Characterisation of angiogenin mediated paracrine neuroprotection in in vitro models

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Citation
Characterisation of angiogenin mediated paracrine neuroprotection in \textit{in vitro} models

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Dissertation submitted to the School of Postgraduate Studies, Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfillment of the degree of Doctor of Philosophy

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Dr. Marion C. Hogg

June 2017
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Student Number 12191833

Date June 22, 2017
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<td>AA-free DMEM</td>
<td>amino acid-free dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>AHA</td>
<td>L-Azidohomoalanine</td>
</tr>
<tr>
<td>Akt</td>
<td>serine/threonine-specific protein kinase</td>
</tr>
<tr>
<td>Ala</td>
<td>alanin</td>
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<tr>
<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
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<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
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<tr>
<td>ANG</td>
<td>angiogenin</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptotic protease-activating factor 1</td>
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<td>APS</td>
<td>ammonium persulfate</td>
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<td>AR</td>
<td>autosomal-recessive</td>
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<tr>
<td>Arg</td>
<td>arginine</td>
</tr>
<tr>
<td>Asp</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>AuD</td>
<td>autosomal-dominant</td>
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<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
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<td>Bak</td>
<td>Bcl-2 homologous antagonist/killer</td>
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<td>Bcl-2 associated x</td>
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<td>Bcl-XL</td>
<td>Bcl-2-like protein 1</td>
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<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>Bim</td>
<td>Bcl-2 interacting mediator</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaPO₄</td>
<td>calcium phosphate</td>
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<tr>
<td>CCT complex</td>
<td>chaperonin containing T-complex polypeptide complex</td>
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<td>CHX</td>
<td>cycloheximide</td>
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<tr>
<td>CM</td>
<td>conditioned medium</td>
</tr>
<tr>
<td>cNB</td>
<td>complete neurobasal medium</td>
</tr>
<tr>
<td>Col4a</td>
<td>collagen 4a</td>
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<td>Cyt c</td>
<td>cytochrome c</td>
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<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<td>DDX proteins</td>
<td>DEAD-box (asp-glu-ala-asp) proteins</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DIV</td>
<td>day in vitro</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
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<tr>
<td>EAAT2</td>
<td>excitatory amino acid transporter 2</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF</td>
<td>eukaryotic initiation factor</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>eRF</td>
<td>eukaryotic release factor</td>
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<td>FALS</td>
<td>familiar amyotrophic lateral sclerosis</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FALS</td>
<td>frontotemporal lobar degeneration</td>
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<tr>
<td>FUS/TLS</td>
<td>fused in sarcoma/translocated in liposarcoma protein</td>
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<tr>
<td>G3BP</td>
<td>GTPase activating protein (SH3 domain) binding protein 1</td>
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<tr>
<td>G418</td>
<td>genetin</td>
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<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<td>GFAP</td>
<td>glial fibrillary acid protein</td>
</tr>
<tr>
<td>Gly</td>
<td>glycine</td>
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<tr>
<td>HBSS</td>
<td>hank's balanced salt solution</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HRE</td>
<td>hexanucleotide repeat expansion</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
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<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
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<tr>
<td>ITAF</td>
<td>IRES trans-acting factor</td>
</tr>
<tr>
<td>Mcl-1</td>
<td>BCL2 family apoptosis regulator</td>
</tr>
<tr>
<td>MCM proteins</td>
<td>mini-chromosome maintenance proteins</td>
</tr>
<tr>
<td>miR</td>
<td>micro ribonucleic acid</td>
</tr>
<tr>
<td>MOCK</td>
<td>transfection without DNA/RNA - only transfection reagent</td>
</tr>
<tr>
<td>mRNPs</td>
<td>messenger ribonucleoproteins</td>
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<tr>
<td>NLS</td>
<td>nuclear localisation sequence</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptor</td>
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<tr>
<td>NSun2</td>
<td>tRNA (cytosine(34)-C(5))-methyltransferase</td>
</tr>
<tr>
<td>OptiMEM</td>
<td>reduced-serum minimal essential medium</td>
</tr>
<tr>
<td>p53</td>
<td>tumor suppressor protein p53</td>
</tr>
<tr>
<td>PBs</td>
<td>processing bodies</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson’s disease</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>Pol I &amp; III</td>
<td>RNA polymerase I &amp; III</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PUMA</td>
<td>p53 upregulated modulator of apoptosis</td>
</tr>
<tr>
<td>RAN</td>
<td>repeat associated non-ATG</td>
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<tr>
<td>rhANG</td>
<td>recombinant human angiogenin</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RNH1</td>
<td>placental ribonuclease inhibitor</td>
</tr>
<tr>
<td>RNU6B</td>
<td>U6 spliceosomal RNA</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ROX</td>
<td>carboxy-X-rhodamine</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>S100b</td>
<td>S100 calcium-binding protein B</td>
</tr>
<tr>
<td>SALS</td>
<td>sporadic amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>SGs</td>
<td>stress granules</td>
</tr>
<tr>
<td>SMI-32</td>
<td>neurofilament heavy chain protein</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase 1</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TDP-43</td>
<td>TAR DNA-binding protein 43</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylendiamin</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T-cell intracellular antigen-1</td>
</tr>
<tr>
<td>tiRNA</td>
<td>tRNA-derived, stress-induced RNA</td>
</tr>
<tr>
<td>TOG</td>
<td>terminal oligo-guanine</td>
</tr>
<tr>
<td>tRF</td>
<td>tRNA-derived fragments</td>
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<td>tRNA</td>
<td>transfer ribonucleic acid</td>
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<tr>
<td>Val</td>
<td>valine</td>
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<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>Y-box binding protein 1</td>
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Summary

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disorder affecting motoneurons. Mutations in ANG, encoding a member of the pancreatic RNase A superfamily, segregate with ALS. Angiogenin is an acute-phase protein, which is secreted from motoneurons under stress conditions. Our group demonstrated that it is taken up by astrocytes where it induces RNA cleavage and mediates neuroprotection in paracrine. It has been shown that angiogenin preferably cleaves tRNAs within the anticodon loop to produce tRNA-derived, stress-induced RNAs (tiRNAs). The functions of tiRNAs are unclear; they have been shown to modulate various pathways, including apoptosis, gene expression and protein translation.

This study was performed to gain insights into the functions of angiogenin with focus on ribonucleolytic activity and to investigate whether specific tiRNA are responsible for the function of angiogenin. Stable SH-SY5Y cells overexpressing wild-type angiogenin and ALS-associated mutants ANG K40I and R31K were generated to investigate the effect of these mutations on angiogenin activity, tiRNA generation, and secretion. We demonstrated for the first time that ANG R31K exhibits an aberrant secretion, which is a novel function and adds new insights into R31K-missense associated ALS pathology.

Overexpressed wild-type angiogenin and R31K mutant were ribonucleolytic active and generated specific tiRNAs, such as 5´Val, whereas other tiRNAs (5´Ala) were not detected. We illustrated that 5´Val tiRNA was increased in the supernatant of the ANG WT and R31K cell lines compared to ribonucleolytic-inactive K40I mutant and control cell lines. Our findings reveal that tiRNAs are secreted, and may exert functions similar to miRNAs, which can be delivered to neighbouring cells where they regulate multiple targets. Furthermore, secreted tiRNAs might be employed as biomarkers to identify early stages of stress-induced neuronal degradation.
Functional analysis of tiRNAs demonstrated that 5`Val tiRNA protected SH-SY5Y cells against proteasomal stress. However, this protection was not mediated by SG formation. Investigations into whether angiogenin functions are mediated by tiRNAs illustrated that neither protection against hypoxia in primary motoneurons nor enhanced protein translation in primary astrocytes was mediated by synthetic tiRNAs. These results suggest that either tiRNA modifications are absent in synthetic tiRNAs or that a pool of tiRNAs are essential for mimicking the effect of angiogenin.
Acknowledgements

I would like to thank Prof. Jochen H. M. Prehn for his support and giving me the opportunity to carry out this research and complete my PhD. I would also like to thank Dr. Marion C. Hogg for all the advice, discussions, and re-reading and correcting the thesis.

