. 2011). It has also been discovered, that there are plenty of brain-specific miRNAs which are crucial for higher cognitive functions and learning, regulating translation locally in the synapses, for example by stabilizing a transcript and thus increasing its half-life (Schratt 2009).

When pri-miRNAs are transcribed they usually fold into a hairpin structure composing of a stem containing the mature miRNA guide strand and a loop structure which then adjoins the complimentary stem strand, including the passenger strand of the miRNA (Figure 3). The selection of passenger strand is not very clear and either -5´ or -3´ can be mature, depending on the particular miRNA. Pri-miRNAs additionally contain mismatches in their stems, and wobble pairs. MiRNAs can also be differently processed (miRNA biogenesis is covered in section 2.1.1) by Drosha or Dicer due to varying cleavage sites to form isomiRs (Morin et al. 2008). They may additionally be edited by adenosine deaminase enzymes (Yang et al. 2006). MiRNAs don't require full complementarity of strands to exert their function and only the seed region (6-8 nt) is enough to recognize their target (Lewis et al. 2005; Ellwanger et al. 2011). Besides the Translin/Trax mediated degradation of pre-

miRNAs, the levels of miRNAs may also be controlled for example by circular RNAs which can act as sponges for certain miRNAs suppressing their activity, and possibly acting as stabilizers (Hansen et al. 2013).

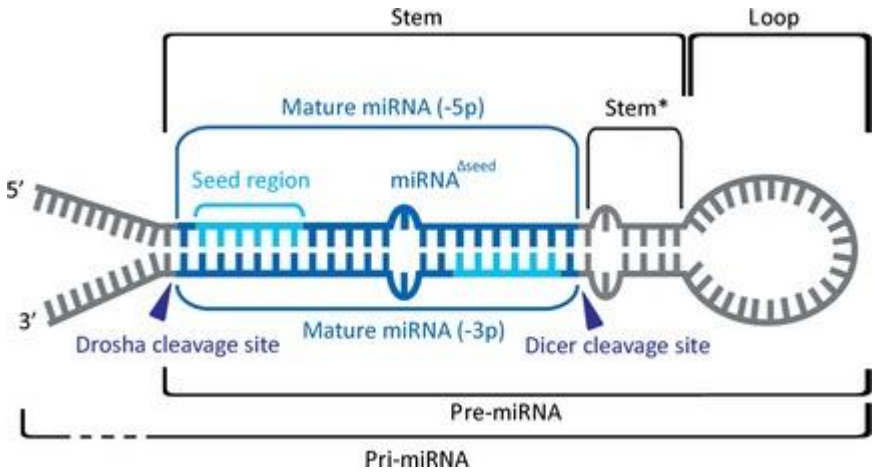

Figure 3. Structure of a primary microRNA. It is composed of -5´ capped and -3´ polyadenylated ends folding into a hairpin structure with the mature miRNA strand in the stem. The stem may contain mismatches and wobble pairs. Drosha cleaves off the cap and polyA ends and Dicer cleaves off the loop (Jevsinek Skok et al. 2013). miRNA = microRNA, pre / pri = primary / precursor.

PiRNAs are 24–32 nt long endogenous sncRNAs (Ross et al. 2014; Weick and Miska 2014). The etymology behind piRNAs derives from the finding that they interact with the PIWI (P-element-induced wimpy testis) subfamily of Ago proteins. They are best known for regulating transposable elements in germline cells. More specifically, piRNAs target transposons and cleave them in complexes with PIWI-proteins. The cleavage may generate additional piRNAs cleaving additional transposons, thus keeping transposon at bay from making detrimental mutations to the genome. Additionally, they are important regulators of chromatin. Recently the function of piRNAs in somatic cells has been further elucidated and they may have important functions in gene regulation and various biological roles, for example in memory formation and cancer (Ross et al. 2014; Abell et al. 2017).

2.2.2 Short interfering RNAs

RNAi can also be achieved with classes of artificial RNA which mimic an intermediate step in the RNAi pathway biogenesis (Cullen 2006). These include siRNA duplexes and ss siRNAs incorporated into pri/pre-miRNA backbones, or shRNAs.

The function of siRNAs, as well as miRNAs, was discovered around two decades ago (Elbashir et al. 2001). They are 20-25 nt in length and can efficiently silence genes in mammalian cells. This is because, in contrast to miRNAs, they mostly degrade mRNA instead of deadenylating or repressing it. In particular, the reason behind this lies in differences between the complementarity of siRNA/miRNA and mRNA sequences (Piatek and Werner 2014). Another significant difference of endogenous miRNAs and siRNAs is the biogenesis, miRNAs are transcribed as capped and polyadenylated pri-miRNA transcripts, siRNAs are usually transcribed as part of various length non-hairpin dsRNAs (Cai et al. 2004; Yuan et al. 2006; Piatek and Werner 2014). In mammals, endogenous siRNAs are not as common as miRNAs and piRNAs due to evolutionary divergence in these different sncRNA classes.

Artificial siRNAs, however, are widely used in biotechnological or medical applications where efficient silencing and high target specificity are desired and thus they are designed to have perfect, or close to perfect, complementarity to target sequence (Boudreau and Davidson 2010; Rossbach 2010). SiRNAs can be designed using various web-based tools optimising for specificity and knockdown efficiency. Besides used as ss siRNA strands, siRNAs can be expressed as duplexes or in a pri/pre-miRNA or shRNA backbone. Even though artificial shRNAs are usually expressed by pol III promoters, such as the U6 promoter, they can also be designed to be transcribed by pol II (Yuan et al. 2006). MiRNAs, as pri-miRNAs, are designed to be expressed usually with pol II promoters such as cytomegalovirus (CMV) promoter.

2.3 Dicer: a central enzyme in the miRNA biogenesis pathway

Dicer, encoded in humans by the *DICER1* gene, is a multidomain RNA-binding protein (Foulkes et al. 2014). It belongs to the family of type III RNase enzymes (Carmell and Hannon 2004). Type III RNase enzymes are categorized into three groups according to their structure. The first group includes the *Escherichia coli* (*E. coli*) RNase III, the second Drosha, and the third Dicer. The common feature of RNase III enzymes is their specificity towards dsRNA.

Many factors may regulate Dicer levels (Figure 7), but the precise mechanisms and transcription factors regulating *DICER1* transcription are poorly known. However, at least in adult T-cell leukemia cells, Dicer transcription is regulated by two AP-1 transcription factors, c-Jun and JunD (Gazon et al. 2016).

As the significance of sncRNAs processed by Dicer was earlier established, it is conceivable that levels of Dicer are tightly regulated e.g. in development. Alas, *Dicer1* is regulated by let-7a, which as a miRNA forms a negative feedback loop on the regulation of all miRNAs by downregulating Dicer (Mayr and Bartel 2009). In general, factors which affect the lin28/let-7 axis affect Dicer, as has been demonstrated by the rapid elevation in Dicer levels and inducing of lin28 by brain-derived neurotrophic factor (BDNF) (Huang et al. 2012). Moreover, the levels of Dicer and its transcript do not always correlate, suggesting additional regulation. For example, treatment with a histone deacetylase inhibitor, trichostatin A, downregulates Dicer protein levels but not the transcript (Wiesen and Tomasi 2009). However, valproate, another histone deacetylase inhibitor, did not reduce Dicer levels, and was, in contrast, shown in another study to rescue low Dicer expression (Gazon et al. 2016)

2.3.1 Structure, localization and function of Dicer

Dicer's main function in RNAi is the cleaving of pre-miRNAs into mature miRNAs. More specifically, Dicer cleaves dsRNA and pre-miRNA hairpin structures into short siRNAs or miRNAs (Hutvagner et al. 2001; Ketting et al. 2001). As a part of the RLC, it helps with loading the processed single-stranded (ss)RNAs into RISC (Figure 4).

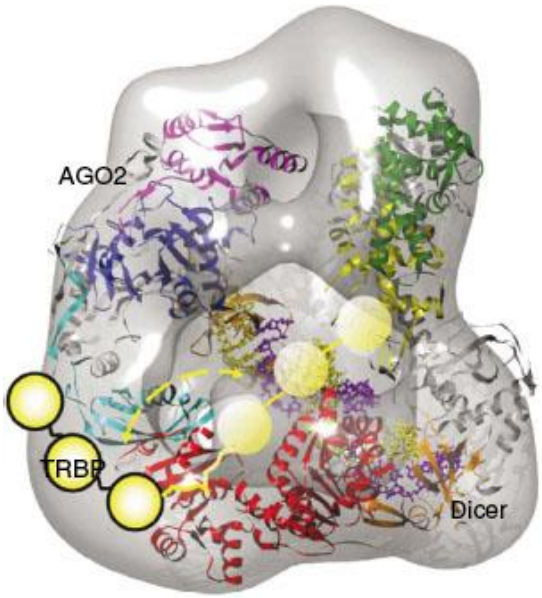

Figure 4. Three dimensional density map of a reconstituted RISC-loading complex from single particle image analysis and cryo electron microscopy data of the DE×H/D domain (red ribbon), the *Giardia* Dicer atomic model (gray-yellow-green-orange ribbon) and the *Thermus thermophilus* Argonaute (gray-cyan-orange-pink-blue). TRBP is presented as a string of three yellow spheres with a linker connecting it to the DE×H/D domain of Dicer. It is hypothesized that the flexibility of TRBP enhances transfer efficiency and selection of correct strands. (Wang et al. 2009). AGO2 = Argonaute2, TRBP = the human immunodeficiency virus transactivating response RNA-binding protein.

The crystal structure of *Giardia intestinalis* Dicer has been determined, and a three-dimensional model of human Dicer based on electron microscopy (Figure 5). The molecular weight of *G. intestinalis* Dicer is 82 kilodaltons (kDa) while the human Dicer is 219 kDa (Macrae et al. 2006; Lau et al. 2012). Human Dicer is composed of different functional subunits: a platform domain, a PAZ-domain (Piwi-Argonaute-Zwille), an amino (N)-terminal helicase (homologous to ATPase/DExD helixes) domain, a DUF283 (domain of unknown function) domain, a dsRNA-binding domain and two conserved RNase III domains, IIIa and IIIb (Carmell and Hannon 2004; Macrae et al. 2006; Wang et al. 2009; Park et al. 2011). The *Giardia* Dicer lacks the helicase and DUF283 domains, but is quite similar in structure regarding the platform-, PAZ- and RNase III domains (Macrae et al. 2006; Lau et al. 2012). The PAZ-domain binds the ends of pre-miRNAs, adjusting the pre-miRNA to be cleaved by the RNase domains thus acting as a 22 nt molecular ruler between these domains (Lau et al. 2009; Wang et al. 2009; Lau et al. 2012). Dicer cleavage is magnesium-dependent and human Dicer does not require adenosine triphosphate

compared to e.g. *Drosophila* Dicer (Zhang et al. 2002). Dicer's function is assisted by additional RBPs, mainly the two already mentioned, TRBP and PACT (Lee et al. 2006). Dicer binding to TRBP and PACT is mediated by the helicase domain (MacRae et al. 2008; Lau et al. 2009). The helicase domain is additionally important for repressing catalysis by attenuating the rate of dsRNA cleavage, and mutations and/or deletion of the helicase domain in human Dicer increases cleaving activity of non-hairpin dsRNAs up to 65-fold compared to the intact enzyme (Ma et al. 2008).

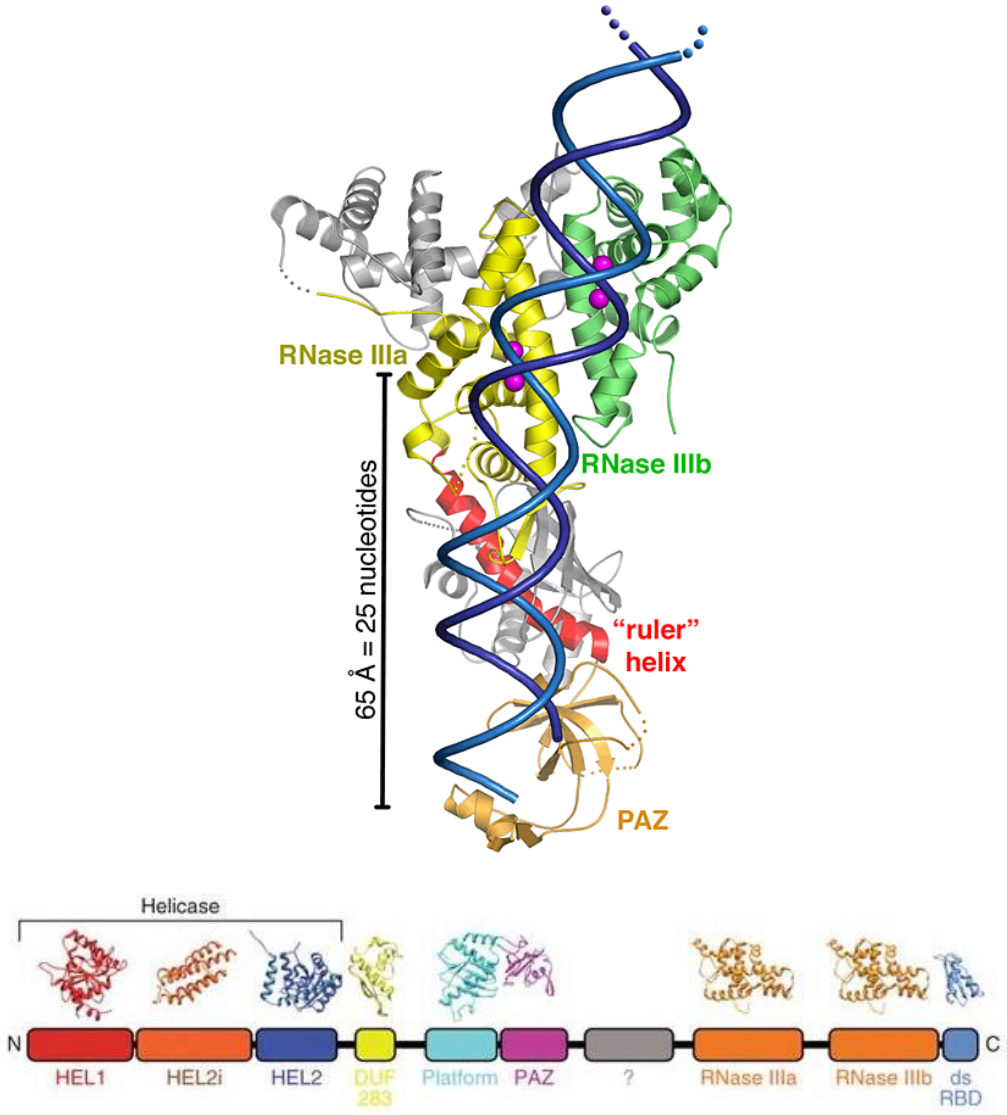

Figure 5. Above is a crystal structure of eukaryotic Dicer from *Giardia intenstinalis*. The structure is about 82 kDA and is composed of two type III RNase domains, a platform (helix) domain and a PAZ-domain. The distance (65 Å) between the PAZ-domain and the RNAse domains is perfectly suited for positioning the dsRNA to be correctly cleaved (Macrae et al. 2006). Below is a schematic sequence annotating the different domains of human Dicer. The main difference to *Giardia* Dicer is the additional helicase and DUF283

domains (Lau et al. 2012). dsRBD = double-stranded RNA-binding domain, DUF283 = domain of unknown function, PAZ = Piwi-Argonaute-Zwille, Å = Ångström.

In addition, recent studies suggest that the helicase of *Drosophila* Dicer may be involved in antiviral responses, binding viral dsRNA differently than endogenous dsRNA (Sinha et al. 2017). Indeed, Dicer's role in viral defence has also been demonstrated in mammalian cells (Machitani et al. 2016).

Dicer can also bind ssRNAs. Although the function of the DUF283 domain is still quite unknown, as the name states, recent studies have shown that the DUF283 domain is responsible for binding ssRNA, acting as an annealer of strands enhancing base-pairing and may facilitate the hybridization of sncRNAs and their targets (Kurzynska-Kokorniak et al. 2016).

Dicer processes pre-miRNAs in the cytosol, and thus its steady-state localization is cytoplasmic (Doyle et al. 2013). However, Dicer can also shuttle to the nucleus and its C-terminal dsRNA-binding domain acts as a nuclear localization sequence. This is further supported by the finding that Dicer is involved in chromatin remodelling in mammals, which additionally links RNAi regulation of heterochromatin to Dicer (Sinkkonen et al. 2010).

2.3.2 Significance of Dicer in diseases

As Dicer is highly important in many cellular processes, it is not surprising that Dicer deficiencies have been associated with many diseases. Global miRNA loss promotes tumorigenesis and *DICER1* dysregulation, mutations or even hemizygous deletion is found in a number of tumours (Kumar et al. 2007; Foulkes et al. 2014; Jiang et al. 2015). In contrast, upregulation of Dicer is found in certain tumours (Chiosea et al. 2006). *DICER1* deficit can cause retinal cell degeneration in an advanced form of age-related macular degeneration in humans, and conditional ablation of Dicer, but not seven other miRNA-processing enzymes, causes degeneration of the same cells in mice (Kaneko et al. 2011). Dicer is additionally downregulated in aged tissue, such as adipocytes (Mori et al. 2012).

Dicer dysfunction or downregulation are also apparent in many central nervous system related disorders and diseases. Decreased *DICER1* in the blood has been detected in psychiatric conditions e.g. post-traumatic stress disorder and depression patients (Wingo et al. 2015). Particularly neurodegenerative diseases have been linked to Dicer deficiencies. For example, Dicer ablation in oligodendrocytes promotes neuronal degeneration in mice and Dicer is downregulated in the blood of multiple sclerosis patients (Shin et al. 2009; Magner et al. 2016). Moreover, miRNAs are downregulated in motor neurons of ALS patients and Dicer stimulation treatment delays the onset of symptoms in mouse ALS models (Emde et al. 2015). Additionally, *DICER1* was found to be downregulated in an mRNA profiling on *post-mortem* laser-microdissected dopaminergic neurons of PD patients (Simunovic et al. 2010). Furthermore, at least in two separate studies, conditional Dicer knockout (CKO) mice have been shown to cause severe nigrostriatal dopaminergic cell loss, demonstrating the importance of Dicer for the survival of these dopaminergic neurons (Pang et al. 2014; Chmielarz et al. 2017). The 2017 study by Chmielarz et al., also demonstrated that 87-weeks old mice show a significant reduction in *Dicer1* in the ventral midbrain compared to 6.5-weeks old mice (Figure 6).

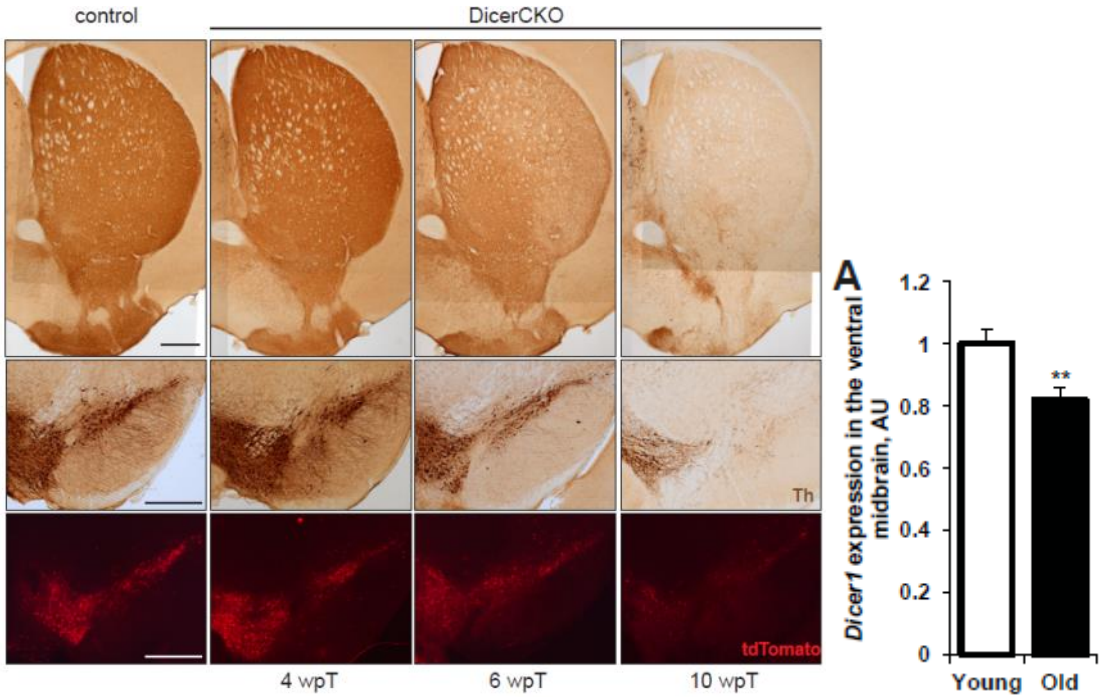

Figure 6. Immunohistochemical analysis of conditional Dicer knockout mice. Dicer knockout induced with tamoxifen in adult dopaminergic neurons causes nigral TH+ cell

death and loss of striatal innervation. A) *Dicer1* mRNA levels in the ventral midbrain of 6.5-weeks old (young) and 87-weeks old (old) mice (Chmielarz et al. 2017). TH = tyrosine hydroxylase, tdTomato = tandem dimer Tomato.