Thanks to Heiko Duessmann for the introduction into fluorescent imaging and his help in the image processing and analysis. I would like to thank Sean Kilbride, Caoimhin Concannon and Christian Hellwig for the introduction into flow cytometry. Furthermore, many thanks to Karen, Marion and Sinead for teaching me the preparation and purification techniques of primary cultures.

Special thanks to all members and former-members of the ALS group HG, Marion, Karen, Sinead, Megan, Saidhbhe, Luise and Martin. Big thanks to Bea, Sinead, Damir, Janis, Robbie, Niamh, Franzi, and Steven for correcting the thesis as well.

I would like to thank all the friends I made in the department and the rest of RCSI though all the years! Thanks to the Gym buddies Fede, Damir and Jonny, and my Dicey’s partner James for being patient with me during the last months.

Thanks to my family, im besonderen „Jateka Haspa”. Danke, dass du stets fuer unsere Eltern da bist und dich um all ihre kleineren und groesseren Probleme kuemmerst. Und naturlich danke ich meiner „Baraschka”! Danke, dass du es mit mir ausgehalten hast wahrend dieser Zeit, mich stets ermutigt hast am Ball zu bleiben und mit Gelassenheit an Schwierigkeiten zu gehen.

Finally, thanks to the Thierry Latran Fondation for funding this research.
1. Chapter 1: Introduction

1.1 Etiology of ALS

Amyotrophic lateral sclerosis (ALS) was first described by J. M. Charcot and A. Joffory in 1869 (Charcot, J. M. & Joffory 1869). ALS is a relentlessly progressive, neurodegenerative disease that affects various parts of the motor system. The loss of motoneurons causes rapidly progressive weakness due to muscle atrophy, and muscle spasticity (Mulder 1982). Different disease onset phenotypes are reported: (i) limb-onset, that affects the upper motoneurons in the motor cortex with signs in the limbs; (ii) bulbar-onset, presenting difficulties with speech and swallowing, and delayed development of limb features; and the less common phenotypes affecting purely (iii) upper motoneurons or (iv) lower motoneurons (Gordon et al. 2006). ALS patients can also show degeneration in prefrontal and temporal cortex neurons to varying degrees (Ringholz et al. 2005), which results in frontotemporal dementia and is known as ALS with frontotemporal lobar degeneration (FTLD). The age of clinical onset of ALS is highly variable but on average it appears at the age of 55 years with prevalence of 0.6-2.4/100,000 population ((Rippon et al. 2006), (Andersen et al. 1997)). Most patients eventually die from respiratory failure. Survival ranges from months to decades, but is usually less than three years after symptom onset (Logroscino et al. 2008). These large differences in age at disease onset and survival exist even between members of families with the same genetic mutation, suggesting there are influences that modify the phenotype (Régal et al. 2006). A broad spectrum of mechanisms have been shown to contribute to ALS pathogenesis, such as excitotoxicity, mitochondrial dysfunction, protein misfolding and aggregation, impaired axonal transport, oxidative stress, disrupted RNA homeostasis and neuroinflammation (reviewed in (Goodall & Morrison 2006), (Ling et al. 2013)). The only licenced therapy to date is Riluzole, which prolongs survival by a few months on average, although when given at an early stage or to younger patients, it has been shown to
be more effective ((Bensimon et al. 1994), (Lacomblez et al. 1996)). Studies have shown that Riluzole decreases neuronal firing and hyper-excitability by several mechanisms – by reducing glutamate release ((Mizoule et al. 1985), (Jehle et al. 2000)), inhibiting post-synaptic NMDA and kainate glutamate receptors (Debono et al. 1993), and blocking inactivated voltage-dependent sodium channels ((Benoit & Escande 1991), (Hebert et al. 1994)).

1.2 Proposed pathogenic mechanisms in ALS

In an interesting mathematical approach, (Al-Chalabi et al. 2014) showed a linear correlation between log of age-specific incidence (5-year age groups) and log age in all five population registers. Applying the results to an established model used in cancer research (ARMITAGE & DOLL 1954), the authors suggested that ALS is a six-step process. This hypothesis suggests that the pathogenic cause underlies the accumulation of different risk factors such as (1) genetic defects, (2) environmental risk factors, (3) injuries, (4) smoking, (5) age etc. Although this hypothesis has yet to be proven, it may explain the variability of onset age and survival even within the same family, and why genetic changes seemingly in unrelated pathways cause the same pathological phenotype in ALS.

1.2.1 Genetic causes of ALS

ALS is traditionally categorised in two groups: familial ALS (FALS) and sporadic ALS (SALS). Approximately 5% of all ALS cases are FALS and 95% are SALS (Byrne et al. 2011). Patients with no familial ALS background are classified to have the sporadic form. Misclassification can result from incomplete family history, non-paternity and incomplete penetrance. However, both groups have similar symptoms and progression. FALS is predominantly inherited in an autosomal-dominant (AuD) manner. Nevertheless, a few very rare cases also demonstrated autosomal-recessive (AR) inheritance, since only mutations in both alleles contributed to ALS pathogenesis (most common missense mutations are summarised in Table 1-1).
### Table 1-1: Most common genetic causes of FALS.

<table>
<thead>
<tr>
<th>Cell biological functions</th>
<th>Gene</th>
<th>Protein</th>
<th>Mode of inheritance</th>
<th>Estimated % of FALS</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA regulation</td>
<td><strong>TARDBP</strong></td>
<td>TDP-43</td>
<td>AuD</td>
<td>1–5%</td>
<td>(Sreedharan et al. 2008), (Rutherford et al. 2008)</td>
</tr>
<tr>
<td>FUS/TLS</td>
<td><strong>FUS/TLS</strong></td>
<td>FUS/TLS</td>
<td>AuD</td>
<td>3-5%</td>
<td>(Kwiatkowski et al. 2009), (Vance et al. 2009)</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiogenin</td>
<td>AuD</td>
<td>&lt;1%</td>
<td></td>
<td>(Greenway et al. 2006), (Wu et al. 2007), (Gellera et al. 2008), (Paubel et al. 2008)</td>
</tr>
<tr>
<td>SETX</td>
<td>Senataxin</td>
<td>AuD</td>
<td>&lt;1%</td>
<td></td>
<td>(Rabin et al. 1999), (De Jonghe et al. 2002)</td>
</tr>
<tr>
<td>C9ORF72</td>
<td>C9ORF72 protein</td>
<td>AuD</td>
<td>23%-40%</td>
<td></td>
<td>(DeJesus-Hernandez et al. 2011), (Renton et al. 2011)</td>
</tr>
<tr>
<td>Proteostasis, vesicle</td>
<td>OPTN</td>
<td>Optineurin</td>
<td>AR</td>
<td>&lt;1%</td>
<td>(Maruyama et al. 2010)</td>
</tr>
<tr>
<td>proteostasis, vesicle</td>
<td><strong>UBQLN2</strong></td>
<td>Ubiquilin 2</td>
<td>X-linked dominant</td>
<td>&lt;1%</td>
<td>(Deng et al. 2011)</td>
</tr>
<tr>
<td>dynamics,</td>
<td><strong>VCP</strong></td>
<td>Valosin-containing</td>
<td>AuD</td>
<td>&lt;1%</td>
<td>(Johnson et al. 2010)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>VAPB</strong></td>
<td>Vesicle-associated</td>
<td>AuD</td>
<td>&lt;1%</td>
<td>(Nishimura et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>membrane protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>ALS2</strong></td>
<td>Alsin</td>
<td>AR</td>
<td>&lt;1%</td>
<td>(Hentati et al. 1994), (Yang et al. 2001)</td>
</tr>
<tr>
<td></td>
<td><strong>SOD1</strong></td>
<td>Superoxide</td>
<td>AuD</td>
<td>20%</td>
<td>(Rosen et al. 1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dismutase 1 (Cu-Zn)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
It is important to highlight how the discovery of specific mutations influenced ALS research. The identification of a SOD1 mutation in ALS patients (Rosen et al. 1993) pushed the research to develop different SOD1 models such as the transgenic SOD1\textsuperscript{G93A} mouse model (Gurney et al. 1994). However, many therapies evaluated in preclinical models failed in clinical trials (Ludolph & Jesse 2009). Indeed, the discovery of TDP-43 and FUS pathologies and the fact that SOD1 mutations were excluded (detailed in chapters 1.2.2 and 1.2.3) suggested that SOD1 pathology is distinct from other types of ALS ((Mackenzie et al. 2007), (Maekawa et al. 2009)). Identification of the C9ORF72-repeat expansion in ALS and FTLD cases linked the two clinical syndromes together ((DeJesus-Hernandez et al. 2011), (Renton et al. 2011)). C9ORF72-repeat length typically contains 23 G\textsubscript{4}C\textsubscript{2} repeats in health controls, whereas expansions in ALS/FTD patients range from hundreds to more than 1000 repeats. This was the first time that a large intronic repeat expansion had been associated with ALS. Since repeat expansions are known to disrupt RNA metabolism in other neurodegenerative diseases, such as Huntington’s disease, fragile X syndrome of mental retardation, and myotonic dystrophy type1&2 (reviewed in (Gatchel & Zoghbi 2005)), it highlighted the importance of RNA homeostasis in motor and frontal cortex neurons. Moreover, the fact that the repeat expansion has been also found in SALS cases (~7%) implicates a genetic etiology and suggests familial and sporadic classification should not be viewed in absolute terms.

Another interesting aspect was driven by the finding of (van Blitterswijk et al. 2012). The authors screened FALS and SALS patients for mutations in TARDBP, FUS/TLS, SOD1, ANG and C9orf72 and identified five FALS families and three “SALS” patients with identical TARDBP mutation (p.N352S). Genealogical and haplotype analyses revealed that these individuals shared a common ancestor. Further analysis revealed that 50% of these individuals (7 patients) had an additional mutation in ANG, C9ORF72 or TARDBP. The authors proposed a model for oligogenic inheritance, which suggests that mutations in two or more genes are required for an individual to develop the disease. This finding also led to discussions about the design of genome-wide association studies (GWAS).
design of the new studies is changing from minor allele frequency of >5% in the general population, which aimed to determine the maximum variation across the genome, to minor allele frequency of <5% located in the coding regions of the genome of ALS patients, which examines whether multiple rare variants responsible for ALS pathogenesis (reviewed in (Renton et al. 2014)).

1.2.2 Protein misfolding and aggregation

Several neurodegenerative diseases reveal abnormal protein aggregations in the central nervous system, such as Parkinson’s disease (PD), Alzheimer’s disease (AD), Huntington’s disease and ALS. In all of these diseases, aggregated proteins are sequestered in discrete structures called inclusion bodies in the brain, which can be located in the cytoplasm, nucleus or extracellularly (reviewed in (Ross & Poirier 2004)). Protein aggregation can result from different factors: (i) mutation in the sequence of the disease-related protein; (ii) genetic alterations that cause an elevation in the protein amounts; (iii) missing- or hyper-post-translational modification; (iv) proteasomal dysfunction; or (v) triggered by environmental stress or aging. Presently it is uncertain whether inclusion bodies cause the disease associated toxicity or whether their formation is a consequence and represents a protective cellular response (reviewed in (Ross & Poirier 2005)). The consequence of protein sequestration is the loss or reduction of specific functions and, in some cases, formation of inclusion bodies leads to gain of novel toxic properties.

Superoxide dismutase 1 (SOD1) is an antioxidant enzyme. Missense and C-terminal truncation mutations of SOD1 lead to misfolding and prion-like aggregations, resulting in a gain of toxicity in the cell ((Bruijn et al. 1998), (Bendotti et al. 2012)). Research conducted on SOD1-mediated toxicity revealed that those aggregations could lead to proteasome stress, impaired ion-homeostasis, neuroinflammation, and disrupted axonal transport (reviewed in (Goodall & Morrison 2006)).

A striking finding was reported by (Neumann et al. 2006), where the authors identified hyper-phosphorylated, ubiquitinated, and N-terminal cleaved TAR DNA-
binding protein 43 (TDP-43) as a major component of neuronal cytoplasmic inclusions in FTD cortical neurons and in ALS spinal motoneurons. Later studies revealed that TDP-43 positive inclusions are present in 97% of ALS and ~40% of FTLD cases ((Mackenzie et al. 2007), (Maekawa et al. 2009), (Cairns et al. 2007)). Fused in sarcoma/translocated in liposarcoma protein (FUS/TLS) is another DNA/RNA-binding protein which has been detected as component of cellular inclusions in 1-5% of ALS and up to 10% of FTLD ((Vance et al., 2009), (Kwiatkowski et al., 2009)). Interestingly, the main exceptions for both proteins were cases which exhibited SOD1 mutations, authors hypothesised that the SOD1-associated pathogenic pathway is distinct from FUS/TLS and TDP-43 proteinopathies (Deng et al. 2010). However, the low prevalence of FUS/TLS and TDP-43 mutations in ALS and rare incidences in FTLD cases cannot be the only reason for abnormal TDP-43 and FUS/TLS aggregations. Furthermore, because FUS/TLS and TDP-43 are predominantly nuclear proteins with DNA and RNA binding properties associated with regulating transcription, splicing and RNA transport ((Buratti & Baralle 2008), (Rogelj et al. 2012)), it was suggested that errors in RNA processing may be central to ALS and FTD pathogenesis (Ling et al. 2013).

1.2.3 RNA homeostasis

Identification of TDP-43 and FUS/TLS mutations and abnormal TDP-43 and FUS/TLS aggregations in ALS and FTLD initiated a research paradigm shift to the investigation of RNA metabolism dysfunction as underlying pathogenesis of multiple neurodegenerative diseases (Lagier-Tourenne et al. 2010). Together with ALS-linked mutations in proteins involved in RNA processing and binding such as angiogenin (Greenway et al. 2006) and senataxin (Chen et al. 2004), and the discovery of the hexanucleotide repeat expansion (HRE) mutation in the C9ORF72 gene ((DeJesus-Hernandez et al. 2011), (Renton et al. 2011)), impaired RNA homeostasis might play a central role in ALS pathogenesis.