Dicer also seems to be critically involved in stress responses and, in turn, is subject to stress conditions itself. Certain cellular stressors such as reactive oxygen species, hypoxia and ultraviolet light, may downregulate Dicer (Figure 7) (Wiesen and Tomasi 2009). In contrast, Dicer protein levels are elevated in response to mild hyperthermia (Devasthanam and Tomasi 2017). Beta-catenin, which is implicated in stress-related psychiatric disorders, has been shown to interact with Dicer and mediate pro-resilient effects (Dias et al. 2014).

2.3.3 Small-molecule modulators of Dicer

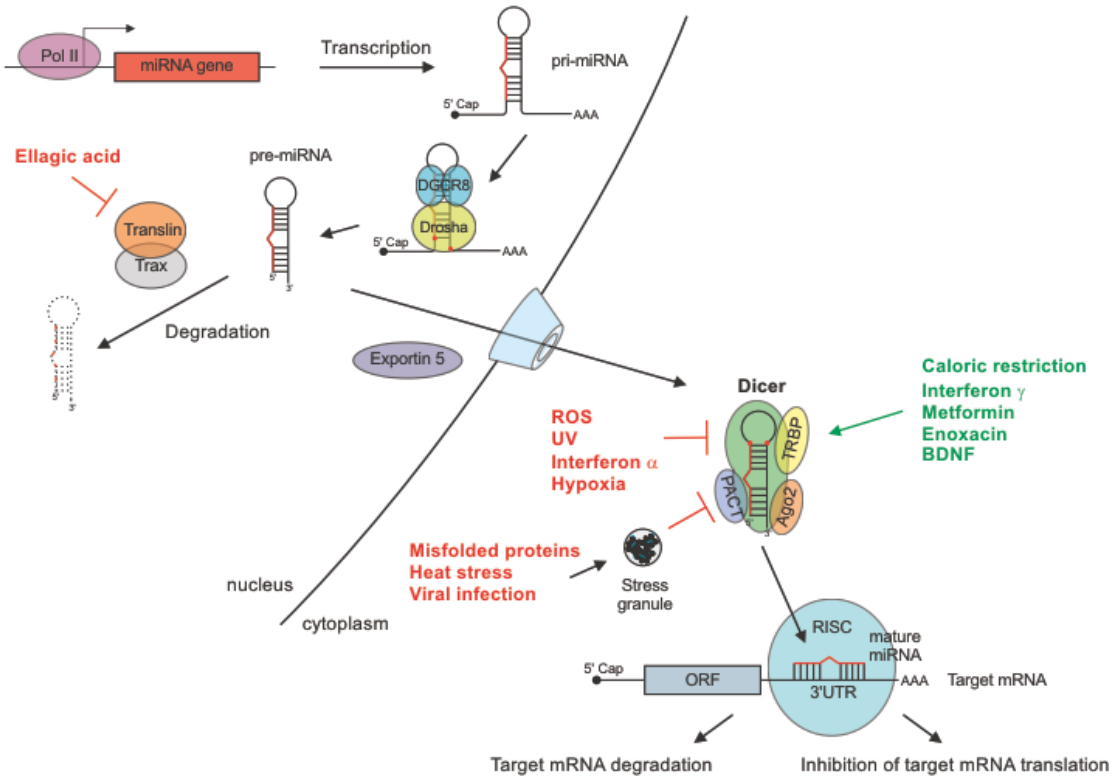

Figure 7. Factors affecting RNAi. Factors which enhance Dicer are marked green, such as enoxacin and metformin, and factors which inhibit RNAi or Dicer are marked red such as ellagic acid and ultraviolet light. Detailed miRNA biogenesis is covered in section 2.1.1. (Vinnikov and Domanskyi 2017). BDNF = Brain-derived neurotrophic factor, ORF = open

reading frame, RISC = RNA-induced silencing complex, ROS = reactive oxygen species, 3'UTR = untranslated region of the -OH end of and RNA, UV = ultraviolet.

There are many factors and compounds shown to affect RNAi and a few known compounds which modulate Dicer activity (Figure 7). A good example of an RNAi enhancing compound is Ellagic acid. It is a natural compound found in many fruits which has antioxidative and pro-apopotic effects and was recently found to rescue miRNA biogenesis through inhibiting the pre-miRNA degrading complex, Translin/Trax (Salimi et al. 2015; Asada et al. 2016). RNAi inhibitors on the other hand, such as polylysine and trypaflavine, can function by suppressing miRISC activity (Watashi et al. 2010). However, this sub-section will focus on small-molecule compounds which have a clear association with Dicer activity.

One of the best studied small-molecule compounds which activates Dicer is the fluoroquinolone antibiotic enoxacin (Figure 8). Enoxacin is a bacterial DNA gyrase and topoisomerase IV inhibitor, which was one of the main reasons it was chosen to be studied for its RNAi enhancing abilities, since inhibiting RNA helicases may stabilize dsRNAs and thus enhance RNAi (Zhang et al. 2008). Enoxacin activates Dicer indirectly through enhancing the interaction with TRBP (Shan et al. 2008). Enoxacin has neuroprotective effects on midbrain dopaminergic neurons and protects them against endoplasmic reticulum (ER) stress (Chmielarz et al. 2017). Additionally, it corrects ALS-associated miRNA-defects *in vitro* and is beneficial for neuromuscular function in mouse ALS models (Emde et al. 2015). Furthermore, an orphan designation for enoxacin in the treatment of ALS was granted by the European Medicines Agency (Public summary of opinion on orphan designation EMA/COMP/125722/2015). Enoxacin also prevents learned helplessness in the rat supporting the hypothesis that the correction of miRNA dysregulation by activating Dicer could be beneficial in treating psychiatric disorders (Smalheiser et al. 2014).

Enoxacin has many targets and activates Dicer at relatively high concentrations compared to its antibiotic activity, so it is prone to causing many unwanted side effects reducing its usability as an RNAi enhancer. These side effects include not only convulsions, but enoxacin may also affect bone formation by inhibiting c-Jun-N-terminal kinase (JNK) signaling, for example (Delon et al. 1999; Liu et al. 2014). Furthermore, due to its antibiotic

activity, throwing the microbiome out of balance can have many secondary adverse effects such as possibly causing neurodegeneration, as a dysfunctional microbiome in the gut has been linked to PD (Sampson et al. 2016).

Metal chelators, mainly pyridine-based ligands and some salen ligands, can also significantly enhance RNAi (Li et al. 2012). Metal chelating compounds which enhance RNAi include deferoxamine mesylate, thiosemicarbazide and 2,2′-dipyridyl, for example. In the same study, the authors showed that intracellular iron can inhibit RNAi and that cytosolic iron disrupts the interactions between PCBP2, pre-miRNAs and Dicer. Therefore, the effect of metal chelators in enhancing RNAi is mediated indirectly by promoting Dicer processing of pre-miRNAs.

Dicer is upregulated by a common diabetes drug, metformin, and is required for metformin's action in cellular senescence models (Hooten et al. 2016). Metformin is also protective in an animal model of PD, however whether this effect is mediated by metformin's ability to upregulate Dicer, is not known, since metformin, as enoxacin, is a polypharmacological compound with many effects (Bayliss et al. 2016).

Thapsigargin, a useful pharmacological research tool is a natural product produced by the plant *Thapsia garganica* and a known regulator of ER stress (Rogers et al. 1995). It acts by depleting ER calcium and there is some evidence showing it can inhibit Dicer (Emde et al. 2015).

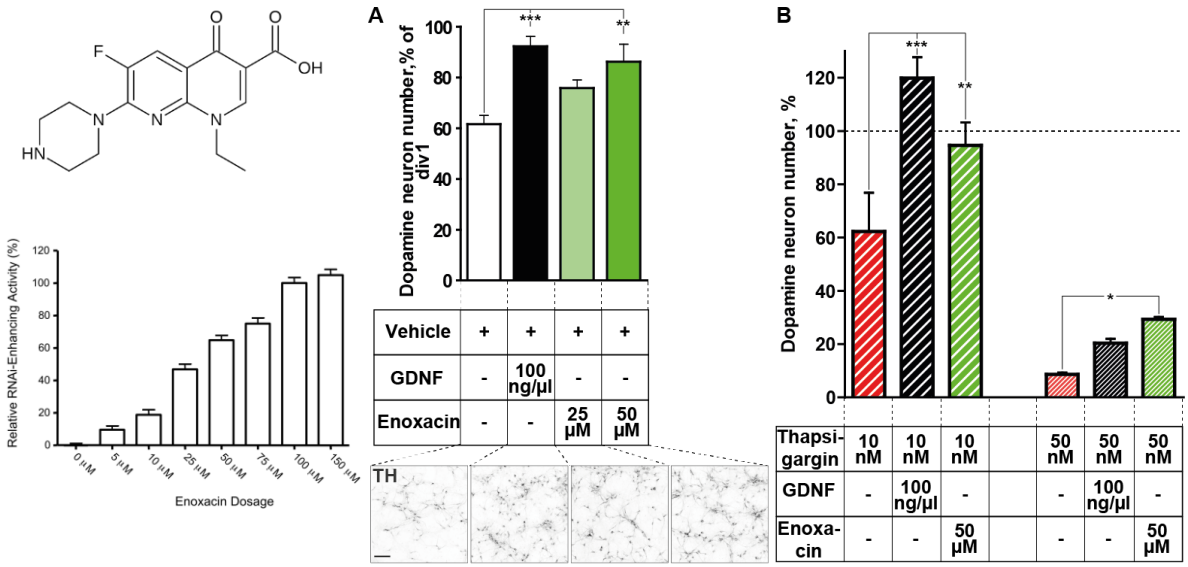

Figure 8. Structure of enoxacin, its relative RNAi enhancing activity between 0-150 µM concentrations and its protective effects in primary dopamine neuron cultures compared to GDNF only (A), which is neuroprotective to dopamine neurons, or rescuing from thapsigargin induced ER stress (B) (Shan et al. 2008; Chmielarz et al. 2017). GDNF = glial-derived neurotrophic factor, RNAi = RNA interference.

2.4 Screening assays for RNAi enhancement

Screening assays are usually enzyme- or cell-based but can also be done in whole-organisms e.g. *C. elegans*, *D. Melanogaster* or *D. Rerio* (Giacomotto and Segalat 2010; Podolska et al. 2014; Xiao et al. 2016). Screening assays can be done in different scales having different levels of throughput. The important things to consider in HTS assays are the use of reporter proteins, controls and internal normalization, how many replicates to use and the assay set-up in itself (Bray and Carpenter 2004). In an optimal assay, there is a clear negative and positive control, and the positive control is the same type as the screened reagents (small-molecule for a small-molecule screen *etc.*). Choosing the right number of replicates is very important to reduce false hits. Usually HTS is performed in duplicates and with subsequent confirmation assays for the hits but can be done with more replicates depending on the quality of the assay. For example, to confirm findings, a three-tiered approach was implemented in finding small molecule ER proteostasis regulators by first screening for

proteostasis activators, then counterscreening to remove global ER stressors and finally multiplex transcriptional profiling was done to find specific regulators (Plate et al. 2016).

The plate setup used for the screening should take, depending on the used plate, into account plate-based edge effects when planning the layout and orientation of screened compounds. That is, compounds and controls should be screened as randomly as possible and avoid using peripheral wells. This is very important also when choosing the reporter(s), as luminescence measurements are typically done in white plates to maximize emission and fluorescence in black plates to minimize background.

Both fluorescent and luminescent proteins have been successfully implemented in high-throughput screening and neither is generally better than the other, but depends on the nature of the assay. For instance, fluorescent proteins can be more toxic but, in contrast, more stable than luciferase proteins, which can be beneficial or detrimental depending on the purpose and conditions (time) of the assay (Fan and Wood 2007). However, in many studies both have been found comparable and engineering of better fluorescent/luminescent protein variants is solving issues related to both methods (Fan and Wood 2007; Lemus-Diaz et al. 2017; Shen et al. 2017)

HTS assay results are often analysed and displayed as % of the measured effect (inhibition/activation). Statistical parameter for quality control are used such as the Z-factor (Z´, number of standard deviations from the mean, see the formula in section 3.2.5 of materials and methods) and signal-to-noise (S/N) or signal-to-background ratio (S/B) (Bray and Carpenter 2004; Birmingham et al. 2009; Xiao et al. 2016). An assay with a Z-factor of 0.5-1 is considered an ideal and robust HTS assay, 0-0.5 is considered marginal but doable and if the assay scores under 0 it is not useful since there is not enough difference between the positive and negative controls. The Z-factor is extremely conservative due to high number of tests and screened compounds and may not always be appropriate for small-scale screening. Variability is measured usually by standard deviations and coefficient of variation (CV) (see section 3.2.5). A CV of less than 10 is usually considered as acceptable variation.

RNAi activity has been measured in enzyme- and cell-based assays (Chiu et al. 2005; Davies and Arenz 2006; Shan et al. 2008; Li et al. 2013; Podolska et al. 2014). The current assays are not optimal to effectively screen for Dicer-activating compounds because they either: 1)

measure Dicer activity *in vitro*, disregarding its natural microenvironment (Davies and Arenz 2006; Podolska et al. 2014), 2) measure the dsRNA or shRNA processing by Dicer instead of a pri-miRNA, which is more relevant to human disease biology than dsRNA/shRNA and the processing of these two classes is significantly different in terms of kinetics (Chiu et al. 2005; Chakravarthy et al. 2010; Li et al. 2012), 3) do not have a second reporter protein for normalisation to enhance sensitivity and rule out non-specific, and potentially toxic, effects (Shan et al. 2008) or 4) they are not optimal for high-throughput (HT) screening due to transient transfection of plasmids, which may additionally cause variability due to multiple copies of plasmid per cell, instead of stably transfected cells (Zhang et al. 2008; Shan et al. 2008). Additionally, in some cases quantification has to be done by measuring protein or mRNA levels. Therefore, there aren't any sensitive, internally normalized HT feasible screening assays available which take mammalian Dicer context into account.

2.4.1 Biochemical enzyme-based assays

Biochemical enzyme-based Dicer cleaving assays have been used to analyse Dicer function, but also to screen for Dicer-activating compounds. A Dicer cleavage assay was developed already a year after the characterization of Dicer, but this was a standard cleavage assay using radiolabelling and electrophoresis and thus is not suitable for HTS (Zhang et al. 2002). Davies and Arenz (2006) established the first homogenous fluorescence assay for Dicer by creating a double labelled pre-let-7 that has a fluorescence emitter and a quencher in the 5' and 3' ends of the pre-let-7 RNA respectively, to find Dicer inhibitors and/or pre-miRNA binding compounds. Almost a decade later, Podolska and colleagues (2014) revised *in vitro* fluorescence Dicer assays by creating a similar assay to Davies and Arenz, but instead of employing a pre-miRNA construct, they added the fluorescence emitters and quenchers on to a 27 nt RNA duplex. The reason was that Dicer cleaves non-hairpin RNA duplexes much slower than short hairpin RNAs (pre-miRNA) and therefore it would be even better for a HTS assay.

Although these assays do enable HT screening, they main issue is that they only measure Dicer binding *in vitro* ruling out Dicer's multi-faceted nature and functions in a cell which

may have compensatory effects. Besides this, they do not give information on other properties, such as cytotoxicity, or inhibition of growth, of the compounds. However, this is easily solvable with secondary screening for toxicity.

2.4.2 Cell-based assays

Fluorescent assays

A few notable cell-based assays for measuring RNAi and Dicer activity have been created and used to find compounds which inhibit or enhance RNAi. An assay developed by Chiu and colleagues (2005) works by transiently co-transfecting EGFP and RFP containing plasmids accompanied by a siRNA targeting EGFP into HeLa cell and measuring EGFP/RFP fluorescence in the presence of siRNA and normalized to the EGFP/RFP ratio of mock-treated cells. The system was able to identify RNAi inhibitors, but the combination of co-transfection of plasmids, siRNA and drugs may lead to too much variation and is not feasible to be used for high-throughput screening. Later, Shan et al. (2008) configured a live HTS applicable assay which was done by selecting HEK-293 clones stably expressing EGFP and short hairpin RNA targeting EGFP with modest knockdown to measure elevated and decreased EGPF levels, thus being able to screen for inhibitors and enhancers. With this assay, the authors found that enoxacin can activate Dicer. They also demonstrated that it not only enhanced processing of shRNA to siRNA but promoted loading of the siRNA into RISC. The authors also showed that enoxacin may promote processing of miRNAs by measuring increased levels of mature miRNA-125a and decreased levels of pri- and pre-miRNA-125a, but did not implement them to target EGFP and use them as a part of the assay.

The main problem is the fact that this assay only has one reporter protein and thus the reduction cannot be normalized to another factor, showing the specificity of the reduction and accounting for other influencing factors. However, they later developed a modified version of the assay by infecting the cells stably co-expressing EGFP and shEGP, with RFP to create a cell line with an additional reporter (Li et al. 2012). This was used to find the relations between metal chelating compounds, PCBP2, pre-miRNAs and Dicer.

This assay was the basis of the assay developed in the experimental part of this work, with the important difference being the processed construct to be more "physiological" and relevant for mammalian Dicer and miRNA dysregulations.

Bioluminescent assays

Enoxacin was identified by another group also in 2008 independently, using a dual luciferase approach (Zhang et al. 2008). They used a siRNA duplex containing a firefly luciferase targeting siRNA (siFL867-885). Another study later used a similar system, however, they had a shRNA targeting firefly luciferase (sh-Fluc). These systems have a good linear range, however, the main problem is, as mentioned, that these assays were done by transient transfection possibly causing variability due to different number of plasmids per cell and stable cell lines expressing these constructs were not generated.

3 EXPERIMENTAL PART

3.1 Aims of the study

The primary aim of this study was to design and develop a functional, sensitive, reliable and scalable cell-based fluorescent reporter assay to measure the pre-miRNA processing activity of Dicer. The secondary aims were to use the assay to screen for compounds which would enhance Dicer activity and optimise the assay for high-throughput screening and *in vivo* studies. To be more precise, the specific aims were (primary aims underlined):

1) Design the assay *in silico*
2) Clone the desired constructs
3) Validate the assay with Dicer modulating compounds in different cell lines
4) Optimise the assay for sensitivity if possible
5) Make stable cell lines, inducible and non-inducible for feasible screening

6) Make a lentiviral vector for transducing post-mitotic cells and to be used as an *in vivo* reporter
7) Screen for Dicer-activating compounds

3.2 Materials and methods

All cell lines, plasmids for cloning, kits, reagents and compounds used in this study are commercially available (Table 1 and 3). All PCR primers and other oligonucleotides were purchased from Metabion GmbH (Steinkirchen, Germany) (Table 2).

Table 1. Cell-lines, plasmids used as linearized vector backbones or templates for PCR and antibiotics for cell culture (incl. bacterial) used in the work. CHO = Chinese hamster ovarian cells, FLP-IN 293 T-Rex = FRT site engineered HEK293T cells, HEK293T = human embryonic kidney cells, SH-SY5Y = neuroblastoma cells

Cell lines	Plasmids	Antibiotics
CHO	psiCHECK2	Ampicillin
FLP-IN 293 T-Rex	pLenti-CRISPR-mCherry	Blasticidin S
HEK293T	pBlockIT-EmGFP	Hygromycin B
SH-SY5Y	pTO-HA-Strep-GW-FRT	Normocin™
	pCDH-CMV-T2A-GFP pCDH-hSYN-T2A-GFP	Spectinomycin
	pMDLg/pRRE	
	pRSV/REV	
	pMD2.G	

3.2.1 Molecular cloning

The reporter construct to measure Dicer activity was first designed *in silico* and a cloning strategy was done based on what genetic elements the final reporter plasmid would need to contain (Figure 9). More specifically, the reporter plasmid is an expression vector with psiCHECK2 (Promega) as the backbone, having *Amp*-resistance gene as the antibiotic selection marker, and is designed to express three main elements: 1) a primary fluorescent reporter protein, a modified red fluorescent protein known as modified Cherry (mCherry), which additionally has a nuclear localization sequence (NLS) and a regulatory element, the Woodchuck virus posttranscriptional regulatory element (WPRE), to stabilize and increase expression, in its mRNA transcript 2) a secondary fluorescent reporter protein, enhanced green fluorescent protein (EGFP) for normalization and 3) a siRNA designed to target the transcript of mCherry inserted into a murine pri-miR155 hairpin (Moreland et al. 1985; Reichel et al. 1996; Zhang et al. 1996; Ludtke et al. 1999; Zufferey et al. 1999; Klein et al. 2006). All of these three main elements have different pA signal sequences and different promoters which are optimal for mammalian gene expression (Gruss et al. 1981; Post et al. 1982; Reyes et al. 1982; Stringer 1985; Kobayashi et al. 1997). When the mCherry-targeting siRNA is expressed in a pri-miR155 hairpin, it must first be processed by the Drosha/DGCR8 microprocessor complex, and then by Dicer to knockdown mCherry. When mCherry, along with the mCherry/EGFP ratio, decreases, it gives an indication on Dicer processing. The hypothesis is that when Dicer is activated, the knockdown, and decrease of mCherry/EGFP ratio should be further decreased.