The HRE GGGGCC (G4C2) C9orf72 mutation induces disruption of nucleocytoplasmic transport by forming nuclear RNA foci ((Zhang et al. 2015),
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(Mizielinska et al. 2013)). (Fratta et al. 2012) showed that G-quadruplexes are formed out of G$_4$C$_2$-sequences (sketch shown in Figure 1.1). Furthermore, it was demonstrated that G$_4$C$_2$ expansions can be translated into multiple dipeptide repeat proteins (DRP) by a non-ATG translation mechanism (Gendron et al. 2013). Repeat associated non-ATG (RAN) translation was initially discovered in spinocerebellar ataxia type 8, a slowly progressive neurodegenerative disorder caused by CAG and CTG repeat expansions (Zu et al. 2011). The authors suggested a hairpin-forming structure, similar to IRES sites. However, translation occurred in all the three reading frames without an ATG initiation codon. RAN translation is a novel mechanism and more research has to be conducted to determine precise RNA structure and associated factors. To investigate the DRP pathology, (Y.-J. Zhang et al. 2016) generated C9ORF72 expansion carrying mice which showed a poly(GA) aggregation phenotype. Furthermore, the authors demonstrated that this aggregation led to sequestration of nucleocytoplasmic transport proteins and impaired nuclear import. Since nuclear RNA foci and DRP generation both result in destruction of nuclear transport, C9orf72 mutations might be linked with FUS/TLS and TDP-43 cytosolic localisation and aggregation.

Another interesting finding is that FUS/TLS and TDP-43 aggregates co-localise with stress granules (SG) markers ((Dormann et al. 2010), (Liu-Yesucevitz et al. 2010)). SGs are cytoplasmic foci, induced by several stress conditions, that repress cap-dependent protein translation (discussed in detail in section 1.6). However, it is still uncertain whether FUS/TLS and TDP-43 aggregation is SG independent or whether SG formation initiates/"seeds" the pathological inclusions (reviewed in (Dewey et al. 2012)).
Figure 1.1: Proposed G-quadruplexes C9orf72 HRE structure. Four separate guanosines are predicted to form a G-quartet. A (GGGGCC)$_4$ sequence will form an antiparallel G-quadruplex structure. G-quartets layers are formed between the guanosines, whereas cytosines form loop structures (adapted from (Haeusler et al. 2014)).

1.2.4 Excitotoxicity in ALS

It is well known that glutamate is the principal excitatory transmitter in the vertebrate nervous system (reviewed in (Meldrum 2000)). Activation of postsynaptic-neuronal glutamate receptors results in the influx of sodium and calcium ions into the cell, leading to depolarization and subsequently to the generation of an action potential (Rothman 1985). Since its discovery, experimental studies have suggested that excitotoxicity could contribute to neuronal damage in stroke, neurotrauma, epilepsy, and a number of neurodegenerative disorders including ALS (reviewed in (Doble 1999), (Coyle & Puttfarcken 1993)). Motoneurons are especially vulnerable to glutamate and concomitant calcium influx, since they have low calcium buffering properties and high AMPA/kainate receptor density (Vandenberghe et al. 2000). Excitotoxicity can be distinguished into two types (i) classical and (ii) slow progression. Classical excitotoxicity is the death of neurons that occurs after acute elevation of extracellular glutamate, whereas slow excitotoxicity refers to neuronal degeneration of weakened postsynaptic neurons during normal glutamate concentrations.

A mechanism of a classical excitotoxicity mediated form of ALS could be caused by oral intake of excitotoxins. In the early 1950s, (KURLAND & MULDER 1954)
reported an ALS prevalence ratio in the Guam population of about 200/100,000 Guam, and therefore 50-100 times higher than in the rest of the world. Subsequent analysis of post-mortem ALS brain tissue revealed an increase of β-methylamino-L-alanine (BMAA) (Murch et al. 2004). BMAA is a neurotoxin which is produced by cyanobacteria (Cox et al. 2005) and functions as a NMDA and AMPA receptor agonist ((Ross et al. 1987), (Copani et al. 1991)). Oral administration of BMAA to monkeys resulted in corticomo-neuronal dysfunction, parkinsonian features, and behavioural anomalies, with degenerative changes of motor neurons in cerebral cortex and spinal cord (Spencer et al. 1987) (Figure 1.2A).

For slow excitotoxicity, there are several examples which demonstrate how these mechanisms could weaken neurons. Disturbance of mitochondrial function is a well known example. (Jung et al. 2002) and (Mattiazzi et al. 2002) reported reduced activity in several mitochondrial respiratory chain complexes and diminished ATP synthesis in SOD1<sup>G93A</sup> mice, an established ALS mouse model with a mutation in the superoxide dismutase 1 gene (SOD1). These deficits were prominent in the spinal cord and became more evident during disease progression. As a consequence, ATP levels were insufficient to address the high ATP-demand, especially in the maintenance of ion-homeostasis, and this may lead to slow excitotoxicity-mediated cell death (reviewed in (Bittigau & Ikonomidou 1997)). Mitochondrial dysfunction is also associated with the calcium buffering capacity of these organelles (Damiano et al. 2006). Increased intracellular calcium concentration results in over-activation of calcium-dependent enzymes and this could also lead to neuronal death (reviewed in (von Lewinski & Keller 2005)) (Figure 1.2B).

Studies reported that glutamate levels were elevated in the cerebrospinal fluid of ALS patients ((Spreux-Varoquaux et al. 2002), (Shaw, Forrest, et al. 1995)). Glutamate elevation might be caused by impaired or lost reuptake of glutamate, since (Rothstein et al. 1995) demonstrated that the levels of excitatory amino acid transporter 2 (EAAT2) were severely decreased in motor cortex and spinal cord of ALS patient tissue. These findings might explain abnormalities in glutamate metabolism in ALS patients (Plaitakis & Caroscio 1987) (Figure 1.2D).
1.2.5 Oxidative stress

Another pathogenic mechanism in ALS is due to increased oxidative stress. Several studies reported markers of elevated oxidative stress in ALS, such as oxidative-damaged protein, lipids, and DNA in post-mortem tissue of ALS patients (Shaw, Ince, et al. 1995), (Ferrante et al. 1997)). The source of oxidative stress results from production of reactive oxygen species (ROS). The generation of cellular ROS, primarily the superoxide radical and subsequently hydrogen peroxide, arises due to “leakage” of electrons from the mitochondrial respiratory chain (reviewed in Lenaz et al. 2002). Cells counter this continuous generation of harmful ROS with several anti-oxidant defence mechanisms: (i) catalytic removal of reactive species by enzymes such as superoxide dismutase, catalase, and peroxidase; (ii) purging by agents either synthesised in vivo (such as glutathione, α-keto acids, lipoic acid and coenzyme Q) or incorporated from food (including vitamin C and vitamin E); (iii) utilising proteins and bio-molecules which minimise the availability of pro-oxidants such as transition metals; and (iv) utilising heat-shock proteins which protect or mediate repair of damaged proteins (reviewed in Barry Halliwell and John M. C. Gutteridge 2015).

Investigation into the relationship between dietary factors and ALS revealed that people who regularly consume antioxidant vitamins E and C supplements have reduced risk of developing ALS (Ascherio et al. 2005), (Wang et al. 2011)). Furthermore, epidemiological studies have shown association between self-reported occupational exposure to metals, such as aluminium, arsenic, cadmium, cobalt, copper, lead, manganese, uranium, vanadium, and zinc, and an increased risk of developing ALS (Beckman et al. 1993), (Kamel et al. 2003), (Roos et al. 2013)).

(Dykens 1994) demonstrated a direct link between mitochondrial calcium uptake and ROS production. Since then several studies demonstrated that excessive stimulation of glutamate receptors results in mitochondrial calcium overload and consequently overproduction of ROS and oxidative stress (Urushitani et al. 2001), (Dykens 1994), (Carriedo et al. 2000)), which created a cross-link between
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oxidative stress and excitotoxicity. ROS production could act as a self-reinforcing process - secreted ROS could reduce glial glutamate clearance by damaging the glutamate transporter directly or by aberrant RNA processing (reviewed in (Maragakis & Rothstein 2001)) (Figure 1.2C).

Figure 1.2: Excitotoxic and oxidative cross-talk between motoneurons and astrocytes in ALS pathogenesis. (A) Elevated extracellular glutamate levels or excitotoxins exposure could induce excessive Ca^{2+} uptake through AMPA/kainate channels in motoneurons. (B) Mitochondrial function could be exhausted through Ca^{2+}-overload or SOD1 dysfunction and aggregation, which would result in increased ROS production, reduced ATP generation, and potential activation of apoptotic pathways. (C) The ROS could pass across the motoneuron plasma membrane and impair the function of astrocytic glutamate transporters, in particular excitatory amino acid transporter 2 (EAAT2). (D) Glutamate uptake would be impaired causing an increase in extracellular glutamate levels.
1.2.6 Risk factors for ALS

Epidemiology studies have identified increasing age, gender and a family history of ALS as risk factors for ALS occurrence (reviewed in (Armon 2003), and (Ingre et al. 2015)).

(Huisman et al. 2011) reported that ALS onset before the age of 40 years is rare, but that incidence increases exponentially with increasing age, with peak incidence at the age of 70-79 for men (~12/100,000) and 65-74 for women (~8/100,000). At later ages, the incidence drops again drastically. (Manjaly et al. 2010) correlated age and sex and illustrated a male-to-female incidence ratio of 2.5 (pre-menopause) and a drop to 1.4 (post-menopause) in Italian and English cohorts. These results suggested that hormonal changes associated with menopause might be an explanation for a sex ratio change in ALS. Similar results were observed by (de Jong et al. 2013), where the authors demonstrated positive association of a longer reproductive time-span to susceptibility and survival of ALS patients in a Netherland’s cohort.

1.3 Angiogenin

Angiogenin was first isolated from culture medium conditioned by colon carcinoma cells (Fett et al. 1985). It exhibits 33% sequence identity and 65% sequence homology with bovine pancreatic RNase A and belongs to the pancreatic RNase A superfamily ((Strydom et al. 1985); (Kurachi et al. 1985)). Genes encoding for all pancreatic RNase A superfamily members are located on human chromosome 14 (reviewed (Dyer & Rosenberg 2006)). Each member has a ~20-28 amino acid signal pro-domain, which is common among secretory proteins. Mature angiogenin consists of 123 amino acids and has a molecular weight of 14.1 kDa. The catalytic site between the RNase A superfamily member is conserved - angiogenin exhibits 3 catalytic residues (Figure 1.3, in red) which mediate phosphodiester bond cleavage (Shapiro et al. 1986). However, angiogenin misses the 4th disulfide bond in comparison to bovine pancreatic RNase A, which results in structural changes and a loop formation \(^{60}\text{NKNGNPHREN}^{68}\) (receptor-binding site; Figure 1.3, in
yellow). Site-specific mutations revealed that this loop is essential for internalisation of angiogenin (Moroianu & Riordan 1994b). It was observed that angiogenin binds cell surface actin and an uncharacterised 170-kDa cell surface protein in endothelial cells ((Hu et al. 1993), (Hu et al. 1997)). Furthermore, our group demonstrated that angiogenin is secreted by motoneurons under stress conditions, and selectively internalised by astroglial cells through binding to syndecan 4 followed by clathrin-mediated endocytosis (Skorupa et al. 2012). (Moroianu & Riordan 1994a) identified the nuclear localisation sequence $^{31}$RRRGL$^{35}$ (NLS; Figure 1.3, in green) required to translocate angiogenin to the nucleus. It has been reported that all three sites (receptor-binding site, NLS, and catalytic site) are essential for angiogenin to mediate angiogenesis and cell proliferation (Moroianu & Riordan 1994b).

**Figure 1.3: 3D structure of human angiogenin and chosen ALS related mutations.** High-resolution structure of mature hANG (PDB ID: 4AOH). Active site residues (in red), receptor-binding site (in yellow), and nuclear localisation sequence (in green) are shown. The figure was created using the software PyMOL (http://www.delanoscientific.com).
1.3.1 Angiogenic activity

Angiogenesis is the growth of blood vessels from the existing vasculature. It is a complex process, which requires migration of endothelial cells across the basement membrane into neighbouring tissue to form new blood vessels (Adair & Montani 2010). It was suggested that angiogenin binds the cell surface actin under conditions which require neovascularisation, and activates protease cascades that mediate the mobilisation of endothelial cells. Reduced cell density stimulates the expression of the 170-kDa cell surface protein, internalisation of angiogenin and subsequent cell proliferation (Hu et al. 1997). It has been shown that angiogenin is rapidly translocated to the nucleus in endothelial cells (Hu et al. 2000). (Xu et al. 2003) revealed that angiogenin stimulates rRNA synthesis by binding CT repeats, which are abundant in the non-transcribed region of the ribosomal RNA gene. The minimum number of CT repeats required for angiogenin binding has been shown to be about six. It was reported that down-regulation of endogenous angiogenin resulted in a decrease of rRNA transcription, ribosomal biogenesis and proliferation (Kishimoto et al. 2005). Furthermore, the authors showed that angiogenin-mediated rRNA transcription is essential for basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) to stimulate angiogenesis (compare Figure 1.4).
Figure 1.4: **Angiogenin-stimulated rRNA transcription is essential for angiogenesis stimulation.** Endocytosis of angiogenin is mediated by a 170 kDa cell surface protein in endothelial cells. Angiogenin is translocated to the nucleus where it enhances rRNA transcription and subsequently simulation of bFGF- and VEGF-mediated cell proliferation.

### 1.3.2 Stress-induced tRNA cleavage

(Shapiro et al. 1986) showed that the RNase activity of ANG is $10^5$–$10^6$ lower than that of RNase A in a cell-free system, which may be caused by reduced substrate accessibility through Gln-117 and Thr-44 interaction (Russo et al. 1996).