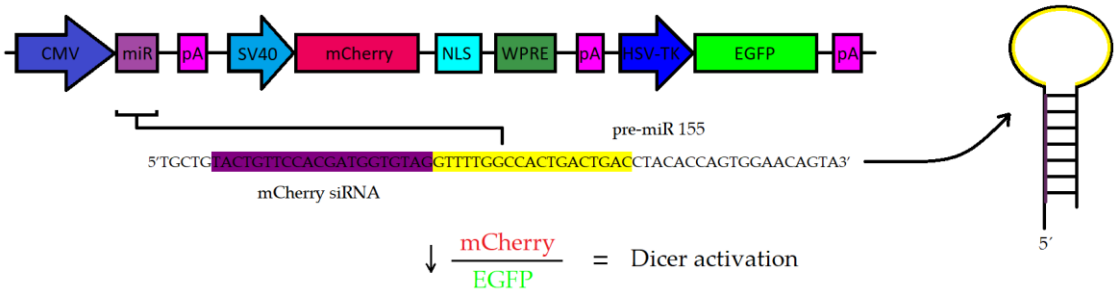

Figure 9. The designed construct which is the basis for the assay. The construct expresses two fluorescent reporter proteins, EGFP and mCherry, and a mCherry targeting siRNA in

a murine pre-miR155 backbone. They have different promoters and different pA sequences, and the mCherry transcript has a nuclear localization sequence (NLS) and is further stabilized by WPRE. A reduction in mCherry/EGFP ratio, due to mCherry silencing, indicates Dicer processing of pre-miR155. CMV = cytomegalovirus, EGFP = enhanced green fluorescent protein, HSV-TK = Herpes simplex virus thymidine kinase, mCherry = modified Cherry (red fluorescent protein), miRNA = microRNA, NLS = nuclear localization sequence, pA = polyadenylation, siRNA = short interfering RNA, SV40 = simian virus 40, WPRE = Woodchuck virus posttranscriptional regulatory element.

Cloning of the reporter plasmid

The backbone for the constructs, psiCHECK2 was digested with restriction enzymes *Xba*I and *Nhe*I and the 2768 bp fragment was purified by electrophoresis (100-120 volts, constant voltage) of the restriction reaction mixture in a 1% agarose gel, cutting out the linearized DNA containing band, and extracting the DNA form the gel by using a gel-extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. For all subsequent linearized fragments, or PCR amplicons, purifications were done in the same manner using the same extraction kit. pA-HSV-TK fragment was PCR amplified from psiCHECK2 using primers pA_for and HSVTK_rev with 15 nts overlapping on both ends, mCherry was amplified by PCR from pLenti-CRISPR-mCherry with primers Cherry_for and NLS_rev with 15 nts overlapping on the 5´ end conserving *Nhe*I restriction site, EGFP from pCDH-hSYN-T2A-GFP with EGFP_for and EGFP_rev with 15 nts overlapping on 3´end conserving *Xba*I restriction site (Table 2). All PCRs were performed with Phusion Hot-Start polymerase (ThermoFisher Scientific, Waltham, MA). All these fragments together with the *Xba*I/*Nhe*I-digested psiCHECK2 vector were recombined in a single In-Fusion (In-Fusion® HD Enzyme Premix, Clontech, Mountain View, USA) reaction for 15 min at 50°C (Irwin et al. 2012). Next, a BsmBI-MscI-BsmBI spacer, obtained by annealing (95°C for 4 min and the allowed to cool at RT for 10 min) the oligonucleotides 5´-TGCTTGAGACGTATGGCCATACGTCTCT-3´ and 5´-CCTGAGAGACGTATGGCCATACGTCTCA-3´, was ligated into pBlockIT vector to get pBlockIT-EmGFP-BMB which was then digested with *Dra*I and re-ligated to remove EmGFP. All ligations were done using T4 DNA ligase (ThermoFisher Scientific, Waltham, USA) incubating the mix for 60 min at RT. Then a CMV-miRNA-pA fragment was amplified from pBlockIT-BMB with CMV_for and pA_rev primers conserving *Bgl*II restriction site (Table 2). The amplified fragment was recombined with In-Fusion into *Bgl*II

digested psiCHECK-SV40-mCherry-NLS-pA-HSVTK-EGFP-pA to obtain psiCHECK-CMV-miRNA-pA-SV40-mCherry-NLS-pA-HSVTK-EGFP-pA.

The mCherry siRNA strands were designed with the Invitrogen BLOCK-iT™ RNAi Designer (https://rnaidesigner.thermofisher.com/rnaiexpress/) and two different siRNAs (highlighted by violet in the final full pre-miR155 strand containing the siRNA) were added by first annealing (95°C for 4 min and the allowed to cool at RT for 10 min) the top and bottom oligonucleotides, siRNA1 top = 5´TGCTGTTGATGTTGACGTTGTAGGCGGTTTTGGCCACTGACTGACCGC CTACAGTCAACATCAA3´ and bottom = 5´CCTGTTGATGTTGACTGTAGGCGGTCAGTCAGTGGCCAAAACCGCCTAC AACGTCAACATCAAC3´ siRNA2 top = 5´TGCTGTACTGTTCCACGATGGTGTAGGTTTTGGCCACTGACTGACCTAC ACCAGTGGAACAGTA3´ and bottom = 5´CCTGTACTGTTCCACTGGTGTAGGTCAGTCAGTGGCCAAAACCTACACC ATCGTGGAACAGTAC3´ and next, ligating them with digested (FastDigest-*Esp*3I (*Bsm*BI) for 1 hour at 37 °C) and gel purified psiCHECK-CMV-miRNA-pA-SV40-mCherry-NLS-pA-HSVTK-EGFP-pA.

WPRE was added to the constructs by amplifying it from pCDH-CMV-T2A-GFP with WPRE_for and WPRE_rev primers conserving *Pme*I restrictions site and recombining it with In-Fusion into *Pme*I digested psiCHECK-CMV-siRNA1-pA-SV40-mCherry-NLS-pA-HSVTK-EGFP-pA and psiCHECK-CMV-siRNA2-pA-SV40-mCherry-NLS-pA-HSVTK-EGFP-pA.

Table 2. Primers used for PCRs in cloning.

Name	Sequence
Cherry_for	5´-CTCACTATAGGCTAGCCACCATGGTGAGCAAGGGCGAGGAG-3´
CMV_for	5´-ATGGCTCGACAGATCGTACGCGTTGACATTGATTATTGACTAG-3´
EGFP_for	5´-GTAAAGCGCTCACCATGGTGAGCAAGGGCGAG-3´
EGFP_rev	5´-CCGCCCCGACTCTAGATTACTTGTACAGCTCGTCCATGC-3´
HSVTK_rev	5´-TGGTGAGCGCTTTACCAACAGTACCGGAATGC-3´
LV_for	5´-TTCAAAATTTTATCGATCGTACGCGTTGACATTGATTATTGAC-3´
LV_rev	5´-TCATTGGTCTTAAAGTATCGATTTTACCACATTTGTAGAGGTTTTAC-3´
NLS_rev	5´-TCAGTCTAGTTTAACGCGTTTGG-3´
pA_for	5´-GTTAAACTAGACTGATTCTAGGCGATCGCTCGAGC-3´
pA_rev	5´- GGTGCTGCGCAGATCACGTGCTATGGCAGGGCCTG-3´
TRex_for	5´-GTGGCGGCCGCTCGAGGGAGGTAGTGAGTCGACCAG-3´
TRex_rev	5´-CAGCGGGTTTAAACGGGCCCGACTCTAGATTACTTGTACAGCTCG-3´
WPRE_for	5´-CCCGGGAATTCGTTTAAACAATCAACCTCTGGATTACAAAATTTGTG-3´
WPRE_rev	5´-GGCCGCTCTAGGTTTAAACAGGCGGGGAGGC-3´

Cloning of the Tet-On inducible expression plasmid

psiCHECK2-CMV-siRNA2-mCherry-WPRE-EGFP was amplified with TRex_for and TRex_rev primers conserving *Xho*I and *Apa*I restriction sites (Table 2). pTO-HA-StrepIII-GW-FRT vector was linearized with *Xho*I and *Apa*I. Purified products were recombined with In-Fusion to form pTO-GW-FRT- CMV-siRNA2-mCherry-WPRE-EGFP.

Cloning of the lentiviral genome insert plasmid

psiCHECK2-CMV-siRNA2-mCherry-WPRE-EGFP was amplified with LV_for and LV_rev primers conserving *Cla*I and *Kpn*I restriction sites (Table 2). pCDH-CMV-MCS-T2A-EGFP was linearized with *Cla*I and *Kpn*I. Purified products were recombined with In-Fusion to form pCDH-CMV-siRNA2-mCherry-WPRE-EGFP.

Ligation and recombination reactions were transformed into competent Stellar or Stbl3 bacterial (*E. coli*) cells, with ampicillin (psiCHECK2) or spectinomycin (pBlockIT) used to

select for correct clones. Transformed cells were plated and grown overnight in 37°C. Various amounts of colonies were selected and cultured for 12-18 hours (37°C in a 222 rpm shaking platform) in LB-medium with correct antibiotic (ampicillin 100 µg/ml or spectinomycin 50 µg/ml). Plasmid extraction and purification from cells was done with a plasmid purification kit (Macherey-Nagel, Düren, Germany) according to the manufactures protocol. Diagnostic digestions were performed with selected restriction enzymes and, in addition, all plasmids were sequenced with primers designed to obtain sequences of the recombination and/or ligated sites. The design of the plasmids, primers and restriction digestions, and analysis of sequencing data was done with A plasmid Editor (ApE) software.

3.2.2 Cell culture and producing stable cell lines

Cell culture

Human embryonic kidney (HEK293T) cells, Chinese hamster ovarian (CHO) cells, human SH-SY5Y neuroblastoma cells and FLP-IN 293 T-Rex (T-Rex) (ThermoFisher Scientific, Waltham, MA) cells were maintained in humidified incubator (37°C, 5% CO_2, saturated humidity, RH 80-100%) and re-plated 2-3 times a week (Tjio and Puck 1958; Biedler et al. 1973; Graham et al. 1977). Commercially available Dulbecco's modified eagle medium (DMEM, pH 7.4) supplemented with 10% fetal bovine serum (FBS) and 100 µg/ml Normocin™ (N, InvivoGen, San Diego, USA) was used as the growth medium. When the cells reached approximately 80-90% confluency, they were re-plated into Greiner CellStar 96-well clear-bottom cell culture plates for a density of ca 10000 cells/well in DMEM, pH 7.4 supplemented with 10% FBS and 100 µg/ml N and incubated at 37°C, 5% CO_2 for 24 hours before transfection or administration of compounds in all assay validation and compound screening experiments. Cells were used from passage 2-20.

Stable transfection of FLP-IN 293 T-REX cells

After T-Rex cells reached 80-90% confluency, they were re-plated into Greiner CellStar dishes in DMEM supplemented with 10% FBS and 100 µg/ml N. 1 µg of pTO-GW-FRT-CMV-siRNA2-mCherry-WPRE-EGFP was transfected simultaneously with 5 µg of flippase

(FLP) expressing plasmid pOG44 using 1 μg/μl polyethylenimine (PEI) (4:1 ratio of PEI/DNA) as the transfection reagent, diluted in OptiMEM (ThermoFisher Scientific, Waltham, MA). Cells were incubated for 48 hours in 37°C, 5% CO_2 before changing medium to DMEM supplemented with 10% FBS, 100 μg/ml N, 15 μg/ml Blasticidin HCl and 100 μg/ml Hygromycin B. Media was changed every 2-4 days for 2 weeks until cells with the correct insertion were left.

VSV.G pseudotyped lentiviral packaging and lentiviral transduction of SH-SY5Y cells

HEK293T were allowed to grow in a humidified incubator (37°C, 5% CO_2), using DMEM supplemented with 10% FBS and 100 μg/ml N as growth medium, until they were 80-90% confluent and then re-plated into Greiner CellStar dishes with DMEM supplemented with 10% FBS, 100 μg/ml N and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Cells were grown to 70-80% confluency and transfected with 4 μg of pCDH-CMV-siRNA2-mCherry-WPRE-EGFP 2 μg of pMDLg/pRRE, pRSV/REV and pMD2.G each using 1 μg/μl PEI (4:1 PEI/DNA) as the transfection reagent, in OptiMEM. Cells were incubated (37°C, 5% CO_2) for 72 hours, transferred into tubes and spun down 1100 rpm/200-300 ×g for 5 min. The supernatant was filtered through a 0.45 μm filter into metal centrifuge tubes (Beckman UltraClear #344058) fitted into metal rotor tubes (Beckman AH-629 rotor). Tubes were spun for 1.5 hrs 25000 rpm (AH-629 (36 mL) Swinging Bucket Rotor, 120000 ×g) at 4°C. Supernatant was discarded, pellets were dried for 2-3 min and resuspended into sterile Dulbecco's phosphate-buffered saline (PBS). Finally, the resuspended mixture was centrifuged for 1 min at maximum speed on a benchtop microcentrifuge (Wealtec E-Centrifuge) and the supernatant was collected, aliquoted and stored at -80°C.

SH-SY5Y cells were maintained in an incubator (37°C, 5% CO_2) in DMEM supplemented with 10% FBS and 100 μg/ml N. The cells were plated into a Greiner CellStar 6-well cell culture plate and transduced the following day with different concentrations of the virus. Cells were observed for fluorescence 72 hours after transduction and were selected by hand-picking fluorescent cells and re-plating them in fresh medium. This was done by aspirating the medium, washing once with PBS and then using a small volume of trypsin to trypsinize cells with a sterile pipette and aspirating them to the pipette tip. The selected cells

were cultured for 1 week, re-plated and sorted with a fluorescence-assisted cell sorting FACSAria machine (BD Biosciences, Franklin Lakes, USA).

3.2.3 Validation of the assay

Plasmid testing

CHO cells were transfected after 24 hours with 10 ng, 30 ng or 100 ng of plasmid DNA of the following constructs, using 1 µg/µl PEI (4:1 PEI/DNA) as the transfection reagent, in OptiMEM: psiCHECK2-CMV-siRNA1-mCherry-EGFP, psiCHECK2-CMV-siRNA1-mCherry-WPRE-EGFP, psiCHECK2-CMV-siRNA2-mCherry-EGFP or psiCHECK2-CMV-siRNA2-mCherry-WPRE-EGFP. Before transient transfection, the cells were deprived of serum by changing the medium to DMEM supplemented with 100 µg/ml N. Cells were treated with enoxacin (in DMSO) or 1% DMSO 1 hour after transfection. After 72 hours, cells were fixed with 4% paraformaldehyde for 20 min, stained with 200 ng/ml 4',6-diamidino-2-phenylindole (DAPI), incubating 10 min in dim light and rinsed once with PBS all at room temperature (RT).

Inducible cell line

T-Rex cells were treated with 10 ng/ml doxycycline hyclate (Cat#D9891, Sigma-Aldrich, St. Louis, USA, DOX, in ethanol) to induce mCherry siRNA expression. This was done by changing the medium to 100 µl of doxycycline containing medium.

T-Rex cells were additionally treated with 50 µM or 100 µM enoxacin sesquihydrate (Cat#94426 Sigma-Aldrich, St. Louis, USA, ENX, in DMSO), 10 nM or 100 nM thapsigargin (Cat#T9033, Sigma-Aldrich, St. Louis, USA, TG, in DMSO), or 0.01-0.2% DMSO diluted in DMEM supplemented with 100 µg/ml N or DMEM supplemented with 100 µg/ml N and 10 ng/ml DOX.

Enoxacin concentration (100 µM) was based on studies done by Shan et al. on the relative RNAi enhancing activity of enoxacin (Figure 8). Thapsigargin (10-100 nM) concentration

was chosen according to literature and previous tests done in thapsigargin assays on primary neurons (Chmielarz et al. 2017).

All validation experiments shown in results were confirmed by 2-3 independent, similar or exact repeat experiments from different passages, unless otherwise stated (pooled data).

Imaging, fluorescence quantification and cell counting

Cells were imaged at RT live, or fixed for 20 min with 4% paraformaldehyde, stained 10 min with 200 ng/ml DAPI and washed with PBS all at RT before imaging. Varying time points were determined, ranging from 24 hours to 120 hours in 24 hour intervals. Imaging was done with Thermo Scientific CellInsight™ CX5 High-content screening (HCS) platform at ×10 magnification at RT. Channels (λ =) 389 nm, 485 nm and 549 nm were used. In experiments where cells were fixed, 9 images (fields) were taken per well and in live cell experiments 3 images were taken per well to reduce stress caused to cells due to exposure to prolonged periods in sub-optimal culture conditions. Images were processed and analysed quantifying fluorescence intensities with CellProfiler & CellProfiler analyst (Carpenter et al. 2006; Jones et al. 2008). Custom-made CellProfiler pipelines were used with thresholding strategies account for the cytosol and nuclei. Quality control was done by visually inspecting images and clearly over/under-exposed images, due to equipment error, were excluded from analysis. Mean fluorescence intensities from all cells (from all images per well) were aggregated per well with custom-made scripts using R Studio. The ratio of mCherry and EGFP fluorescence was calculated in Microsoft Excel 2016. In experiments with transient transfection, background fluorescence values were additionally subtracted before calculating ratios.

Cell counting was done by classifying cells with CellProfiler Analyst using RandomForest classifier and LogisticRegression algorithms and training them to identify cells. After positive cells were identified, the total number of positive cells from each image from each well was summed per group using CellProfiler Analyst and Microsoft Excel 2016.

3.2.4 Small-scale screening of compounds

T-Rex cells were kept and plated as described in section 3.2.2. Briefly, the cells were plated into Greiner CellStar 96-well clear-bottom plates for a density of ca 10000 cells/well in DMEM supplemented with 10% FBS and 100 µg/ml N and incubated for 24 hours in a humidified incubator (37°C, 5% CO_2).

After 24 hours various compounds, which were diluted into DMEM supplemented with 100 µg/ml N and 10 ng/ml DOX, were administered to the cells by changing 100 µl of corresponding compound containing medium to the cells or vehicle as a negative control. The compounds were diluted from the following stocks: 10 mM 6-OHDA (in DMSO), 10 µM bafilomycin A1 (in DMSO), 50 ng/µl BDNF (in DMEM), 5,7725 mM corticosterone (in ethanol), 5 mM ellagic acid (in DMSO), 50 mM enoxacin sodium salt (Cat#557305 Millipore, Burlington, USA, in PBS), 500 mM metformin (Cayman Chemical Company, Ann Arbor, USA, cat#13118, in ddH_2O), 20 mM PD98059 (in DMSO), 1 mM staurosporine (in DMSO), 10 mM SU6656 (in DMSO) or 20 mM UO126 (in DMSO). The final concentrations of the compounds used for the screening can be found from Table 3, except for enoxacin which was used as 100 µM (sodium salt) concentration.

The effects of the higher doses of BDNF and metformin were further studied in two duplicate repeat experiments (quadruplicate if considering the groups only) done in the same manner as described without all of the other compounds.

Compounds were chosen either based on literature to have an effect on Dicer (see section 2.3.3), or arbitrarily from what was conveniently available (Table 3). Concentrations were chosen according to literature and unpublished experiments by colleagues. Two concentrations were used, the compound's effective concentration regarding its main mechanism of action and a higher concentration inferred by enoxacin's Dicer-activating concentration.

Cells were incubated in 37°C, 5% CO_2 and imaged live 24 hours, 72 hours and 120 hours after administration of compounds. Imaging, processing of images, data processing and statistical analysis was done as described in section 3.2.3.

Table 3. Compounds used in the small-scale screening. 6-OHDA = 6-hydroxydopamine, BDNF = brain-derived neurotrophic factor, MEK1/2 = mitogen-activated protein kinase kinase 1/2, Src = proto-oncogene tyrosine-protein kinase Src.

Drugs & Compounds	Mechanism of action	Reference	Concentrations
6-OHDA	Oxidative agent / Dopaminergic cell toxin	(Michel and Hefti 1990)	10 and 100 µM
Bafilomycin A1	Autophagy inhibitor	(Redmann et al. 2017)	10 and 100 nM
BDNF	Neurotrophic factor	(Kalcheim and Gendreau 1988)	5 and 50 pg/µl
Corticosterone	Glucocorticoid (hormone)	(Kurek et al. 2016)	1 and 5 µM
Ellagic acid	Inhibits Translin/Trax	(Salimi et al. 2015)	10 and 100 µM
Metformin	Anti-diabetic compound	(McKiney et al. 2010)	100 µM and 1 mM
PD98059	MEK1/2 inhibitor	(Koivisto et al. 2014)	10 and 100 µM
Staurosporine	Apoptosis inducer	(Koh et al. 1995)	100 nM and 1 µM
SU6656	Src kinase inhibitor	(Blake et al. 2000)	2 and 20 µM
UO126	MEK1/2 inhibitor	(Burkhard and Shapiro 2010)	10 and 100 µM

3.2.5 Statistical analysis

All statistical analysis and graphs were done with GraphPad Prism version 6.01 or 7.03 (GraphPad Software, Inc., La Jolla, San Diego, USA). A p value of less than 0.05 was considered statistically significant. All data are expressed as mean and error bars are standard deviations. Statistical analysis was done within plates in the validation experiment and the preliminary small-scale screen to assess initial effects. However, to be fully certain of an effect, experiments were repeated and pooled data of independent (*i.e.* different

plates from different passages and in some cases freeze/thaw cycles) experiments was analysed by calculating the mean value of all wells per group per plate and using the mean values from each independent experiment for statistical analysis.