Angiogenin catalyses a transphosphorylation reaction, which produces a 2’,3’-cyclic phosphate at the 3’-end and a hydroxyl-group at the 5’end of the new generated 3’tRNA ((Raines 1998), (Leland et al. 2002)). Our group and others demonstrated that angiogenin generates specific cleavage patterns with distinct fragments of nucleotide lengths between 30 nt and 47 nt RNA fragments, unlike RNase A which has no sequence specificity ((Skorupa et al. 2012), (Yamasaki et al. 2009)). (Fu et al. 2009) revealed that angiogenin cleaves tRNA in or near the anticodon loop in mammalian cells. The function of cleaved tRNA was guided by the finding that stress-induced cleavage of tRNA promoted survival against starvation in *Tetrahymena thermophila* (Lee & Collins 2005). It was already known
that angiogenin is an acute-phase protein (Verselis et al. 1999) which is up-regulated during hypoxia ((Hartmann et al. 1999), (Nakamura et al. 2006)). Our group demonstrated that angiogenin prevents neuronal death induced by tunicamycin, exposure to 1% O₂, and AMPA induced excitotoxicity, and increased the lifespan of ALS SOD1<sup>G93A</sup> transgenic mice ((Kieran et al. 2008), (Sebastià et al. 2009)). Furthermore, (Saikia et al. 2014) reported that angiogenin-induced accumulation of tiRNA increased the survival of mouse embryonic fibroblasts during hyperosmotic stress. The authors illustrated that specific 5` and 3` tiRNAs bind cytochrome c, and it was proposed that this interaction inhibits apoptosome formation and activity. The Anderson laboratory illustrated in several in vitro experiments that a subset of tRNA-derived, stress-induced RNAs (tiRNAs) induced stress granule formation, and inhibited cap-dependent protein translation by interfering with the assembly of the cap binding complex eukaryotic initiation factor 4F (eIF4F), detailed in section 1.6 ((Yamasaki et al. 2009), (Emara et al. 2010), (Ivanov et al. 2011)). Since internal ribosome entry site (IRES) mediated translation was less affected, and it is known to be employed by genes involved in pro-survival and anti-apoptosis, the Anderson laboratory proposed that the protective properties of angiogenin are mediated by generation of tiRNAs and that these tiRNAs reprogram protein translation to save anabolic energy, enhance damage repair and promote survival. The question is how the ribonucleolytic activity is regulated. (Blanco et al. 2014) revealed that hypomethylation of tRNAs, such as observed in microcephaly patients, results in accumulation of 5`tiRNAs generated by angiogenin, and subsequently leads to decreased cell size and enhanced apoptosis of cortical, hippocampal and striatal neurons. This finding suggests that methylation of tRNA prevents abnormal tRNA cleavage. Another mechanism might be associated with angiogenin localisation and inhibition. (Shapiro & Vallee 1987) demonstrated that angiogenic and ribonucleolytic activities of angiogenin can be inhibited by placental ribonuclease inhibitor (RNH1). RNH1 is a 50-kDa leucine-rich repeat protein which exhibits strong affinity to angiogenin (Kᵢ= 0.29 fM) (Lee & Vallee
(Pizzo et al. 2013) reported dynamic cellular localisation of angiogenin and RNH1. Under growth conditions cytoplasmic angiogenin associates with RNH1, whereas nuclear-located angiogenin did not co-localise with RNH1. In contrast, under stress conditions RNH1 was enriched in the nucleus, whereas angiogenin was mainly localised in the cytoplasm. These findings suggest that RNH1 suppresses ribonucleolytic activity in the cytoplasm during growth conditions, and angiogenic activity in the nucleus during stress conditions to avoid unnecessary rRNA production.

1.3.3 ANG mutations in ALS

The first hint that angiogenin was associated with ALS was the finding that VEGF is a modifier gene associated with motoneuron degeneration in individuals with ALS and in a mouse model of ALS (Lambrechts et al. 2003). In 2004, (Greenway et al. 2004) suggested the first direct link between ANG and ALS, and in 2006 our group identified seven ANG missense mutation in ALS patients (Greenway et al. 2006). Since then, several genetic studies have shown that mutations affecting the ribonucleolytic activity or the nuclear localisation sequence of angiogenin may cause ALS (summarised in Table 1-2 and Figure 1.5). Since ANG mutations cause a loss-of-function, it is likely that impaired function of angiogenin is the reason for ANG associated ALS cases. Furthermore, studies also identified mutations in the signal peptide of the angiogenin precursor. The implications of those mutations were not investigated, although it was suggested that they may affect the secretion of angiogenin (Wu et al. 2007).
Table 1-2: ALS associated missense mutations in human mature ANG. Reported missense mutations of FALS and SALS individuals, and in vitro investigated mutational effects. Ribonucleolytic activity was measured towards tRNA cleavage in a buffered system adapted from (Shapiro et al. 1986).

<table>
<thead>
<tr>
<th>ANG mutation</th>
<th>Residue function</th>
<th>Mutation effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q12L</td>
<td>Interaction with the active site residue Lys40</td>
<td>Structural changes, impaired nuclear translocation (mainly in cytosol), catalytically inactive (3%)</td>
<td>(Greenway et al. 2006), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>K17E/I</td>
<td>Positioned close to active site</td>
<td>Structural change, impaired nuclear translocation (mainly in cytosol), reduced ribonucleolytic activity (19% - K17E; K17I - 13%)</td>
<td>(Greenway et al. 2006), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>D22G</td>
<td>Interaction with catalytic triad member His114</td>
<td>Reduced ribonucleolytic activity (21%)</td>
<td>(Brown et al. 2012), (Padhi et al. 2014)</td>
</tr>
<tr>
<td>S28N</td>
<td>Located in a helix near the active site and close to NLS</td>
<td>Impaired nuclear translocation (mainly in cytosol), reduced ribonucleolytic activity (~20%)</td>
<td>(Wu et al. 2007), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>R31K</td>
<td>Nuclear localisation sequence (NLS - R31-R-R-G-L35)</td>
<td>Delayed nuclear translocation, ribonucleolytic functional (91%)</td>
<td>(Greenway et al. 2006), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>L35P</td>
<td>Interaction with catalytic triad member His114; Nuclear localisation sequence</td>
<td>Structural change, reduced ribonucleolytic activity (35%); not transported into nucleus</td>
<td>(Ueki et al. 2008), (Padhi et al. 2014)</td>
</tr>
<tr>
<td>C39W</td>
<td>Disulfide bridge formation with Cys92 - protein folding/stability</td>
<td>Structural change, catalytically inactive (4%)</td>
<td>(Greenway et al. 2006), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>K40I</td>
<td>Ribonucleolytic activity (catalytic triad His13, Lys40 and His114)</td>
<td>Catalytically inactive (&lt;1%)</td>
<td>(Greenway et al. 2006), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>I46V</td>
<td>Not fully conserved</td>
<td>Catalytically inactive (9%)</td>
<td>(Greenway et al. 2006), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td><strong>ANG mutation</strong></td>
<td><strong>Residue function</strong></td>
<td><strong>Mutation effect</strong></td>
<td><strong>Reference</strong></td>
</tr>
<tr>
<td>------------------</td>
<td>----------------------</td>
<td>---------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>K54E</td>
<td>Protein folding/stability</td>
<td>Structural change, ribonucleolytic functional (80%)</td>
<td>(Fernández-Santiago et al. 2009)</td>
</tr>
<tr>
<td>T80S</td>
<td>Part of a peripheral subsite for binding polynucleotide substrates</td>
<td>Increased ribonucleolytic activity (T80A 1010%; T80D 280%)</td>
<td>(van Es et al. 2011), (Shapiro 1998)</td>
</tr>
<tr>
<td>F100I</td>
<td>Protein folding/stability</td>
<td>Not examined, ribonucleolytic functional (predicted)</td>
<td>(van Es et al. 2011), (Padhi et al. 2013)</td>
</tr>
<tr>
<td>V103I</td>
<td>Protein folding/stability</td>
<td>Not examined, ribonucleolytic functional (predicted)</td>
<td>(Zou et al. 2012), (Padhi et al. 2013)</td>
</tr>
<tr>
<td>P112L</td>
<td>Close proximity to catalytic triad member His114</td>
<td>Impaired nuclear translocation (mainly in cytosol), reduced ribonucleolytic activity (28%)</td>
<td>(Wu et al. 2007), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>V113I</td>
<td>Close proximity to catalytic triad member His114</td>
<td>Ribonucleolytic functional (75%)</td>
<td>(Gellera et al. 2008), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>H114R</td>
<td>Ribonucleolytic activity</td>
<td>Catalytically inactive (2%)</td>
<td>(Gellera et al. 2008), (Thiyagarajan et al. 2012)</td>
</tr>
<tr>
<td>R121H/C</td>
<td>Part of a peripheral subsite for binding polynucleotide substrates</td>
<td>Increased ribonucleolytic activity (155% - R121H)</td>
<td>(Paubel et al. 2008), (Luigetti et al. 2011), (Thiyagarajan et al. 2012)</td>
</tr>
</tbody>
</table>

**Figure 1.5: Schematic representation of mature angiogenin mutations.** All the reported missense mutations in mature angiogenin coding sequence are shown.
1.4 Cap-dependent and cap-independent protein translation

Since angiogenin and angiogenin products have been shown to influence protein translation, it is necessary to cover the major mechanisms and stages of protein translation. Protein translation can be divided in three stages: initiation; elongation; and termination. Protein synthesis is mainly regulated at the initiation stage. There are two major mechanisms of protein synthesis: (i) cap-dependent; and (ii) cap-independent translation pathway. The difference is in the recruitment of 40S ribosome to the internal codon location (reviewed in (Jackson et al. 2010)).