One-way analysis of variance (ANOVA) was used when comparing three or more groups simultaneously, when variances were assumed statistically equal and did not differ significantly according to the Brown-Forsythe test, with Tukey's multiple comparison test or Bonferroni's multiple comparison test as post-hoc tests. Conserved post-hoc tests were chosen to minimize false discovery rate and increase the reliability of results. In experiments where variances were not statistically equal, a non-parametric test, the Kruskal-Wallis test, was used, with Dunn's multiple comparison test. When comparing two groups, Student's two-tailed unpaired *t*-test was used if variances were statistically equal, and when not (invalid F-test), the Mann-Whitney U test was used. Comparisons were done by comparing the means of each group to the mean of the negative and/or positive control. Assessing the quality of the assay regarding HTS was done by calculating the Z-factor (1) and CV (2) as follows (Adapted from Xiao et al. 2016):

$$Z' = 1 - 3 \times \frac{SD_{PC} + SD_{NC}}{|Mean_{PC} - Mean_{NC}|} \quad (1)$$

$$CV\ (\%) = SD / Mean \times 100 \quad (2)$$

Where PC is positive control wells, NC is negative control wells and SD is standard deviation.

3.3 Results

Plasmids

All of the produced plasmids were verified by DNA sequencing and they functioned as planned which was assessed in validation experiments (Appendix 1 and Figure 12).

Stable cell lines

T-Rex cells expressed both fluorescent proteins and doxycycline induction of mCherry siRNA was shown to be functional in all experiments where T-Rex cells were used (Appendix 2, Figures 11, 14, 15, 16, 19).

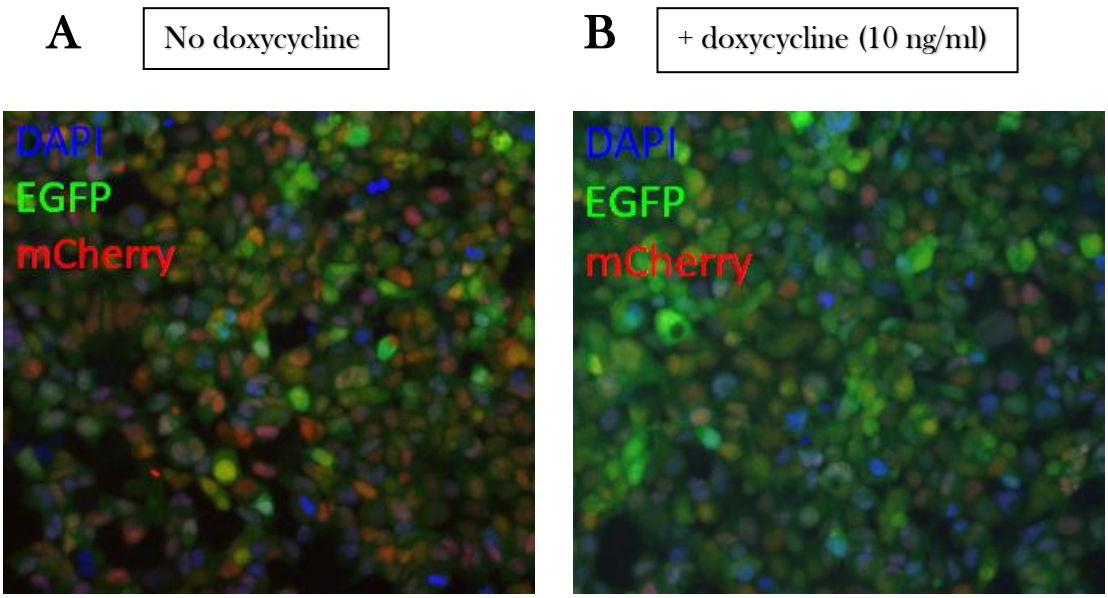

Figure 10. Doxycycline induction to validate the proper functioning of the Tet-On system in FLP-IN 293 T-Rex cells. Representative images of doxycycline treated and vehicle treated cells. Reduced mCherry fluorescence can be visually observed by comparing the nuclei of cells treated with doxycyline (B) to non-treated cells (A). DAPI stains the nucleus, EGFP is localized in the cytosol and mCherry is localized in the nucleus due to the NLS integrated to the mCherry transcript. DAPI = 4',6-diamidino-2-phenylindole, EGFP = Enhanced green fluorescent protein, mCherry = modified Cherry (red fluorescent protein).

Lentiviral vector transduced SH-SY5Y cells expressed EGFP but a clear majority did not express mCherry and, therefore, the cells were not selected for further use (Figure 11).

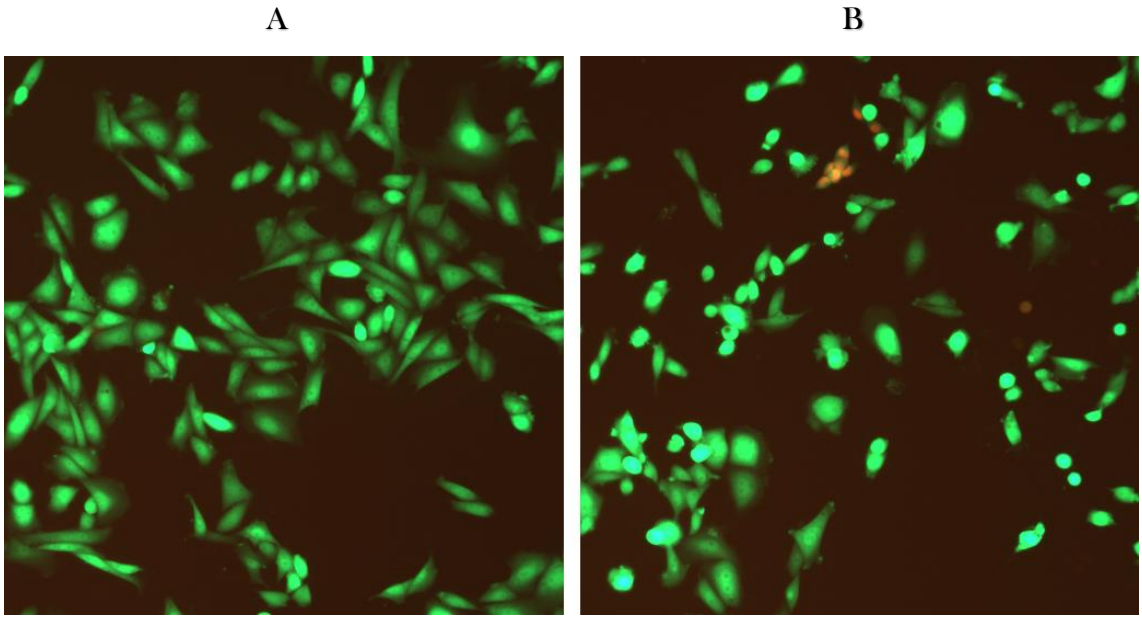

Figure 11. Representative images of lentiviral vector transduced SH-SY5Y neuroblastoma cells. A) Cells robustly expressed EGFP (green) B) Only a few cells had quantifiable mCherry (red).

3.3.1 Validation with enoxacin and thapsigargin

A plethora of assay parameters that may affect the signal of Dicer activation and noise were considered and studied during this thesis work.

To start, plasmids were tested with transient transfection for transient protein expression. Transfecting the plasmids psiCHECK2-siRNA1 and psiCHECK-siRNA2 both with, or without WPRE in CHO cells, the mCherry siRNA was highly efficient in knocking down mCherry expression ($H=31.07$, $p=0.0003$, Kruskal-Wallis test) after 72 hours of transfection, as the mean mCherry/EGFP fluorescence ratio was very low compared to control (Figure 12). The plasmid psiCHECK2-CMV-siRNA2-mCherry-WPRE-EGFP produced the highest mean mCherry fluorescence and was thus chosen to be implemented into stable cell lines (Figure 12 and additional data not shown).

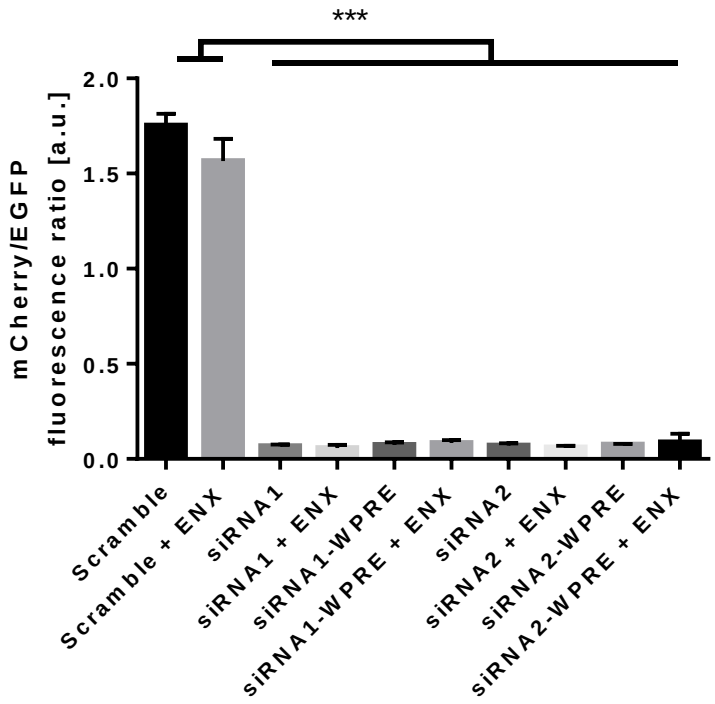

Figure 12. Testing the plasmids by transient transfection. Mean mCherry/EGFP fluorescence ratio measured in Chinese hamster ovarian cells 72 h after administration of vehicle (1% DMSO) or enoxacin as the positive control (100 μM). Scramble is a non-targeting miRNA which serves as a negative control. Data are expressed as mean +/- SD. Kruskal-Wallis test, ***p<0.001. n = 5 (wells). ENX = enoxacin.

Thereafter, stable cell lines were produced and an isogenic, inducible T-Rex cell line was chosen for further experiments (Figure 10). Enoxacin, as the positive control, (10-100 mM) did not reduce mCherry/EGFP ratio significantly after 24-48 hours of induction with doxycycline compared to DMSO in experiments where cells were fixed (data not shown). Therefore, thapsigargin was tested if it could inhibit Dicer and if enoxacin treatment could rescue this inhibition (Figure 13). Tests showed that already after 24 hours, thapsigargin treatment significantly increased mCherry/EGFP ratio (One-way ANOVA, p=0.005 $F_{2,12}$=8.493). However, the effect was significant only in the 100nM arm (p=0.0049, Bonferroni's multiple comparison test) compared to the negative control (DMSO), and furthermore, both concentrations reduced cell viability, killing the cells. This was assessed by visual inspection with a microscope, a significant reduction in mean EGFP fluorescence (One-way ANOVA, p=0.0001 $F_{2,12}$=20.70) in TG 10 nM and 100 nM compared to DMSO treated cells (p=0.0241 and p<0.0001, respectively, Bonferroni's multiple comparison test)

and significant reduction in the number of cells in both arms (One-way ANOVA, p<0.0001 $F_{2,12}$=90.50 10 nM: p=0.0034, 100n nM: p<0.0001, Bonferroni's multiple comparison test).

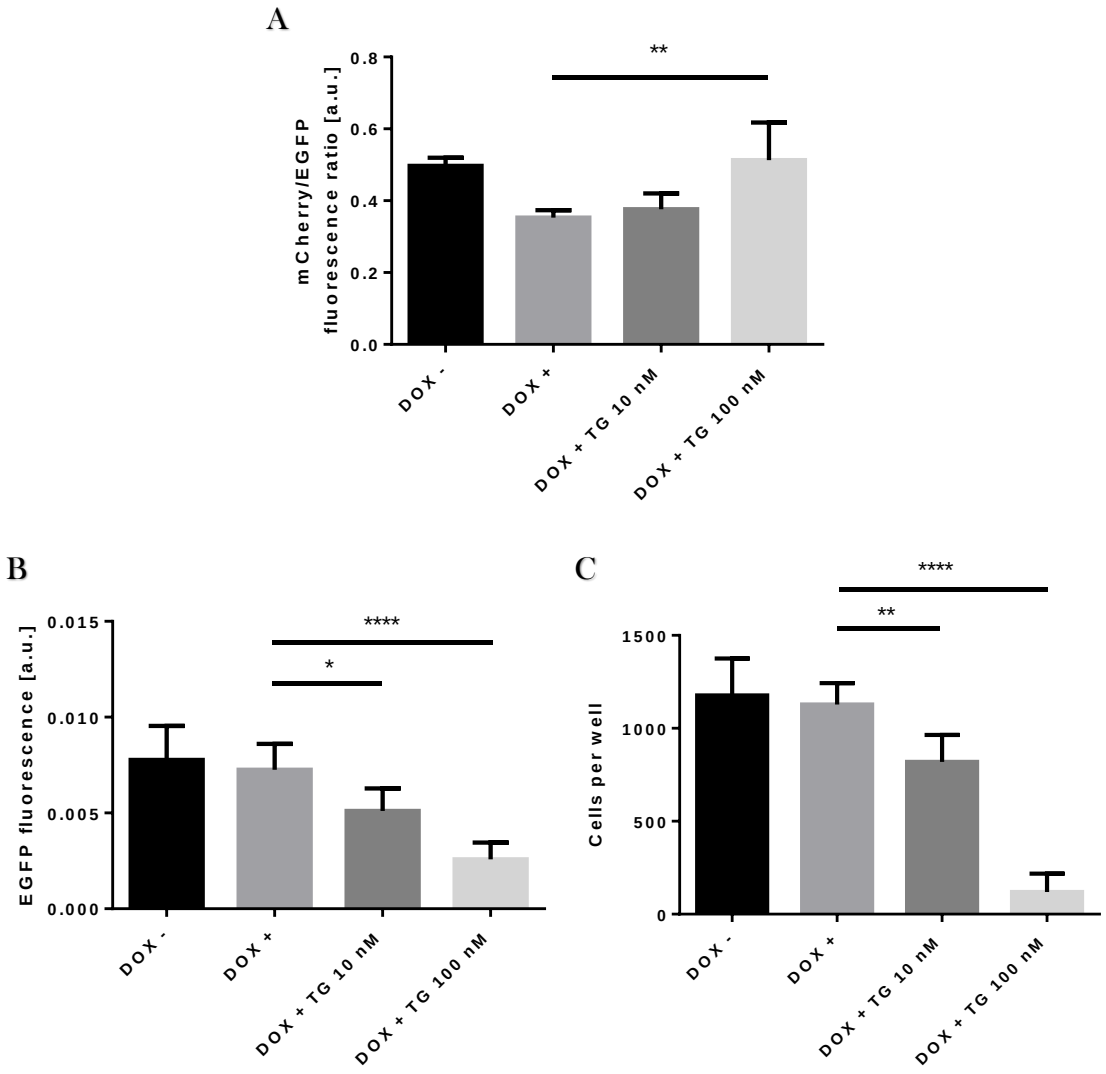

Figure 13. Testing thapsigargin to optimise a rescue assay. Results after 24 hours of administration of vehicle (0.01% DMSO), doxycycline and vehicle or doxycycline and either 10 nm or 100 nm of thapsigargin in FLP-IN 293 T-Rex cells. Measured A) Mean mCherry/EGFP fluorescence ratio. B) Mean EGFP fluorescence C) Mean number of cells per well. Non-doxycycline treated cells (DOX -) here is to show that the siRNA was expressed and DOX+ serves as the control for the assay. Data are expressed as mean +/- SD. One-way ANOVA with Bonferroni's multiple comparison test, *p<0.05, **p<0.01 ****p<0.0001. n = 5 (wells). DOX = doxycycline, TG = thapsigargin.

Next, live-cell imaging with different time points were assessed. Enoxacin (100 μM) did not reduce mCherry/EGFP ratio significantly after 24-48 hours of induction with doxycycline

compared to DMSO, similarly to previously mentioned experiments fixing cells (Figures 14 and 15). After 72 hours enoxacin significantly reduced mCherry/EGFP fluorescence ratio compared to DMSO (Unpaired Student's *t*-test, p=0.0036, t=4.073 df=8).

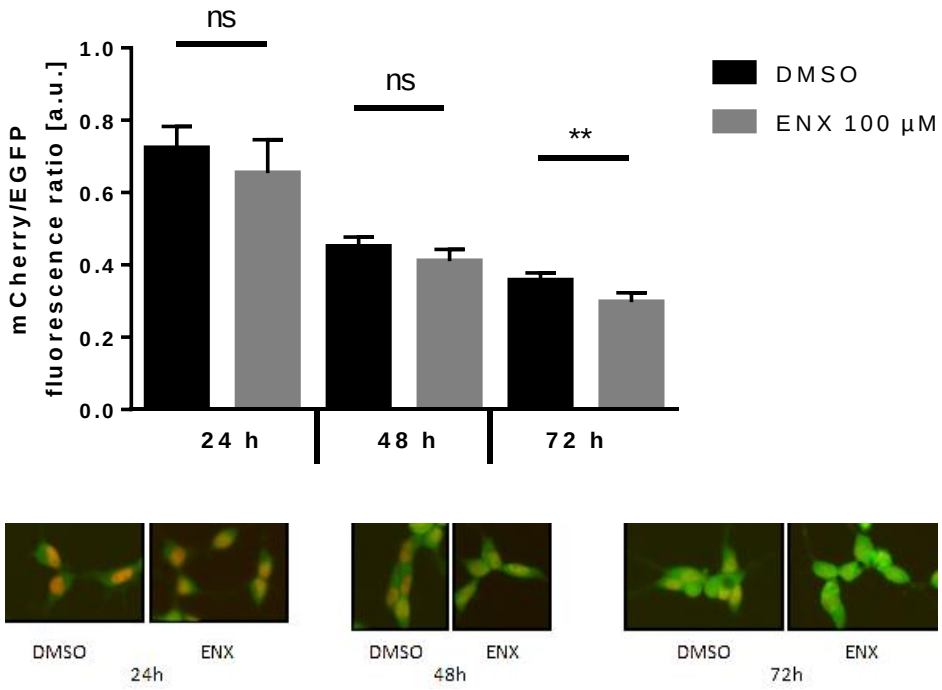

Figure 14. Validating the assay using enoxacin as a positive control. Mean mCherry/EGFP fluorescence ratio measured after three time points. FLP-IN 293 T-Rex cells were treated with either doxycycline and vehicle (0.2% DMSO) or doxycycline and enoxacin (100 µM). Representative images of cells quantified in the assay shown below. EGFP (green) is localized in the cytosol and mCherry (red) is localized in the nucleus. Reduced mCherry expression due to doxycycline and enoxacin is apparent. Data are expressed as mean +/- SD. Unpaired Student's *t*-test, **p=0.0036, ns=not significant. n=5 (wells). DMSO = dimethyl sulfoxide, ENX = enoxacin.

The effect was confirmed in two subsequent experiments and even further time points were also examined (Figure 15 and data not shown). Besides comparing the positive and negative control head on (Student's *t*-test), results were also compared to non-doxycycline induced cells to see whether variation here would make a difference. Using one-way ANOVA ($p<0.0001$, $F_{2,15}=1175$), the effect of enoxacin in reducing mCherry/EGFP fluorescence ratio was significant compared to DMSO after 72 hours of treatment (p=0.0005, Tukey's

multiple comparison test). After 120 hours, the effect was even more significant ($p<0.0001$ Tukey's multiple comparison test, One-way ANOVA, $p<0.0001$, $F_{2,15}=1896$).

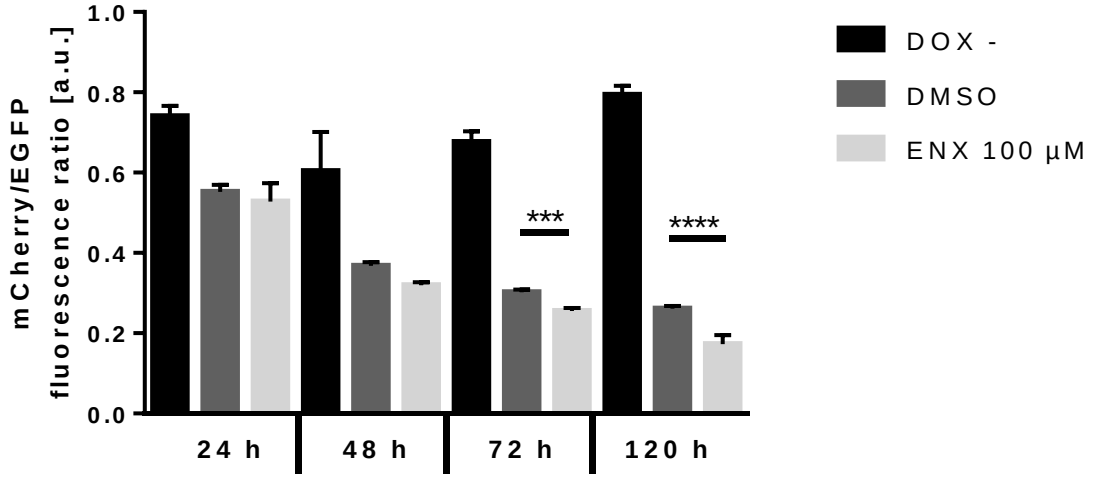

Figure 15. Further validation of the assay using different time points. Mean mCherry/EGFP fluorescence ratio measured after four time-points in FLP-IN 293 T-Rex cells treated with vehicle only (0.2% DMSO), doxycycline and vehicle or doxycycline and enoxacin (100 µM) combined. Non-doxycycline treated group (DOX -) are displayed to show that the siRNA was expressed and to serve as an additional control for the assay. Data are expressed as mean +/- SD. One-way ANOVA with Tukey's multiple comparison test, ***$p<0.001$, ****$p<0.0001$. n=6 (wells). DMSO = dimethyl sulfoxide, DOX = doxycycline, ENX = enoxacin.