Cap-dependent translation initiation can be divided into two stages and it requires the 7-methylguanylate (m$^7$G) cap at 5’ end of mRNA. (i) mRNA activation through formation and binding of eIF4F complex to m$^7$G cap of the mRNA. eIF4F complex consists of the cap-binding protein eIF4E, the adapter protein eIF4G, and the ATP-dependent RNA helicase eIF4A, which unwinds the secondary structure in the 5′-untranslated region (5′-UTR) of the mRNA. (ii) Formation of the 43S complex followed by the attachment to activated mRNA. 43S complex is composed of: (1) eIF2 ternary complex (eIF2-GTP-tRNA$^{\text{Met}}$); (2) 40S ribosome; (3) eIF-3, which stimulates mRNA recruitment; (4) eIF1 and eIF1A, which induce an open conformation of the 40S ribosome (Figure 1.6A). In the next step 43S complex scans the mRNA downstream to recognise the initiation codon (AUG). After the initiation codon was found (nomenclature changes to 48S complex), 48S complex changes the conformation to a “closed”-formation and eIF2-bound GTP gets hydrolysed. In the next steps 80S ribosome assembles by binding of 60S complex and eIF5B-mediated displacement of eIF1, eIF2-GDP, eIF3, eIF4B, eIF4F and eIF5. Hydrolysis of eIF5B-bound GTP releases eIF1A and eIF5-GDP, and elongation-competent 80S ribosome translates the mRNA. After elongation-competent 80S ribosome reaches the stop codon (UAA/UAG/UGA) eukaryotic release factors and GTP (eRF1, and 3) bind to the A-site of the ribosome, and eRF 3-mediated GTP hydrolysis release the newly synthesized protein from the ribosome. Translation is a cyclical process, mRNA-bound 80S ribosome dissociates into 60S ribosome, and mRNA-bound 40S complex is disassembled.
by ATP-binding cassette sub-family E member 1 (ABCE1) (reviewed in (Jackson et al. 2012)).

Cap-independent translated mRNA contains an internal ribosome entry site (IRES), which allows recruitment of 43S or 40S complex independent from m⁷G cap and several other eIFs. IRES sites are long, GC-rich, and highly structured domains located in the 5’UTR area of mRNAs (Park et al. 2005). The elongation, termination and recycling steps are the same as during cap-dependent translation. IRES sequence was firstly discovered in the viral RNA genome of poliovirus (PV) and encephalomyocarditis virus (EMCV) ((Pelletier & Sonenberg 1988), (Jang et al. 1988)). Viruses use the cap-independent translation to ensure that their RNA templates are translated, whereas products of these templates often inhibit the translation of the host (reviewed in (Walsh & Mohr 2011)). Although, this pathway was discovered in viral gene expression, an increasing number of cellular IRES elements suggests that cap-independent protein translation has its place in eukaryotic gene expression (reviewed in (Mokrejs et al. 2010)). There are several IRES types, which have different requirements of eIFs to initiate cap-independent protein translation. Furthermore, IRES trans-acting factors (ITAFs) have been identified which enhance the internal initiation in cellular IRES elements (compare Figure 1.6B) (reviewed in (Komar & Hatzoglou 2005)). This variation might correlate with the architecture of the IRES structure. Cryo-electron microscopy determined structures revealed that IRES types which require no eIFs to initiate protein translation, such as Cricket paralysis virus (CrPV), exhibit a compact structure in the free form and complex into the 40S inter-subunit space reaching formation when IRES domains are bound to 40S complex (Spahn et al. 2004) (Figure 1.6D). Whereas, IRES types which require a subset of eIFs, such as hepatitis C virus (HCV), present a more extended architecture in the free form and interact mostly with the solvent-accessible side of the 40S subunit when IRES domain is bound to 40S complex (Boehringer et al. 2005) (Figure 1.6C). These results suggest that IRES formations may mimic the eIF functions to reduce or in extreme cases to abolish the necessity of eIFs to induce protein translation.
Figure 1.6: Cap-dependent and cap-independent protein translation pathways. (A) Mechanism of mRNA activation, and 43S complex attachment of cap-dependent protein translation (adapted from (Jackson et al. 2010)). (B) Cap-independent ribosomal recruitment to internal locations in mRNA through three types of internal ribosomal entry sites (IRESs) (adapted from (Komar & Hatzoglou 2011)). (C) 43S complex recruitment through IRES structure of hepatitis C virus (HCV). Cryo-electron microscopy determined interaction between 40S complex (yellow) and the HCV IRES (purple) (adapted from (Jackson et al. 2010) and (Boehringer et al. 2005)). (D) 40S complex recruitment through IRES structure of Cricket paralysis virus (CrPV). Cryo-electron microscopy determined interaction between 40S complex (yellow) and the CrPV IRES (purple) (adapted from (Jackson et al. 2010) and (Spahn et al. 2004)).
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(Gingras et al. 1999) revealed that under stress condition the activity of the cap-binding factor eIF4E is down-regulated, which results in impaired cap-dependent protein synthesis. In consequence, IRES-dependent expression is favoured (reviewed in (Stoneley & Willis 2004), (Hellen & Sarnow 2001)). There are several proteins identified which are either translated cap-independently during cellular stress or exhibit an IRES domain in the promoter region (summarised in Table 1-3). Many of these proteins are involved in pro-survival and proliferating pathways, which suggest IRES may be involved in adaptation to physiological or pathological stress.

Table 1-3: IRES translated proteins

<table>
<thead>
<tr>
<th>Cellular function</th>
<th>Protein</th>
<th>Cellular stress</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-apoptosis</td>
<td>BAG-1</td>
<td>Heat shock</td>
<td>(Coldwell et al. 2001)</td>
</tr>
<tr>
<td></td>
<td>Bcl-2</td>
<td>Etoposide and sodium arsenite</td>
<td>(Sherrill et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>HIAP2</td>
<td>Tunicamycin</td>
<td>(Warnakulasuriyarachchi et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>XIAP</td>
<td>Irradiation</td>
<td>(Holcik et al. 1999)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>HIF-1</td>
<td>Hypoxia and serum-starvation</td>
<td>(Lang et al. 2002)</td>
</tr>
<tr>
<td></td>
<td>VEGF</td>
<td>Hypoxia</td>
<td>(Stein et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>Tie2</td>
<td>Hypoxia</td>
<td>(Park et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>FGF-1 &amp; 2</td>
<td>5' UTR analysis (bicistronic system)</td>
<td>(Martineau et al. 2004), (Bonnal et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>IGF-IR</td>
<td>Rapamycin</td>
<td>(Giraud et al. 2001)</td>
</tr>
<tr>
<td>Gene transcription – proliferation</td>
<td>c-Myc</td>
<td>5' UTR analysis (bicistronic system)</td>
<td>(Stoneley et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>c-myb</td>
<td>5' UTR analysis (bicistronic system)</td>
<td>(Mitchell et al. 2005)</td>
</tr>
<tr>
<td>Gene transcription – development, differentiation</td>
<td>Runx1</td>
<td>5' UTR analysis (bicistronic system)</td>
<td>(Mitchell et al. 2005)</td>
</tr>
</tbody>
</table>
1.5 tRNA biology

It has been shown that tRNAs are the substrates of angiogenin. In order to comprehend the generation of tRNA fragments, it is essential to introduce the complex biology of tRNAs.