After this, a small-scale screening was done to further assess the quality of the assay and to possibly find Dicer-activating compounds (see section 3.4.2). However, due to the screening experiments showing that enoxacin did not have a significant effect in reducing mCherry/EGFP fluorescence ratio analysing pooled data from four independent experiments, a more thorough analysis combining these four screening experiment results with the previous two shown here (Figures 14 and 15) and a third one (data not shown) showed that the effect was still significant after 72 hours (Figure 16, Unpaired t-test, $p=0.0272$, $t=2.515$ $df=12$) but not anymore after 120 hours of treatment (Unpaired t-test, $p=0.0834$, $t=1.889$ $df=12$).

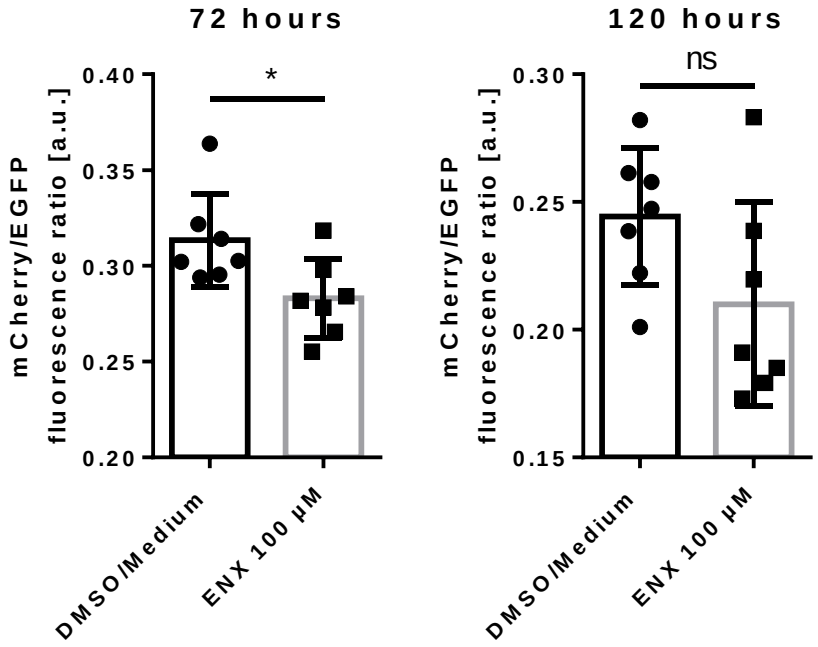

Figure 16. Thorough analysis combining data from validation experiments to data from screening experiments. Mean mCherry/EGFP fluorescence ratio assessed from pooled data from seven independent experiments after 72 hours and 120 hours of either vehicle (0.2% DMSO or plain medium if sodium salt enoxacin stock, diluted in PBS, was used) or enoxacin (100 µM) treatment in FLP-IN 293 T-Rex cells. Data are expressed as mean +/- SD. Unpaired t-test, *p<0.05, ns=not significant. n=7 (independent experiments). DMSO = dimethyl sulfoxide, ENX = enoxacin.

3.3.2 Screening compounds of interest

Screening 10 compounds found no compounds to reduce mCherry/EGFP fluorescence ratio significantly, but a few, namely ellagic acid, staurosporine and UO126, which increased it (Figure 17, One-way ANOVA, p<0.0001 $F_{21,74}$=17.53 and ****p<0.0001 Bonferroni's multiple comparison test). However, many of these compounds which increased mCherry/EGFP ratio also had a significant reduction in mean EGPF fluorescence (One-way ANOVA, p<0.0001 $F_{21,74}$=4.263 and *p<0.05, Bonferroni's multiple comparison test). The compounds which increased EGFP fluorescence most prominently were staurosporine (1 µM) and UO126 (100 µM). Compounds which had a trend in reducing mCherry/EGFP fluorescence ratio compared to the negative control, were BDNF

(50 pg/µl) and metformin (1 mM) and they were chosen for further experiments to assess valid statistical analysis of the effects.

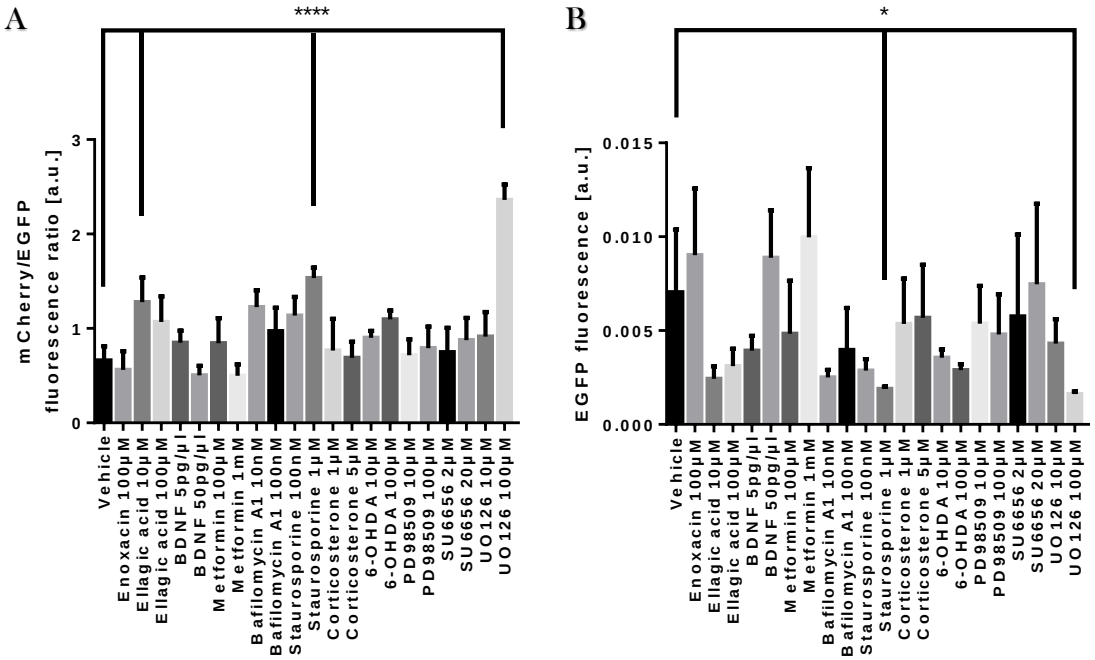

Figure 17. Preliminary screening with ten compounds of interest. A) Mean mCherry/EGFP fluorescence ratio B) mean EGFP fluorescence. Vehicle is doxycycline containing medium. Data are expressed as mean +/- SD. One-way ANOVA with Bonferroni's multiple comparison test, *p<0.05, ****p<0.0001. n=4 (wells). 6-OHDA = 6-hydroxydopamine, BDNF = brain-derived neurotrophic factor.

In the validation screening experiments of BDNF and metformin, there was no statistical significance in the reduction of mCherry/EGFP fluorescence ratio between enoxacin, BDNF and metformin compared to the negative control (doxycycline in plain medium, since none of the three compounds were diluted in DMSO) after 72 hours (Figure 18A and B, One-way ANOVA, p=0.1244 $F_{3,12}$=2.344) nor 120 hours of treatment (One-way ANOVA, p=0.1251 $F_{3,12}$=2.339). However, all of them showed a trend in reduction, with metformin having the largest reduction of the groups after 72 hours and enoxacin after 120 hours following administration of the compounds. The results were also analysed by comparing the relative change in mCherry/EGFP fluorescence ratio (as was done in Li et al. 2012 for example), between the groups compared to the negative control (Figure 18C and D).

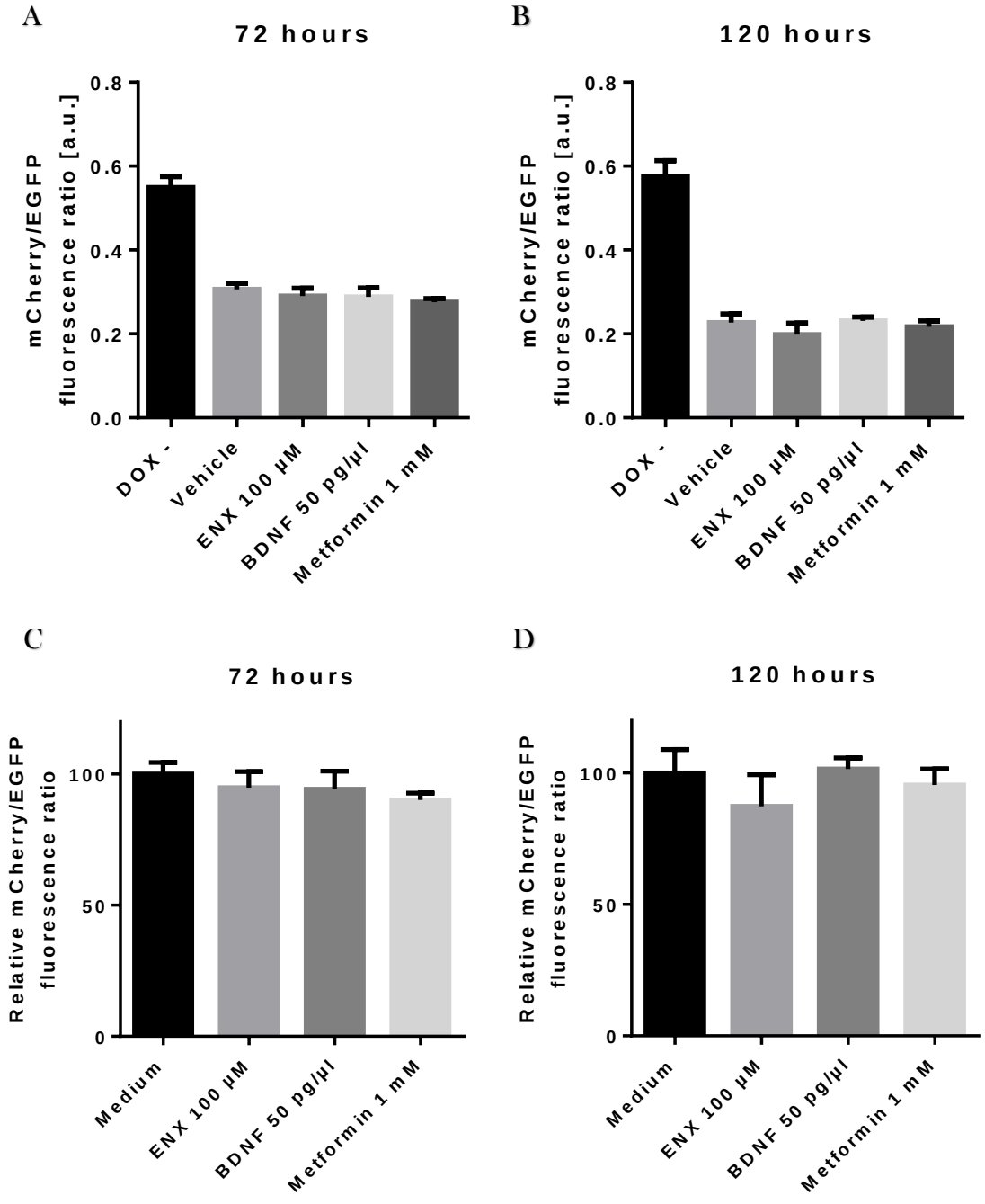

Figure 18. Re-screening BDNF and metformin after 72 hours (A, C) and 120 hours (B, D). Results displayed as mCherry/EGFP ratio (A, B) and relative mCherry/EGFP ratio comparing them to the negative control (medium) (C, D) Non-doxycyline treated cells (DOX-) are displayed to show that the siRNA was expressed and Vehicle (plain medium) serves as the negative control for the assay. One-way ANOVA showed no statistically significant differences between groups. n=4 (independent experiments). BDNF = brain-derived neurotrophic factor, DOX = doxycycline, ENX = enoxacin.

None of the compounds showed significant reduction in mean EGFP fluorescence compared to the negative control (vehicle) 72 hours after treatment (Figure 19, One-way ANOVA, p=0.3486 $F_{3,12}$=1.209). However, after 120 hours there was a significant difference (One-way ANOVA, p=0.0227 $F_{3,12}$=4,622) between the negative control and enoxacin (p=0.04).

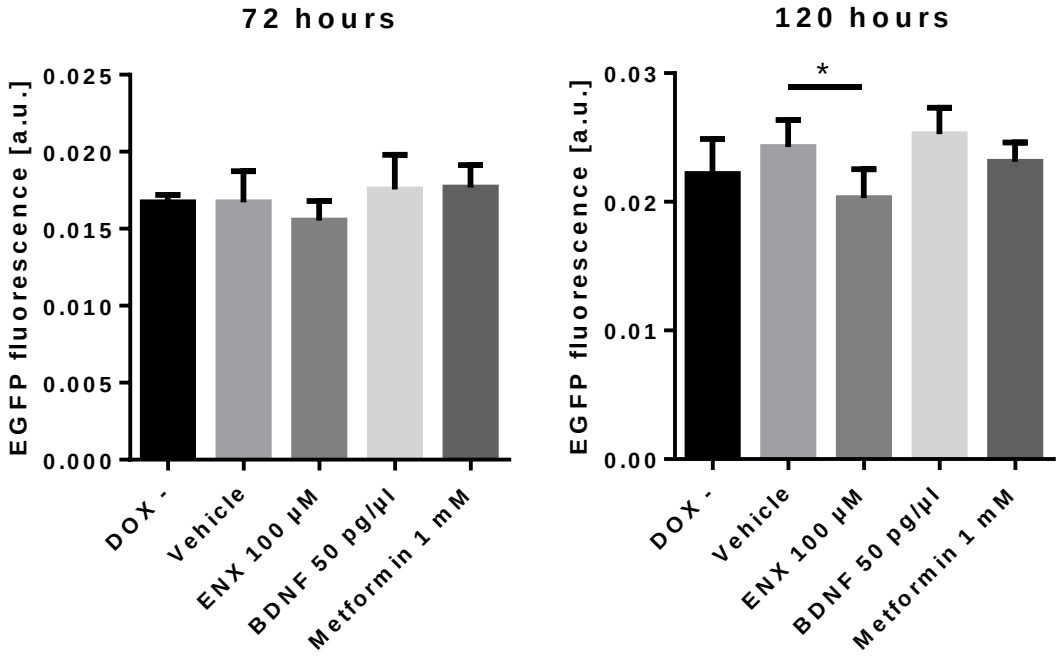

Figure 19. Mean EGFP fluorescence which gives an indication of cell death. After 72 hours the groups had similar mean EGFP fluorescence. After 120 hours enoxacin showed a significant reduction in mean EGFP fluorescence compared to the negative control (vehicle). One-way ANOVA with Tukey's multiple comparison test, *p=0.04. n=4 (independent experiments). BDNF = brain-derived neurotrophic factor, DOX = doxycycline, ENX = enoxacin.

3.3.3 High-throughput screening quality measures

The negative (plain medium or 0.2-1% DMSO) controls and the positive control (enoxacin) were used to calculate the Z-factor and CV (Table 4). The assay based on the pooled, seven experiment (plate), data, gives a Z-factor of -3.45, and CV values of under 10 for both controls.

Table 4. Calculated Z-factors and coefficient variations from seven different experiments (plates) at 72 hours after treatment. Negative control is DMSO (0.2-1%) or plain medium, depending on the used enoxacin stock, and positive control is enoxacin.

Experiment	Z´	CV% (Negative control)	CV% (Positive control)
Plate 1	-5.18994498	12.95	13.95
Plate 2	0.053969431	2.31	3.04
Plate 3	-8.336164584	4.42	12.97
Plate 4	-12.80405038	10.93	11.04
Plate 5	-24.88146876	5.15	3.92
Plate 6	-5.055476146	9.22	11.13
Plate 7	-2.816754611	14.92	9.93
All plates combined	-3.448583992	7.77	7.31

3.3.4 Summary

Transiently transfecting the plasmids, highly efficient silencing due to mCherry siRNA resulted in low mCherry fluorescence. Of all the tested plasmids, psiCHECK2-CMV-siRNA2-mCherry-WPRE-EGFP was chosen due to highest mCherry fluorescence (Figure 12, and other experiments not presented here).

After a number of parameter optimisation experiments, in stable cell lines, enoxacin did not seem to have an effect in reducing mCherry/EGFP fluorescence by optimisation after 24-48 hour experiments in which cells were fixed prior to imaging (data not shown). Therefore, thapsigargin was chosen in order to optimise a rescue assay (Figure 13). 100 nM increased mCherry/EGFP fluorescence ratio significantly, but also induced cell death, demonstrated by the significant reduction in mean EGFP fluorescence and cell viability (Figures 13B and 13C)

In live cell experiments, enoxacin did not reduce mCherry/EGFP fluorescence ratio significantly compared to vehicle (plain medium, 0.2% or 1% DMSO depending on used

enoxacin stock) after 24-48 hours of administration but did so after 72 and 120 hours in three independent experiments (Figures 14, which also display descriptive reduction by images, Figure 15, and additional data not shown). However, after a small-scale screen, followed by four re-screening replicate experiments combined to the validation experiments, a pooled analysis showed that enoxacin's effect in reducing mCherry/EGFP fluorescence ratio was significant only after 72 hours following administration.

Small-scale screening found no compounds to significantly reduce mCherry/EGFP fluorescence ratio (Figures 17 and 18). However, the effects of metformin and BDNF were comparable to that of enoxacin (Figure 18).

Assay quality in HTS terms, was determined by calculating Z-factors and CV values for each independent experiment and for the pooled data of seven experiments (Table 4).

3.4 Discussion

The results of this work demonstrate the development and optimisation of an assay to measure Dicer activity.

One secondary aim was to make a lentiviral vector out of the construct to make other stable cell lines, besides the T-Rex cells, and use it to transduce neurons (or other post-mitotic cells), in addition to possible *in vivo* studies by e.g. administering the virus intracerebrally to mice or rats, to study the biology of Dicer. A virus was produced, but the titer was extremely low, most probably due to multiple polyA sequences in the inserted construct, which produce truncated genomes and inhibit packaging of the virus (Blo et al. 2008). Although there were only trace amounts of the virus, a few SH-SY5Y cells were successfully transduced enabling use of the cells by picking them, expanding them in culture and sorting them. However, nearly all of the cells did not express quantifiable mCherry, or silencing was yet again too efficient for mCherry to be clearly detected so the cells were not usable for assaying Dicer activity (Figure 11).

3.4.1 Assay validation

The fact that some preliminary results (marked "data not shown" in text) were not repeated is not in accordance with the scientific method. However, considering that most validation experiments were purely for optimisation purposes, it would be redundant to repeat experiments with clearly unwanted results, when attempting to develop a reliable assay. All findings showing the assay to work, were further studied by independent, similar (enough to make rational inference on the effects), or replicate experiments from different passages as described in section 3.2.3.

Transiently transfecting the plasmids, mCherry silencing was too efficient and, therefore, differences in mCherry fluorescence between groups was not optimal to quantify (Figure 12). Efficient silencing was caused probably due to CMV being a high expression promoter combined with the prominent knockdown efficiency of full complementary siRNAs. Thus, making inducible stable cell lines, by integrating the assay construct into a cells genome, to control expression was justified, besides the reason for making the assay feasible for HT screening. Also, a small effect of enoxacin reducing mCherry/EGFP ratio in the scramble plasmids was interesting, but with further analysis the difference may have been caused mostly due to background fluorescence being higher in the enoxacin group.

Serum deprivation was chosen due to initial experiments with serum included media showing that cells overgrew in the wells which hampered image analysis. Especially considering the later time points (72-120 h) this would have been a major problem if frequently dividing cells could grow for long periods. Serum deprivation was also decided based on the reason that it has lots of proteins (albumin) capable of binding drugs and other compounds (Huntley et al. 1977). Binding could affect concentrations and diffusion of compounds in to the cytosol of the cell, which could alter the efficacy of the compounds considerably. In hindsight, serum deprivation was a serendipitous decision due to a study showing that serum deprivation downregulates Dicer (Asada et al. 2008). Regarding the assay, this is a fitting finding, since correcting Dicer deficiencies is the main reason for the use of Dicer-activating compounds. In fact, if Dicer were fully functional and correctly regulated, it could be that activating it more would have no significant effect, since processing is already fully saturated. This could also explain why the effect of enoxacin was

rather modest. Furthermore, regarding therapeutic use, activating fully functional Dicer could even be disadvantageous to humans since its efficiency is already naturally attenuated by the helicase domain (Ma et al. 2008; Park et al. 2011).

One problem with the inducible cell line was that expression threshold was reached already with a low dose of doxycycline. In titration experiments, there was no significant difference in using higher concentrations than 10 ng/ml (Appendix 2, Figure 22). For example, 100 ng/ml and 1000 ng/ml induced similar expression, or mCherry knockdown, as 10 ng/ml. In contrast, lower than 10 ng/ml, namely 1 ng/ml, did not produce significant knockdown and thus doxycycline did not leave much room for optimisation since saturation of doxycycline-induced expression was already reached with 10 ng/ml induction (Appendix 2, Figure 23).

The results on using thapsigargin for a rescue assay, would suggest that 100 nM thapsigargin treatment inhibited Dicer, however, the significant cell death suggests, on the contrary, that Dicer may have not been inhibited but was degraded due to cell death. Whether thapsigargin also inhibited Dicer, besides getting downregulated/lost due to cell death, is redundant to figure out in terms of the assay, since 10 nM already killed the cells, but did not significantly increase mCherry/EGFP ratio. Ergo, even if enoxacin would have helped with the survival there would not have been much measurable Dicer inhibition to rescue.

As to why the hypothesized Dicer activating, or more general RNAi enhancing, effect was significant only after 72 hours after treatment, the turnover of miRNA/mRNA could take longer than expected. Kinetic studies and previous studies suggest that 48-72 hours is optimal in RNAi assays (Birmingham et al. 2009; Li et al. 2012; Lemus-Diaz et al. 2017). (Further discussion on RNAi kinetics can be found in section 3.4.4.). 48 hours of incubation was also used in the original studies where enoxacin was found to enhance RNAi, however as noted, the authors' construct does not have a pri-miRNA, but a shRNA which changes the kinetics (Shan et al. 2008; Chakravarthy et al. 2010). Furthermore, in the later study, where they used a dual fluorescence system, the effect of enoxacin in reducing relative GFP/RFP fluorescence ratio was not highly efficient (60-70% relative ratio compared to negative control) and the ca 90% in this work may be due to differences in pri-miRNA and shRNA. Additionally, the pri-miRNA rules out selectivity of the activation to be mediated by Dicer since it is first processed by the Drosha/DGCR8 complex (Figure

1). The reason why Dicer activation was not significant anymore after 120 hours in the pooled data may be due to cells dying in some of the experiments after 5 days and thus increasing the mCherry/EGFP ratio (Figure 19). The use of alternative or additional positive controls could be tested, such as metal chelating compounds e.g. deferoxamine mesylate and thiosemicarbazide, especially that they showed more prominent Dicer enhancement compared to enoxacin (ca 50% reduction in relative GFP/RFP compared to enoxacin's 30-40%) (Li et al. 2012). However, enoxacin was chosen due to its positive effects to the survival of dopaminergic cells and in animal models of neurodegeneration (Emde et al. 2015; Chmielarz et al. 2017).

The precise effect of enoxacin reducing mCherry expression alone, by activating Dicer, should be confirmed by another quantitative method such as qPCR to measure the levels of mCherry mRNA and mCherry siRNA and to see if there is a difference with enoxacin treated cells to control cells. This would thus refute the suggestion that the reduction in mCherry fluorescence is not due to reduced protein levels but something else, e.g. the effect being noise.

Dicer levels should also be determined in serum deprived T-Rex cells and cells grown in medium with serum, by Western blotting or enzyme-linked immunosorbent assay, to confirm previous findings and to assess how Dicer downregulation could affect activation.

General aspects of the assay

Tet-inducible T-Rex cells have been used successfully in HTS assays previously and are optimal in terms of usability regarding their growth rate and growth conditions (Plate et al. 2016). However, one thing to be considered would have been sorting these cells, as was done with the lentiviral vector transduced SH-SY5Y cells, to select for clones that would optimally express the reporter proteins. This may have also helped with the low inducibility achieved by doxycycline, and a clonal line could have had better dose–responsiveness than the used isogenic line.

EGFP was stable and reliable in the experiments, except for the edge effect in screening (see 3.4.2) and some minor variability noticed between EGFP reduction and cell death. mCherry expression (or the measured emission, however this can only be affected by

changing the protein structure) on the other hand was quite low as can be seen from Figures 10 and 14. This is not optimal, and a wider range would be better for quantifying changes in fluorescence.

3.4.2 Compound screening

The ten-compound screen found that ellagic acid, staurosporine and UO126 seemed to inhibit Dicer, however they also seemed to induce cell death which was assessed by the reduction in EGFP and, furthermore, visual inspection by a microscope and the images attained from CellInsight. Indeed, some compounds were chosen due to their ability to effectively induce apoptosis for example, and thus to further assess if the EGFP as an internal normalization factor functioned as it did in the validation experiments (Figure 13B and 13C). However, there were many other compounds which seemed to induce cell death by microscopic assessment but did not produce significant reduction in EGFP fluorescence suggesting that the results from this experiment are not reliable. Microscopic inspection especially is not reliable and thus cells should have been counted. However, cells were not counted in any of the screening experiments due to cells not being equally spread out through the wells and because the imaging took place always in the same spot from the well. In addition, the whole well was not imaged (only 3 images were taken compared to 9 images from which cells were counted as is demonstrated in e.g. Figure 13C) and thus the results would only be indicative. A reduction in relative cell death could have been counted, in which case the total amount would not matter, between time points, but inferring death with visual inspection accompanied by significant reduction in mean EGFP fluorescence is enough to conclude that they would not be optimal study further as Dicer-activating compounds. The use of EGFP fluorescence reduction as a marker for cell viability is supported additionally by the findings that there was a clear link between cell death and EGFP reduction measured in validation experiments, as can be seen from Figures 13B and 13C. Another live cell imaging platform could have been an option but was not considered due to limited resources and time in addition to the convenience of the CellInsight platform. Furthermore, and more importantly, after 96 h of plating (72 h after treatment), the cells were in dense clumps, which CellProfiler could not differentiate and count reliably

by EGFP (Figure 20). Nuclei were separable by mCherry, however cells could not be reliably counted based on them since some cells had non-detectable mCherry and thus would not have been included in counts. However, still, looking at the EGFP for cell death was also not reliable in the preliminary ten-compound screen due to large variability. Variability was caused in part due to using the whole plate and an edge effect was found in the outmost wells. Nevertheless, the variation in EGFP was later reduced in the confirmatory experiments with selected compounds, more wells and not using the outmost wells, which retained the reliability of using EGFP as a proxy for cell death.

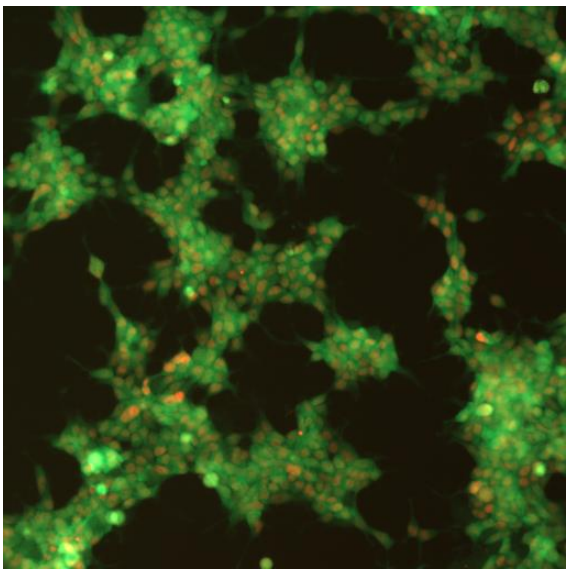

Figure 20. Representative image of densely packed live T-Rex cells 96 hours after plating. Looking at individual cells based on EGFP (green) it is difficult to differentiate between them, while mCherry (red) marked nuclei are more clearly differentiable.

Concentrations used for screening were based on literature (see section 3.2.4) but is based on a speculative conclusion that they may have other effects besides their main activity. A wider titration of, especially lower, concentrations should be considered, particularly with compounds that effectively reduced cell viability.

Taken together, results from the preliminary experiment are not conclusive since it was a single experiment and there was much more variability observed comparing to other experiments. Therefore, the re-screening experiments focusing on BDNF and metformin were done with repeat experiments and valid replicate analysis. The effects of BDNF and

metformin were most intriguing, however, not statistically significant. BDNF and metformin had a tendency in enhancing RNAi as expected according to previous studies shown that they can elevate Dicer levels (Huang et al. 2012; Hooten et al. 2016). However, these findings are not definitive and have not been confirmed in multiple controlled experiments. The findings here, assuming the assay works in the first places, do suggest that they could support their ability to upregulate Dicer in lieu of comparing their effects to other compounds which definitely did not reduce mCherry/EGFP fluorescence ratio. However effects were not statistically significant, so more tests are needed to assess their actual effects as was done with enoxacin.

Since enoxacin did not reduce mCherry/EGFP fluorescence ratio significantly, a pooled analysis from 7 plates was done to assess the integrity of the assay which showed that the effect was only significant after 72 hours. However, the enoxacin batch was changed and a sodium salt was used in the screening experiments which may have had some quality related issues, although this is unlikely, since it was purchased from a trustworthy vendor. Nevertheless, based on this data, the true effect of enoxacin should be studied and validated in further replicates.

3.4.3 Assay quality in terms of high-throughput screening

The combined plate Z-factor and CV state that the variability of the assay is good, but the difference between the effect of positive control (enoxacin) and negative control (DMSO or plain medium) is not different enough to warrant large-scale HTS (Table 4). Looking at the quality of the assay and dissecting individual experiments, one of the plates gives a Z´= 0.053969431, and CVs less than 5, indicating that the assay could be used to some extent for HTS if it were to have such quality. However, one positive experiment out of seven can hardly be met with conviction, and besides on the contrary, one plate had a Z´= -12.80405038 and CVs over 10 which would definitely not be suitable for HTS. Taken together, the quality of the assay does not support large-scale high-throughput screening in terms of looking at the Z-factor of individual or combined experiments. The S/B or the S/N was not calculated because they don't tell anything about the measured phenomena

and because the Z-factor and CV are enough to assess the quality and, thus, calculating the S/B or S/N would be redundant for further quality assessment.

3.4.4 General discussion

One important thing to consider is the mechanism of action of enoxacin. It may be that its neuroprotective activity is not a result from, at least mostly, Dicer activation. Enoxacin has been shown to inhibit JNK, of which inhibition has neuroprotective action in neurodegenerative animal models (Qu et al. 2013; Liu et al. 2014; Le Pichon et al. 2017). Moreover, proteins in the piRNA pathway may repress miRNAs and recently it was shown that enoxacin targets PIWIL3 in cancer cells which may contribute further to its miRNA enhancing activities (Abell et al. 2017). However, in another study it was shown that enoxacin has varying effects on the expression of certain miRNAs, by even downregulating some of them which also may or may not contribute to its neuroprotective effects (Valianatos et al. 2017). Nevertheless, these findings further illuminate the polypharmacological nature of enoxacin and supports the reasoning that its neuroprotective enhancing action should be studied thoroughly. This could be done using *in vitro* and *in vivo* Dicer CKO models and finding out whether it is still has neuroprotective effects without Dicer.

Furthermore, the precise binding mechanism of enoxacin on TRBP-Dicer should be studied. Since the RNAi-enhancing activity of enoxacin depends on TRBP, and because TRBP-Dicer binding is facilitated by the helicase domain of Dicer, in addition to enoxacin's ability to inhibit helicases, it may be that enoxacin acts as a stabilizer of TRBP-Dicer through its helicase binding domain, rather than through allosteric modulation inducing a conformational change on a different binding site (MacRae et al. 2008; Shan et al. 2008; Zhang et al. 2008; Lau et al. 2009; Cao et al. 2017). Adding to support these studies, also the binding of TRBP and pre-miRNAs has been elucidated and, thus, performing biophysical and structural biological studies on the precise binding of enoxacin in complex with Dicer-TRBP and possibly a pre-miRNA, could enable structure-activity relationship assisted screening, or *de novo* design, of better compounds to enhance this interaction (Benoit et al. 2013).

Additionally, the interactions of Dicer with its other notably interacting proteins, such as PACT and PCBP2 should be studied to see if affecting their interaction could have therapeutic effects, as is hypothesized by the TRBP-Dicer enhancing effect of enoxacin (Lee et al. 2006; Shan et al. 2008)).

RNAi kinetics and stoichiometry

Improving the effect size, could be done by changing to a more sensitive (brighter) reporter protein and by expressing additional siRNAs or adding binding sites for the siRNA. As shown by Mukherji et al. (2011), adding binding sites for miRNAs can have up to 10-fold changes in repression on the target mRNA. Or conversely, switching to higher expression of the pre-miRNA, changing the siRNA to a partial complementary miRNA and optimising with additional binding sites if expression were to saturate binding.

However, as can be seen from the studies, the models factor different variables in their predictions and only give estimates on the kinetics, and the notable discrepancies between siRNA and miRNA kinetics should be assessed and taken into account when measuring their effect (Cuccato et al. 2011; Mukherji et al. 2011). This is especially important considering the assay established in this work. The construct has a siRNA, and is subject to siRNA kinetics, but is implemented into a pri-miRNA backbone and thus is processed by Dicer much more efficiently than from a dsRNA (Chakravarthy et al. 2010). This may cause that intact Dicer processing combined to low expression is efficient enough so that additional TRBP-mediated enhancement won't have a significant effect, *i.e.* there isn't enough processable pre-miRNA for Dicer activation to cause any change, in contrast to a situation where Dicer is dysfunctional or deficient. This is supported by the fact that the optimum RNAi-enhancing effect by enoxacin was observed at a siRNA concentration of 8.4×10^{-10} M and to achieve the same RNAi knockdown efficiency, enoxacin can reduce the amount of siRNA by 2-5-fold (Zhang et al. 2008). It is not known if the amount to make this concentration is even produced by the induction of the T-Rex cells herein.

One important thing to consider is when affecting gene expression rather than a protein's mechanistic function, is that the maximal effect takes longer (48-72 h) to reach. This is why the earlier time points (24-48 h) may have not given positive results, due to false negatives and later time points (>72 h) may give false positives (Birmingham et al. 2009). Considering

why the assays created by Shan, Li and colleagues in 2008 and 2012 which used 48 hours of incubation, were validated to work, may be due to the differences in constructs, as they have a shRNA targeting the reporter, opposed to the pri-miRNA used here. These are transcribed (pol II vs pol III) and processed differently (pri-miRNA first by Drosha) which may alter kinetics and affect turnover times and account for the differences in results.

As mentioned, serum deprivation was used, although serendipitously, as means to downregulate Dicer, but it may not be enough to downregulate into Dicer deficient disease mimicking levels. Therefore, one thing to consider would be the use of Dicer deficient cell lines, such as cancer cell lines know to be hemizygous for Dicer (Kumar et al. 2007; Foulkes et al. 2014).

In conclusion, regarding only Dicer kinetics in terms of RNAi and not considering other steps, an ideal assay construct to measure Dicer activity dependent miRNA dysregulations would have 1-3 miRNAs (e.g. could also be expressed with a bidirectional promoter which would produce the right amount of expression) with the miRNAs targeting a bright fluorescent protein, or have more binding sites in the reporter transcript, using a Dicer deficient cell line. Regarding purely Dicer kinetics, a dsRNA would be better than a pri/pre-miRNA so that activation would have a larger effect due to slower processing. However, if this were to be used to screen drugs for miRNA dysregulations, it assumes dsRNA and pre-miRNA binding and processing is similar enough so that compounds which are found to activate in the assay would also work *in vivo* to reduce miRNA dysregulation associated diseases, which may not be the case. As to why it is in any case better to have a miRNA instead of a siRNA, is the reason for the more physiological role of miRNAs in mammals. It is not well known how the functions of miRNAs and siRNAs exerted on mRNA, *i.e.* degradation mostly with siRNAs compared to additional translational repression of miRNAs, may affect cellular function more generally. These may or may not have some further effects in Dicer or miRNA related dysregulations aimed to model with this assay, making miRNAs a better option from a physiological perspective.

When considering the whole RNAi process, the steps upstream and downstream of Dicer processing in miRNA biogenesis, namely Drosha/DGCR8 processing, exp5 transporting, and miRISC mediated repressing should also be studied in terms of kinetics. This would able the assessment of whether they can act as rate-limiting so that Dicer activation is

redundant if any of the upstream or downstream processes were to be much less effective. For example, overexpression of shRNAs may clog up exp5 transport and additional overexpression of exp5 has been shown to enhance shRNA mediated RNAi which could also be the case in Drosha/DGCR8 or miRISC functioning (Yi et al. 2005).

Future prospects for optimisation

Bioluminescence based approach with *renilla* and firefly luciferases as the reporters is also an option, but most luciferase based approached for measuring RNAi have only been done by transient transfection, which as previously mentioned is not optimal for HTS and may induce variation (Zhang et al. 2008; Watashi et al. 2010). However, a dual luciferase system implemented into an adeno-associated virus and used as a miRNA sensor could be repurposed for a Dicer assay (Tian et al. 2012). Nevertheless, Lemus-Diaz et al. (2017) show that a similar fluorescence reporter system is comparable with a luciferase-based reporter system. Thus, advancing with fluorescent reporter systems, one option would be to use a brighter RFP, such as tandem dimer-(td)Tomato may increase sensitivity and therefore effect size. A brighter reporter could circumvent doxycycline inducing minimal expression and not being able to adjust the assay by controlling expression. On the other hand, tdTomato is not as photostable as mCherry, so it should be taken into account, especially when choosing imaging equipment (Graewe et al. 2009).

Moreover, depending on the imaging platform, other options would be to use cyan fluorescent protein (CFP) and enhanced yellow fluorescent protein (EYFP) and different channels, or new blue-shifted engineered variants of fluorescent proteins, if the imaging platform was prone to overlapping of channels, for example (Molina et al. 2017). Additional reporters could allow to monitor even more processes at once and increase amount of data acquired from the assay. However CFP has shown to be photosensitive and subject to bleaching, thus making an RFP/GFP couple much better suited than CFP/EYFP (Tramier et al. 2006). Furthermore, engineered fluorescent proteins, such as mCherry variants, with minimal toxicity should be considered which could help with variability caused by potential toxic effects or reporters on cells (Shen et al. 2017).

Although variation between replicates was already quite low, showed by low standard deviations and good CV values (<10), the variation could have been further reduced by first

calculating the ratio of mCherry and EGFP in single cells and then proceeding to calculate the mean of these ratios, opposed to counting the mean first and ratio after as has been done in these analyses. However, clumping of the cells, as previously mentioned, affected the detection capabilities of the CellProfiler pipeline and could have skewed the results, and therefore calculating whole area or aggregate fluorescence, as was done, from an image would still have been a better option.

Recently published miRNA sensors could be repurposed to measure Dicer activity (Lemus-Diaz et al. 2017). The construct Lemus-Diaz et al. created is quite similar to ours, the main difference being that they express endogenous human miRNAs (miR-27a and miR-19b) and have binding sites for them in the reporter protein transcript (either RFP or CFP), compared to our designed mCherry targeting siRNA. This could help with the physiological relevancy of the assay as mentioned previously.

Taken the previous discussion into consideration, as of right now there is a new and improved construct which has tdTomato instead of mCherry and expresses human miR-19b or human miR-27a and includes binding sites for them in the td-Tomato-NLS-WPRE transcript (Lemus-Diaz et al. 2017). The hypothesis for my work further is that this construct would increase the sensitivity and linear range of the assay, in addition to making it more physiologically relevant.

An important thing for all the dual assays, would be to show further proof of the reporters reporting the actual phenomena aimed to be measured. This could be done by showing the correlation of the expression of reporters and their differences using precise ratiometric bidirectional promoter systems (Sladitschek and Neveu 2016). Further adjusting and optimisation could therefore be done by changing promoters that have similar expression patterns and adding regulatory regions or elements to further stabilize expression, such as was done here with WPRE.

Regarding the lentiviral vector transduced SH-SY5Y cells, further sorting may have enabled selecting an expanding the few mCherry expressing cells but was not continued due to the T-Rex cell line seeming to work. Nevertheless, producing a viral vector would be an interesting avenue in future research for studying the reporter *in vivo*. This could possibly be done by *in vitro* translation, so that the pA sequences would not disturb the packaging (Blo et al. 2008). This is something to be considered when the assay is fully optimised, if

for instance, the new construct with human miRNAs and tdTomato should be more sensitive.

Future prospects for screening

Finding inhibitors, or activators that do not have drug-like properties would not be a loss either, because they could be repurposed to be used as cancer therapeutics, or chemical biology tools to further elucidate Dicer biology, respectively. One thing not to be overlooked is, that not only the activation of Dicer but more generally inducing Dicer, that is upregulating the expression of Dicer instead of, or in addition to, activation, may be beneficial in ameliorating neurodegenerative diseases. As it may be in the case of metformin, although it has not been shown that its neuroprotective effects, e.g. in PD, are mediated through Dicer upregulation (Bayliss et al. 2016; Hooten et al. 2016). Nevertheless, finding compounds which induce, rather than just activate Dicer is desirable, whether it is for therapeutic purposes or not.

It would be interesting to study whether enoxacin, metorfmin or BDNF, would have synergistic effects in the assay, for example metformin upregulating while enoxacin activating and thus enhancing processing further. However, kinetics of miRNA processing and mRNA repression/degradation upstream and downstream of Dicer should be further elucidated, as previously mentioned, to account whether Dicer induction is even meaningful by itself or should also be accompanied by enhancing other key factor of RNAi to have significant effects in non- Dicer deficient states.

Above all, a large-scale screen has been planned in collaboration for 3920 compounds from three different libraries. The assay still needs to be optimised so that the Z-factor is enough to justify large-scale screens, since they are expensive and wasting valuable resources is of interest to no one. However, if the assay cannot be further optimised and the effect of enoxacin is as shown in this study, the screen can be done with more replicates but fewer compounds (5-7 wells and <1000 compounds compared to 2-3 wells and 3920 compounds) without the reliability of the results suffering too much.

Nevertheless, as it is tempting to speculate, if there are hits with drug-like properties found from screening, they will be confirmed and validated with the new construct and additional

in vitro binding studies. It is plausible that hits may be found rather easily, since from a medicinal chemistry perspective, Dicer, due to its RNA-binding interactions, multidomain nature and its actions mediated in concert with the RLC, is a suitable drug target since it may have plenty of binding sites for the drug(s) to exert their action on. The validated hit(s) will be selected as lead compound(s), optimised in collaboration with chemists and studied further in *in vivo* models of PD.

4 CONCLUSIONS

Dicer is a promising drug target for many miRNA dysregulation associated diseases, predominantly cancers linked to Dicer deficiencies and aging-related neurodegenerative disease, particularly ALS and PD (Kumar et al. 2007; Emde et al. 2015; Chmielarz et al. 2017). Inducing, *i.e.* activating or upregulating, Dicer could help prevent global loss of miRNA -related stress to cells and promote the survival of cells with stress-related degenerative phenotypes. Therefore, compounds which selectively induce Dicer could have therapeutic value.

This work aimed to establish an assay to screen for Dicer-activating compounds. Dicer processing of pre-miRNA and the subsequent silencing of a fluorescent reporter was achieved. The assay is functional to some extent, as mCherry/EGFP fluorescence ratio reduction was statistically significant after 72 hours administration of enoxacin, a known Dicer stimulating compound (Shan et al. 2008). Screening ten pharmacologically active compounds with the assay did not find any compounds to significantly reduce mCherry/EGFP fluorescence ratio. However, metformin and BDNF, compounds previously shown to have the ability to elevate Dicer levels, showed similar results to enoxacin, the positive control (Huang et al. 2012; Hooten et al. 2016). Enoxacin, BDNF and metformin should be further studied regarding their possible synergistic effects as well.

Assessing the quality of the assay in terms of HTS, the Z-factor and CV indicate that the assay is robust in terms of variability but does not measure differences between the controls clearly enough to support large-scale screening with thousands of compounds.

Taken together, my thesis work provided further knowledge in the development of Dicer and miRNA processing related screening assays. Regarding the assay, because the effect of enoxacin was not robust, more optimisation is still needed with a better construct, other positive controls, and possibly clonal selection of cells if large-scale high-throughput screening were to be conducted with minimal resources.

5 REFERENCES

Abell NS, Mercado M, Caneque T, Rodriguez R, Xhemalce B: Click Quantitative Mass Spectrometry Identifies PIWIL3 as a Mechanistic Target of RNA Interference Activator Enoxacin in Cancer Cells. J Am Chem Soc 139: 1400-1403, 2017

Adams D, Suhr OB, Dyck PJ, Litchy WJ, Leahy RG, Chen J, Gollob J, Coelho T: Trial design and rationale for APOLLO, a Phase 3, placebo-controlled study of patisiran in patients with hereditary ATTR amyloidosis with polyneuropathy. BMC Neurol 17: 181, 2017

Asada K, Canestrari E, Fu X, Li Z, Makowski E, Wu YC, Mito JK, Kirsch DG, Baraban J, Paroo Z: Rescuing dicer defects via inhibition of an anti-dicing nuclease. Cell Rep 9: 1471-1481, 2014

Asada K, Canestrari E, Paroo Z: A druggable target for rescuing microRNA defects. Bioorg Med Chem Lett 26: 4942-4946, 2016

Asada S, Takahashi T, Isodono K, Adachi A, Imoto H, Ogata T, Ueyama T, Matsubara H, Oh H: Downregulation of Dicer expression by serum withdrawal sensitizes human endothelial cells to apoptosis. Am J Physiol Heart Circ Physiol 295: H2512-2521, 2008

Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004

Bayliss JA, Lemus MB, Santos VV, Deo M, Davies JS, Kemp BE, Elsworth JD, Andrews ZB: Metformin Prevents Nigrostriatal Dopamine Degeneration Independent of AMPK Activation in Dopamine Neurons. PLoS One 11: e0159381, 2016

Benoit MP, Imbert L, Palencia A, Perard J, Ebel C, Boisbouvier J, Plevin MJ: The RNA-binding region of human TRBP interacts with microRNA precursors through two independent domains. Nucleic Acids Res 41: 4241-4252, 2013

Bernstein E, Caudy AA, Hammond SM, Hannon GJ: Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409: 363-366, 2001

Bethune J, Artus-Revel CG, Filipowicz W: Kinetic analysis reveals successive steps leading to miRNA-mediated silencing in mammalian cells. EMBO Rep 13: 716-723, 2012

Biedler JL, Helson L, Spengler BA: Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. Cancer Res 33: 2643-2652, 1973

Birmingham A, Selfors LM, Forster T, Wrobel D, Kennedy CJ, Shanks E, Santoyo-Lopez J, Dunican DJ, Long A, Kelleher D, Smith Q, Beijersbergen RL, Ghazal P, Shamu CE: Statistical methods for analysis of high-throughput RNA interference screens. Nat Methods 6: 569-575, 2009

Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA: SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol Cell Biol 20: 9018-9027, 2000

Blo M, Micklem DR, Lorens JB: Enhanced gene expression from retroviral vectors. BMC Biotechnol 8: 19, 2008

Borchert GM, Lanier W, Davidson BL: RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13: 1097-1101, 2006

Boudreau RL, Davidson BL: RNAi therapeutics for CNS disorders. Brain Res 1338: 112-121, 2010

Bray MA, Carpenter A: Advanced Assay Development Guidelines for Image-Based High Content Screening and Analysis. Assay Guidance Manual, 2004

Burkhard K, Shapiro P: Use of Inhibitors in the Study of MAP Kinases. Map Kinase Signaling Protocols, Second Edition 661: 107-122, 2010

Cai X, Hagedorn CH, Cullen BR: Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10: 1957-1966, 2004

Cao S, Sun R, Wang W, Meng X, Zhang Y, Zhang N, Yang S: RNA helicase DHX9 may be a therapeutic target in lung cancer and inhibited by enoxacin. Am J Transl Res 9: 674-682, 2017

Carmell MA, Hannon GJ: RNase III enzymes and the initiation of gene silencing. Nat Struct Mol Biol 11: 214-218, 2004

Carpenter AE, Jones TR, Lamprecht MR, Clarke C, Kang IH, Friman O, Guertin DA, Chang JH, Lindquist RA, Moffat J, Golland P, Sabatini DM: CellProfiler: image analysis software for identifying and quantifying cell phenotypes. Genome Biol 7: R100, 2006

Chakravarthy S, Sternberg SH, Kellenberger CA, Doudna JA: Substrate-specific kinetics of Dicer-catalyzed RNA processing. J Mol Biol 404: 392-402, 2010

Chennathukuzhi VM, Kurihara Y, Bray JD, Hecht NB: Trax (translin-associated factor X), a primarily cytoplasmic protein, inhibits the binding of TB-RBP (translin) to RNA. J Biol Chem 276: 13256-13263, 2001

Chiosea S, Jelezcova E, Chandran U, Acquafondata M, McHale T, Sobol RW, Dhir R: Up-regulation of dicer, a component of the MicroRNA machinery, in prostate adenocarcinoma. Am J Pathol. 169: 1812-1820, 2006

Chiu YL, Dinesh CU, Chu CY, Ali A, Brown KM, Cao H, Rana TM: Dissecting RNA-interference pathway with small molecules. Chem Biol 12: 643-648, 2005

Chmielarz P, Konovalova J, Najam SS, Alter H, Piepponen TP, Erfle H, Sonntag KC, Schutz G, Vinnikov IA, Domanskyi A: Dicer and microRNAs protect adult dopamine neurons. Cell Death Dis 8: e2813, 2017

Cuccato G, Polynikis A, Siciliano V, Graziano M, di Bernardo M, di Bernardo D: Modeling RNA interference in mammalian cells. BMC Syst Biol 5: 19, 2011

Cullen BR: Transcription and processing of human microRNA precursors. Mol Cell 16: 861-865, 2004

Cullen BR: Is RNA interference involved in intrinsic antiviral immunity in mammals? Nat Immunol 7: 563-567, 2006

Davies BP, Arenz C: A homogenous assay for micro RNA maturation. Angew Chem Int Ed Engl 45: 5550-5552, 2006

Delon A, Bouquet S, Huguet F, Brunet V, Courtois P, Couet W: Pharmacokinetic-pharmacodynamic contributions to the convulsant activity of fluoroquinolones in rats. Antimicrob Agents Chemother 43: 1511-1515, 1999

Devasthanam AS, Tomasi TB: Dicer protein levels elevated by mild hyperthermia promote a pro-survival phenotype. Oncotarget 8: 67001–67016, 2017

Dias C, Feng J, Sun H, Shao NY, Mazei-Robison MS, Damez-Werno D, Scobie K, Bagot R, LaBonte B, Ribeiro E, Liu X, Kennedy P, Vialou V, Ferguson D, Pena C, Calipari ES, Koo JW, Mouzon E, Ghose S, Tamminga C, Neve R, Shen L, Nestler EJ. Beta-catenin mediates stress resilience through Dicer1/microRNA regulation. Nature 516: 51-55, 2014

Doyle M, Badertscher L, Jaskiewicz L, Guttinger S, Jurado S, Hugenschmidt T, Kutay U, Filipowicz W: The double-stranded RNA binding domain of human Dicer functions as a nuclear localization signal. RNA 19: 1238-1252, 2013

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T: Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411: 494-498, 2001

Ellwanger DC, Buttner FA, Mewes HW, Stumpflen V: The sufficient minimal set of miRNA seed types. Bioinformatics 27: 1346-1350, 2011

Emde A, Eitan C, Liou LL, Libby RT, Rivkin N, Magen I, Reichenstein I, Oppenheim H, Eilam R, Silvestroni A, Alajajian B, Ben-Dov IZ, Aebischer J, Savidor A, Levin Y, Sons R, Hammond SM, Ravits JM, Moller T, Hornstein E: Dysregulated miRNA biogenesis downstream of cellular stress and ALS-causing mutations: a new mechanism for ALS. EMBO J 34: 2633-2651, 2015

Ender C, Krek A, Friedlander MR, Beitzinger M, Weinmann L, Chen W, Pfeffer S, Rajewsky N, Meister G: A human snoRNA with microRNA-like functions. Mol Cell 32: 519-528, 2008

Fabian MR, Mathonnet G, Sundermeier T, Mathys H, Zipprich JT, Svitkin YV, Rivas F, Jinek M, Wohlschlegel J, Doudna JA, Chen CY, Shyu AB, Yates JR, 3rd, Hannon GJ, Filipowicz W, Duchaine TF, Sonenberg N: Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell 35: 868-880, 2009

Fan F, Wood KV: Bioluminescent assays for high-throughput screening. Assay Drug Dev Technol 5: 127-136, 2007

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806-811, 1998

Foulkes WD, Priest JR, Duchaine TF: DICER1: mutations, microRNAs and mechanisms. Nat Rev Cancer 14: 662-672, 2014

Gazon H, Belrose G, Terol M, Meniane JC, Mesnard JM, Césaire R, Peloponese JM Jr: Impaired expression of DICER and some microRNAs in HBZ expressing cells from acute adult T-cell leukemia patients. Oncotarget. 7: 30258-30275, 2016

Ghosh S, Kaushik A, Khurana S, Varshney A, Singh AK, Dahiya P, Thakur JK, Sarin SK, Gupta D, Malhotra P, Mukherjee SK, Bhatnagar RK: An RNAi based high-throughput screening assay to identify small molecule inhibitors of Hepatitis B Virus replication. J Biol Chem 292: 12577-12588, 2017

Giacomotto J, Segalat L: High-throughput screening and small animal models, where are we? Br J Pharmacol 160: 204-216, 2010

Graewe S, Retzlaff S, Struck N, Janse CJ, Heussler VT: Going live: a comparative analysis of the suitability of the RFP derivatives RedStar, mCherry and tdTomato for intravital and in vitro live imaging of Plasmodium parasites. Biotechnol J 4: 895-902, 2009

Graham FL, Smiley J, Russell WC, Nairn R: Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J Gen Virol 36: 59-74, 1977

Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R: Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 123: 631-640, 2005

Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R: The Microprocessor complex mediates the genesis of microRNAs. Nature 432: 235-240, 2004

Gruss P, Dhar R, Khoury G: Simian virus 40 tandem repeated sequences as an element of the early promoter. Proc Natl Acad Sci U S A 78: 943-947, 1981

Haase AD, Jaskiewicz L, Zhang H, Laine S, Sack R, Gatignol A, Filipowicz W: TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep 6: 961-967, 2005

Hammond SM, Bernstein E, Beach D, Hannon GJ: An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404: 293-296, 2000

Hansen TB, Jensen TI, Clausen BH, Bramsen JB, Finsen B, Damgaard CK, Kjems J: Natural RNA circles function as efficient microRNA sponges. Nature 495: 384-388, 2013

Harwig A, Kruize Z, Yang Z, Restle T, Berkhout B: Analysis of AgoshRNA maturation and loading into Ago2. PLoS One 12: e0183269, 2017

Hooten NN, Martin-Montalvo A, Dluzen DF, Zhang YQ, Bernier M, Zonderman AB, Becker KG, Gorospe M, de Cabo R, Evans MK: Metformin-mediated increase in DICER1 regulates microRNA expression and cellular senescence. Aging Cell 15: 572-581, 2016

Huang YW, Ruiz CR, Eyler EC, Lin K, Meffert MK: Dual regulation of miRNA biogenesis generates target specificity in neurotrophin-induced protein synthesis. Cell 148: 933-946, 2012

Huntley TE, Neitzel JK, Elson MK: Binding properties of purified adult and fetal bovine serum albumin. Biochim Biophys Acta. 490: 112-119, 1997

Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD: A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293: 834-838, 2001

Irwin CR, Farmer A, Willer DO, Evans DH: In-fusion(R) cloning with vaccinia virus DNA polymerase. Methods Mol Biol 890: 23-35, 2012

Jevsinek Skok D, Godnic I, Zorc M, Horvat S, Dovc P, Kovac M, Kunej T: Genome-wide in silico screening for microRNA genetic variability in livestock species. Anim Genet 44: 669-677, 2013

Jiang Z, Kong C, Zhang Z, Zhu Y, Zhang Y, Chen X: Reduction of protein kinase C alpha (PKC-alpha) promote apoptosis via down-regulation of Dicer in bladder cancer. J Cell Mol Med 19: 1085-1093, 2015

Jones TR, Kang IH, Wheeler DB, Lindquist RA, Papallo A, Sabatini DM, Golland P, Carpenter AE: CellProfiler Analyst: data exploration and analysis software for complex image-based screens. BMC Bioinformatics 9: 482, 2008

Kalcheim C, Gendreau M: Brain-derived neurotrophic factor stimulates survival and neuronal differentiation in cultured avian neural crest. Brain Res 469: 79-86, 1988

Kaneko H, Dridi S, Tarallo V, Gelfand BD, Fowler BJ, Cho WG, Kleinman ME, Ponicsan SL, Hauswirth WW, Chiodo VA, Kariko K, Yoo JW, Lee DK, Hadziahmetovic M, Song Y, Misra S, Chaudhuri G, Buaas FW, Braun RE, Hinton DR, Zhang Q, Grossniklaus HE, Provis JM, Madigan MC, Milam AH, Justice NL, Albuquerque RJ, Blandford AD, Bogdanovich S, Hirano Y, Witta J, Fuchs E, Littman DR, Ambati BK, Rudin CM, Chong MM, Provost P, Kugel JF, Goodrich JA, Dunaief JL, Baffi JZ, Ambati J: DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. Nature 471: 325-330, 2011

Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH: Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev 15: 2654-2659, 2001

Klein R, Ruttkowski B, Knapp E, Salmons B, Gunzburg WH, Hohenadl C: WPRE-mediated enhancement of gene expression is promoter and cell line specific. Gene 372: 153-161, 2006

Kobayashi M, Tanaka A, Hayashi Y, Shimamura S: The CMV enhancer stimulates expression of foreign genes from the human EF-1 alpha promoter. Anal Biochem 247: 179-181, 1997

Koh JY, Wie MB, Gwag BJ, Sensi SL, Canzoniero LM, Demaro J, Csernansky C, Choi DW: Staurosporine-induced neuronal apoptosis. Exp Neurol 135: 153-159, 1995

Koivisto E, Jurado Acosta A, Moilanen AM, Tokola H, Aro J, Pennanen H, Sakkinen H, Kaikkonen L, Ruskoaho H, Rysa J: Characterization of the regulatory mechanisms of activating transcription factor 3 by hypertrophic stimuli in rat cardiomyocytes. PLoS One 9: e105168, 2014

Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N: Combinatorial microRNA target predictions. Nat Genet 37: 495-500, 2005

Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M: Silencing of microRNAs in vivo with 'antagomirs'. Nature 438: 685-689, 2005

Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T: Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet 39: 673-677, 2007

Kurek A, Kucharczyk M, Detka J, Slusarczyk J, Trojan E, Glombik K, Bojarski B, Ludwikowska A, Lason W, Budziszewska B: Pro-apoptotic Action of Corticosterone in Hippocampal Organotypic Cultures. Neurotox Res 30: 225-238, 2016

Kurzynska-Kokorniak A, Pokornowska M, Koralewska N, Hoffmann W, Bienkowska-Szewczyk K, Figlerowicz M: Revealing a new activity of the human Dicer DUF283 domain in vitro. Sci Rep 6: 23989, 2016

Lai EC, Wiel C, Rubin GM: Complementary miRNA pairs suggest a regulatory role for miRNA:miRNA duplexes. RNA 10: 171-175, 2004

Lau PW, Guiley KZ, De N, Potter CS, Carragher B, MacRae IJ: The molecular architecture of human Dicer. Nat Struct Mol Biol 19: 436-440, 2012

Lau PW, Potter CS, Carragher B, MacRae IJ: Structure of the human Dicer-TRBP complex by electron microscopy. Structure 17: 1326-1332, 2009

Le Pichon CE, Meilandt WJ, Dominguez S, Solanoy H, Lin H, Ngu H, Gogineni A, Sengupta Ghosh A, Jiang Z, Lee SH, Maloney J, Gandham VD, Pozniak CD, Wang B, Lee S, Siu M, Patel S, Modrusan Z, Liu X, Rudhard Y, Baca M, Gustafson A, Kaminker J, Carano RAD, Huang EJ, Foreman O, Weimer R, Scearce-Levie K, Lewcock JW: Loss of dual leucine zipper kinase signaling is protective in animal models of neurodegenerative disease. Sci Transl Med 9, 2017

Lee RC, Ambros V: An extensive class of small RNAs in Caenorhabditis elegans. Science 294: 862-864, 2001

Lee RC, Feinbaum RL, Ambros V: The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75: 843-854, 1993

Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN: The nuclear RNase III Drosha initiates microRNA processing. Nature 425: 415-419, 2003

Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN. The role of PACT in the RNA silencing pathway. EMBO J. 25: 522–532, 2006

Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, Kim VN: MicroRNA genes are transcribed by RNA polymerase II. EMBO J 23: 4051-4060, 2004

Lemus-Diaz N, Boker KO, Rodriguez-Polo I, Mitter M, Preis J, Arlt M, Gruber J: Dissecting miRNA gene repression on single cell level with an advanced fluorescent reporter system. Sci Rep 7: 45197, 2017

Lewis BP, Burge CB, Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20, 2005

Li Y, Ji P, Jin P: Probing the microRNA pathway with small molecules. Bioorg Med Chem 21: 6119-6123, 2013

Li Y, Lin L, Li Z, Ye X, Xiong K, Aryal B, Xu Z, Paroo Z, Liu Q, He C, Jin P: Iron homeostasis regulates the activity of the microRNA pathway through poly(C)-binding protein 2. Cell Metab 15: 895-904, 2012

Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM: Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433: 769-73, 2005

Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ: Argonaute2 is the catalytic engine of mammalian RNAi. Science 305: 1437-1441, 2004

Liu X, Qu X, Wu C, Zhai Z, Tian B, Li H, Ouyang Z, Xu X, Wang W, Fan Q, Tang T, Qin A, Dai K: The effect of enoxacin on osteoclastogenesis and reduction of titanium particle-induced osteolysis via suppression of JNK signaling pathway. Biomaterials 35: 5721-5730, 2014

Londin E, Loher P, Telonis AG, Quann K, Clark P, Jing Y, Hatzimichael E, Kirino Y, Honda S, Lally M, Ramratnam B, Comstock CE, Knudsen KE, Gomella L, Spaeth GL, Hark L, Katz LJ, Witkiewicz A, Rostami A, Jimenez SA, Hollingsworth MA, Yeh JJ, Shaw CA, McKenzie SE, Bray P, Nelson PT, Zupo S, Van Roosbroeck K, Keating MJ, Calin GA, Yeo C, Jimbo M, Cozzitorto J, Brody JR, Delgrosso K, Mattick JS, Fortina P, Rigoutsos I: Analysis of 13 cell types reveals evidence for the expression of numerous novel primate- and tissue-specific microRNAs. Proc Natl Acad Sci U S A 112: E1106-15, 2015

Ludtke JJ, Zhang G, Sebestyen MG, Wolff JA: A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. J Cell Sci 112 : 2033-2041, 1999

Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U: Nuclear export of microRNA precursors. Science 303: 95-98, 2004

Ma E, MacRae IJ, Kirsch JF, Doudna JA: Autoinhibition of human dicer by its internal helicase domain. J Mol Biol 380: 237-243, 2008

Machitani M, Sakurai F, Wakabayashi K, Tomita K, Tachibana M, Mizuguchi H: Dicer functions as an antiviral system against human adenoviruses via cleavage of adenovirus-encoded noncoding RNA. Sci Rep 6: 27598, 2016

MacRae IJ, Ma E, Zhou M, Robinson CV, Doudna JA: In vitro reconstitution of the human RISC-loading complex. Proc Natl Acad Sci U S A 105: 512-527, 2008

Macrae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, Adams PD, Doudna JA: Structural basis for double-stranded RNA processing by Dicer. Science 311: 195-198, 2006

Magner WJ, Weinstock-Guttman B, Rho M, Hojnacki D, Ghazi R, Ramanathan M, Tomasi TB: Dicer and microRNA expression in multiple sclerosis and response to interferon therapy. J Neuroimmunol 292: 68-78, 2016

Mayr C, Bartel DP: Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. Cell 138: 673-684, 2009

McDade E, Bateman RJ: Stop Alzheimer's before it starts. Nature 547: 153-155, 2017

McKiney JM, Irwin N, Flatt PR, Bailey CJ, McClenaghan NH: Acute and long-term effects of metformin on the function and insulin secretory responsiveness of clonal beta-cells. Biol Chem 391: 1451-1459, 2010

Michel PP, Hefti F: Toxicity of 6-hydroxydopamine and dopamine for dopaminergic neurons in culture. J Neurosci Res 26: 428-435, 1990

Minones-Moyano E, Porta S, Escaramis G, Rabionet R, Iraola S, Kagerbauer B, Espinosa-Parrilla Y, Ferrer I, Estivill X, Marti E: MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet 20: 3067-3078, 2011

Molina RS, Tran TM, Campbell RE, Lambert GG, Salih A, Shaner NC, Hughes TE, Drobizhev M: Blue-Shifted Green Fluorescent Protein Homologues Are Brighter than Enhanced Green Fluorescent Protein under Two-Photon Excitation. J Phys Chem Lett 8: 2548-2554, 2017

Moreland RB, Nam HG, Hereford LM, Fried HM: Identification of a nuclear localization signal of a yeast ribosomal protein. Proc Natl Acad Sci U S A 82: 6561-6565, 1985

Mori MA, Raghavan P, Thomou T, Boucher J, Robida-Stubbs S, Macotela Y, Russell SJ, Kirkland JL, Blackwell TK, Kahn CR: Role of microRNA processing in adipose tissue in stress defense and longevity. Cell Metab 16: 336-347, 2012

Morin RD, O'Connor MD, Griffith M, Kuchenbauer F, Delaney A, Prabhu AL, Zhao Y, McDonald H, Zeng T, Hirst M, Eaves CJ, Marra MA: Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. Genome Res 18: 610-621, 2008

Mukherji S, Ebert MS, Zheng GX, Tsang JS, Sharp PA, van Oudenaarden A: MicroRNAs can generate thresholds in target gene expression. Nat Genet 43: 854-859, 2011

Mullokandov G, Baccarini A, Ruzo A, Jayaprakash AD, Tung N, Israelow B, Evans MJ, Sachidanandam R, Brown BD: High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries. Nat Methods 9: 840-846, 2012

Pang X, Hogan EM, Casserly A, Gao G, Gardner PD, Tapper AR: Dicer expression is essential for adult midbrain dopaminergic neuron maintenance and survival. Mol Cell Neurosci 58: 22-28, 2014

Park JE, Heo I, Tian Y, Simanshu DK, Chang H, Jee D, Patel DJ, Kim VN: Dicer recognizes the 5' end of RNA for efficient and accurate processing. Nature 475: 201-205, 2011

Piatek MJ, Werner A: Endogenous siRNAs: regulators of internal affairs. Biochem Soc Trans 42: 1174-1179, 2014

Plate L, Cooley CB, Chen JJ, Paxman RJ, Gallagher CM, Madoux F, Genereux JC, Dobbs W, Garza D, Spicer TP, Scampavia L, Brown SJ, Rosen H, Powers ET, Walter P, Hodder P, Wiseman RL, Kelly JW: Small molecule proteostasis regulators that reprogram the ER to reduce extracellular protein aggregation. Elife 5, 2016

Podolska K, Sedlak D, Bartunek P, Svoboda P: Fluorescence-based high-throughput screening of dicer cleavage activity. J Biomol Screen 19: 417-426, 2014

Post LE, Norrild B, Simpson T, Roizman B: Chicken ovalbumin gene fused to a herpes simplex virus alpha promoter and linked to a thymidine kinase gene is regulated like a viral gene. Mol Cell Biol 2: 233-240, 1982

Qiu Y, Xu Y, Zhang Y, Zhou H, Deng YQ, Li XF, Miao M, Zhang Q, Zhong B, Hu Y, Zhang FC, Wu L, Qin CF, Zhou X: Human Virus-Derived Small RNAs Can Confer Antiviral Immunity in Mammals. Immunity 46: 992-1004 e5, 2017

Qu C, Li W, Shao Q, Dwyer T, Huang H, Yang T, Liu G: c-Jun N-terminal kinase 1 (JNK1) is required for coordination of netrin signaling in axon guidance. J Biol Chem 288: 1883-1895, 2013

Redmann M, Benavides GA, Berryhill TF, Wani WY, Ouyang X, Johnson MS, Ravi S, Barnes S, Darley-Usmar VM, Zhang J: Inhibition of autophagy with bafilomycin and chloroquine decreases mitochondrial quality and bioenergetic function in primary neurons. Redox Biol 11: 73-81, 2017

Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Schell J, Reiss B, Maas C: Enhanced green fluorescence by the expression of an Aequorea victoria green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc Natl Acad Sci U S A 93: 5888-5893, 1996

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G: The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403: 901-906, 2000

Reyes GR, Gavis ER, Buchan A, Raj NB, Hayward GS, Pitha PM: Expression of human beta-interferon cDNA under the control of a thymidine kinase promoter from herpes simplex virus. Nature 297: 598-601, 1982

Rodriguez-Blazquez C, Forjaz MJ, Lizan L, Paz S, Martinez-Martin P: Estimating the direct and indirect costs associated with Parkinson's disease. *Expert Rev* Pharmacoecon Outcomes Res 15: 889-911, 2015

Rogers TB, Inesi G, Wade R, Lederer WJ: Use of thapsigargin to study Ca2+ homeostasis in cardiac cells. Biosci Rep 15: 341-349, 1995

Ross RJ, Weiner MM, Lin H: PIWI proteins and PIWI-interacting RNAs in the soma. Nature 505: 353-359, 2014

Rossbach M: Small non-coding RNAs as novel therapeutics. Curr Mol Med 10: 361-368, 2010

Salimi A, Roudkenar MH, Sadeghi L, Mohseni A, Seydi E, Pirahmadi N, Pourahmad J: Ellagic acid, a polyphenolic compound, selectively induces ROS-mediated apoptosis in cancerous B-lymphocytes of CLL patients by directly targeting mitochondria. Redox Biol 6: 461-471, 2015

Sampson TR, Debelius JW, Thron T, Janssen S, Shastri GG, Ilhan ZE, Challis C, Schretter CE, Rocha S, Gradinaru V, Chesselet MF, Keshavarzian A, Shannon KM, Krajmalnik-Brown R, Wittung-Stafshede P, Knight R, Mazmanian SK: Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease. Cell 167: 1469-1480 e12, 2016

Schmiedel JM, Klemm SL, Zheng Y, Sahay A, Bluthgen N, Marks DS, van Oudenaarden A: Gene expression. MicroRNA control of protein expression noise. Science 348: 128-132, 2015

Schratt G: microRNAs at the synapse. Nat Rev Neurosci 10: 842-849, 2009

Shan G, Li Y, Zhang J, Li W, Szulwach KE, Duan R, Faghihi MA, Khalil AM, Lu L, Paroo Z, Chan AW, Shi Z, Liu Q, Wahlestedt C, He C, Jin P: A small molecule enhances RNA interference and promotes microRNA processing. Nat Biotechnol 26: 933-940, 2008

Shen Y, Chen Y, Wu J, Shaner NC, Campbell RE: Engineering of mCherry variants with long Stokes shift, red-shifted fluorescence, and low cytotoxicity. PLoS One 12: e0171257, 2017

Shin D, Shin JY, McManus MT, Ptacek LJ, Fu YH: Dicer ablation in oligodendrocytes provokes neuronal impairment in mice. Ann Neurol 66: 843-857, 2009

Simunovic F, Yi M, Wang Y, Stephens R, Sonntag KC: Evidence for gender-specific transcriptional profiles of nigral dopamine neurons in Parkinson disease. PLoS One 5: e8856, 2010

Sinha NK, Iwasa J, Shen PS, Bass BL: Dicer uses distinct modules for recognizing dsRNA termini. Science 359: 329-334, 2017

Sinkkonen L, Hugenschmidt T, Filipowicz W, Svoboda P: Dicer is associated with ribosomal DNA chromatin in mammalian cells. PLoS One 5: e12175, 2010

Sladitschek HL, Neveu PA: Bidirectional Promoter Engineering for Single Cell MicroRNA Sensors in Embryonic Stem Cells. PLoS One 11: e0155177, 2016

Smalheiser NR, Zhang H, Dwivedi Y: Enoxacin Elevates MicroRNA Levels in Rat Frontal Cortex and Prevents Learned Helplessness. Front Psychiatry 5: 6, 2014

Stringer JR: Construction of a viable simian virus 40 variant that carries a poly[d(GT) . d(CA)] insertion. J Virol 53: 698-701, 1985

Taira E, Finkenstadt PM, Baraban JM; Identification of translin and trax as components of the GS1 strand-specific DNA binding complex enriched in brain. J Neurochem 71: 471-477, 1998

Tian W, Dong X, Liu X, Wang G, Dong Z, Shen W, Zheng G, Lu J, Chen J, Wang Y, Wu Z, Wu X: High-throughput functional microRNAs profiling by recombinant AAV-based microRNA sensor arrays. PLoS One 7: e29551, 2012

Tjio JH, Puck TT: Genetics of somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. J Exp Med 108: 259-268, 1958

Tramier M, Zahid M, Mevel JC, Masse MJ, Coppey-Moisan M: Sensitivity of CFP/YFP and GFP/mCherry pairs to donor photobleaching on FRET determination by fluorescence lifetime imaging microscopy in living cells. Microsc Res Tech 69: 933-939, 2006

Valianatos G, Valcikova B, Growkova K, Verlande A, Mlcochova J, Radova L, Stetkova M, Vyhnakova M, Slaby O, Uldrijan S: A small molecule drug promoting miRNA processing induces alternative splicing of MdmX transcript and rescues p53 activity in human cancer cells overexpressing MdmX protein. PLoS One 12: e0185801, 2017

Varol D, Mildner A, Blank T, Shemer A, Barashi N, Yona S, David E, Boura-Halfon S, Segal-Hayoun Y, Chappell-Maor L, Keren-Shaul H, Leshkowitz D, Hornstein E,

Fuhrmann M, Amit I, Maggio N, Prinz M, Jung S: Dicer Deficiency Differentially Impacts Microglia of the Developing and Adult Brain. Immunity 46: 1030-1044 e8, 2017

Vasudevan S, Tong Y, Steitz JA: Switching from repression to activation: microRNAs can up-regulate translation. Science 318: 1931-1934, 2007

Vinnikov IA, Domanskyi A: Can we treat neurodegenerative diseases by preventing an age-related decline in microRNA expression? Neural Regen Res 12: 1602-1604, 2017

Wang HW, Noland C, Siridechadilok B, Taylor DW, Ma E, Felderer K, Doudna JA, Nogales E: Structural insights into RNA processing by the human RISC-loading complex. Nat Struct Mol Biol 16: 1148-1153, 2009

Wang J, Samuels DC, Zhao S, Xiang Y, Zhao YY, Guo Y: Current Research on Non-Coding Ribonucleic Acid (RNA). Genes (Basel) 8, 2017

Watashi K, Yeung ML, Starost MF, Hosmane RS, Jeang KT: Identification of small molecules that suppress microRNA function and reverse tumorigenesis. J Biol Chem 285: 24707-24716, 2010

Weick EM, Miska EA: piRNAs: from biogenesis to function. Development 141: 3458-3471, 2014

Wen Z, Zhang J, Tang P, Tu N, Wang K, Wu G: Overexpression of miR185 inhibits autophagy and apoptosis of dopaminergic neurons by regulating the AMPK/mTOR signaling pathway in Parkinson's disease. Mol Med Rep 17: 131-137, 2018

Wiesen JL, Tomasi TB: Dicer is regulated by cellular stresses and interferons. Mol Immunol 46: 1222-1228, 2009

Wightman B, Ha I, Ruvkun G: Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855-862, 1993

Wilson RC, Doudna JA: Molecular mechanisms of RNA interference. Annu Rev Biophys 42: 217-239, 2013

Wingo AP, Almli LM, Stevens JS, Klengel T, Uddin M, Li Y, Bustamante AC, Lori A, Koen N, Stein DJ, Smith AK, Aiello AE, Koenen KC, Wildman DE, Galea S, Bradley B, Binder EB, Jin P, Gibson G, Ressler KJ: DICER1 and microRNA regulation in post-traumatic stress disorder with comorbid depression. Nat Commun 6: 10106, 2015

Xiao T, Liu R, Proud CG, Wang MW: A high-throughput screening assay for eukaryotic elongation factor 2 kinase inhibitors. Acta Pharm Sin B 6: 557-563, 2016

Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, Nishikura K: Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol 13: 13-21, 2006

Yi R, Doehle BP, Qin Y, Macara IG, Cullen BR: Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. RNA 11: 220-226, 2005

Yi R, Qin Y, Macara IG, Cullen BR: Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17: 3011-3016, 2003

Yuan J, Wang X, Zhang Y, Hu X, Deng X, Fei J, Li N: shRNA transcribed by RNA Pol II promoter induce RNA interference in mammalian cell. Mol Biol Rep 33: 43-49, 2006

Zhang G, Gurtu V, Kain SR: An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. Biochem Biophys Res Commun 227: 707-711, 1996

Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W: Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J 21: 5875-5885, 2002

Zhang Q, Zhang C, Xi Z: Enhancement of RNAi by a small molecule antibiotic enoxacin. Cell Res 18: 1077-1079, 2008

Zufferey R, Donello JE, Trono D, Hope TJ: Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J Virol 73: 2886-2892, 1999

APPENDIX 1

Plasmid maps of produced plasmids.

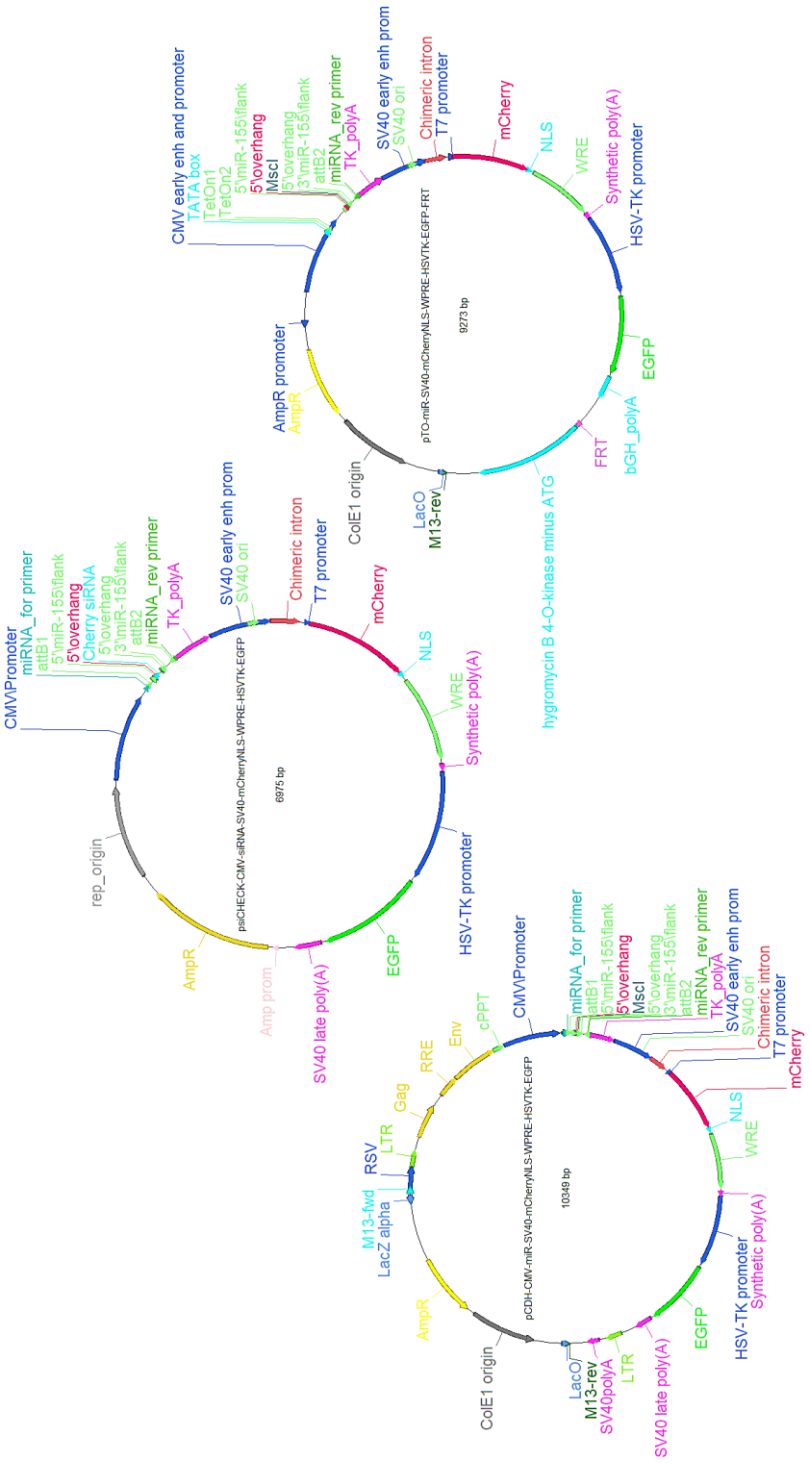

Figure 21. Plasmid maps of plasmids produced in this work as they are displayed in A plasmid Editor software. psiCHECK is the reporter plasmid, pCDH was used for producing the lentiviral vector and pTO was used for integrating the reporter plasmid into the FLP-IN 293 T-Rex cells' genome.

APPENDIX 2

Doxycycline titration experiments using doxycycline-inducible cells. Two independent experiments using FLP-IN 293 T-Rex cells to assess optimal expression of mCherry siRNA.

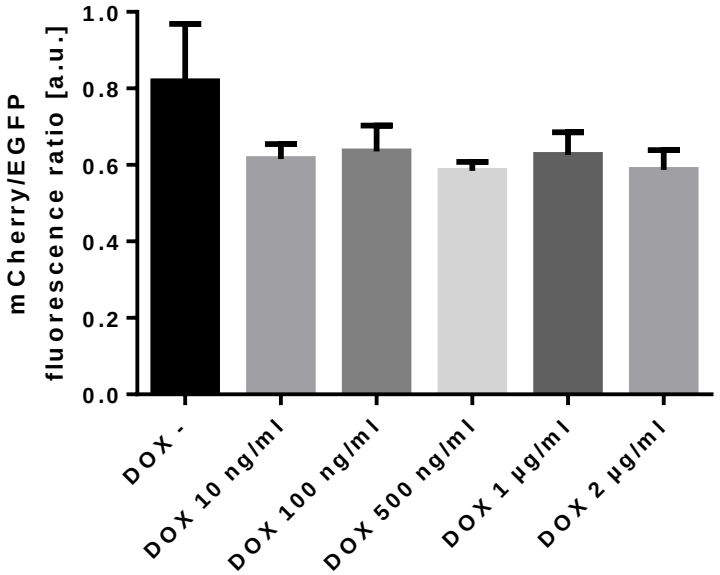

Figure 22. Doxycycline titration using concentrations between 10-2000 ng/ml. There were no significant differences in mCherry/EGFP fluoresence ratio between doxycycline treated groups. Data are expressed as mean +/- SD, n=6 (wells). DOX = doxycycline.

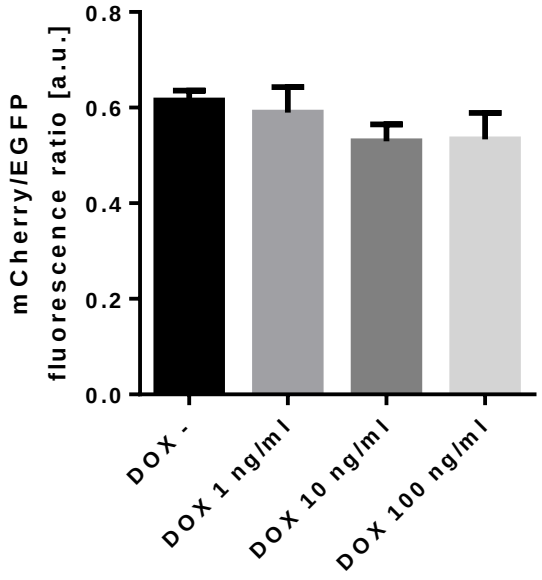

Figure 23. Doxycycline titration using concentrations between 1-100 ng/ml. The difference in mCherry/EGFP fluoresence ratio between groups was minimal. 1 ng/ml was comparable to medium. Data are expressed as mean +/- SD, n=5 (wells). DOX = doxycycline.