1.5.1 Eukaryotic non-coding RNAs

The RNA content of a eukaryotic cell can be divided into categories depending on function. A general division can be made between coding and non-coding RNA (ncRNA). The coding RNAs represent only a small percentage of the total RNA content, and are coding for all proteins in the cell. These are pre-messenger RNAs, which are complexly processed and modified, before the mature messenger RNA (mRNA) is translated to proteins and eventually degraded. The whole content of coding RNA is defined as transcriptome, and the composition of transcriptome varies between cell types and the stages of cell development. However, the ncRNA group makes up the greater part of the total RNA content (~98%), and has more subgroups with different functions (Mattick 2005). The most investigated ncRNA with well-established functions are microRNAs (miRNAs), small interfering RNAs (siRNAs), transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), and a variety of short non-coding RNAs which are usually divided into three categories based on their distribution - small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), and small cytoplasmic RNAs (scRNAs). The "main" function of these RNAs is as follows:

- microRNAs (miRNAs), suppress translation via non-perfect pairing with target mRNA.
- small interfering RNAs (siRNAs), regulates the expression of a perfect pairing double stranded RNA.
- ribosomal RNAs (rRNAs), are components of ribosomes, and involved in protein synthesis.
Introduction

- transfer RNAs (tRNAs), are transferring amino acids to ribosomes and ensure correct linkage of amino acids, based on nucleotide sequence of the mRNA.
- small nuclear RNAs (snRNAs), are involved in mRNA processing.
- small nucleolar RNAs (snoRNAs), are playing a central role in rRNA processing.
- small cytoplasmic RNAs (scRNAs), a diverse group of molecules with wide range and the functions are not fully known

1.5.2 tRNA biogenesis

Around 4-10% of all RNA are tRNAs, which makes them the most abundant of all small non-coding RNA molecules (reviewed in (Kirchner & Ignatova 2015)). They deliver amino acids to ribosomes in a codon-dependent manner corresponding to the mRNA template. tRNAs biogenesis can be divided in three processes: (i) transcription; (ii) processing, splicing and modification; and (iii) aminoacylation. Precursor tRNA is transcribed by RNA polymerase III (Pol III) in the nucleolus. This process involves co-factors such as transcription factor TFIIIC, and transcription initiation factor IIIB (TFIIB) consisting of B double prime 1 (BDP1), B-related factor 1 (BRF1) and TATA-binding protein (TBP). In the maturation step the 5`leader and 3`trailer are cleaved in the nucleolus and the tRNA is transported to the nucleoplasm. In the nucleoplasm pre-tRNA introns are spliced, tRNAs are modified and a CCA-tail is added. Post-transcriptional modifications are crucial for tRNAs structure, stability and accuracy in protein translation. Typical modifications are incorporations of hydroxyl, methyl, and thiol groups, and nucleotide editing, such as adenosine-to-inosine. They mostly observed in the loop regions of tRNAs. At the last tRNA biogenesis step, the specific amino acid is added. tRNA aminoacylation can occur in the nucleoplasm as well as in cytosol and mitochondria. Aberrantly processed pre-tRNAs are degraded from the 3` end through the nuclear surveillance pathway, whereas mature tRNAs lacking modifications or hyper modified are degraded from the 5` end in the cytosol (reviewed in (Phizicky & Hopper 2010)) (Figure 1.7).
Figure 1.7: tRNA biogenesis. tRNA biogenesis begins with the pre-tRNA transcription in the nucleolus of the cell. Several proteins are involved in the transcription of tRNA-encoding gene, such as RNA polymerase III (Pol III in purple), TATA-binding protein (TBP in orange), B double prime 1 (BDP1 in blue), B-related factor 1 (BRF1 in green) and transcription initiation factor IIIB (TFIIB in red). The 5’ leader and 3’ trailer are cleaved in the nucleolus and pre-tRNA is transported to the nucleoplasm. In the next steps the pre-tRNA introns are spliced, tRNAs are modified (green circles) and a CCA-tail is added. In the last maturation step codon specific amino acid is added by aminoacyl tRNA synthetases (blue circles). This step can emerge in the nucleus as well as in the cytoplasm. Furthermore, tRNA surveillance and degradation processes also occur in both compartments nucleus and cytoplasm (modified after Kirchner & Ignatova 2015)).

tRNAs code for 20 standard amino acids and up to 4 different triplet anticodons for the same amino acid isotype. In human, 597 different gene loci are coding for the tRNAs with gene loci on different chromosomes for the same tRNA anticodon (Genomic tRNA Database, http://gtrnadb.ucsc.edu/). This variety might be a result of genomic expansion from single-celled organism to vertebrate and represent the evolutionary versatility (reviewed in McFarlane & Whitehall 2009)). However, it is remarkable that tRNA expression has a tissue-specific pattern and might represent the translational need of the different cell types (Dittmar et al. 2006). Furthermore, it has been observed that tRNA pools differed between proliferating and
differentiating human cells, suggesting the existence of transcriptional programs coordinating tRNA supply and demand (Gingold et al. 2014). Manipulation of the natural tRNA pool has been reported to alter the folding and solubility of many cellular proteins and decreased proliferation (Fedyunin et al. 2012). Taken together, tRNAs play an important role in controlling gene expression.

1.5.3 Stress-induced dynamics of tRNA pools

It has been observed that under suboptimal growth conditions and in response to different types of stress (e.g. oxidative and thermal stress, and viral infection), cells adapt by reprogramming gene expression (reviewed in (Kirchner & Ignatova 2015)). (Whitney et al. 2007) revealed that under amino acid and glucose starvation cytoplasmic tRNA is rapidly re-imported into the nucleus, indicating a mechanism which reduces global translation.

A quantitative systems approach in yeast illustrated stress-related alterations in tRNA modification with a specific signature for each type of stress (Chan et al. 2010). The authors suggested a model of dynamic control of tRNA modifications in cellular response pathways as mechanism controlling protein translation. Furthermore, recent findings indicate that tRNA modifications are not only fine-tuning of stress-associated translation regulation but rather have direct effects in stress signalling (Zinshteyn & Gilbert 2013). Although these findings represent results in single cell eukaryotes, mutations in elongator complex protein 3, which exhibits methyltransferase activity, have been associated with increased risk of developing ALS (Simpson et al. 2009).

Another regulatory mechanism that controls gene expression is the stress-associated tRNA cleavage. As mentioned earlier, angiogenin is a well-known ribonuclease which cleaves mature tRNA in the anticodon loop and generates 5` and 3` tiRNAs during stress conditions in mammals with fragment lengths between 30-47 nt ((Fu et al. 2009), (Saikia et al. 2012)) (Figure 1.8). It has been shown that 5`tiRNAs are phosphorylated at their 5`end, whereas 3`tiRNAs contain a hydroxyl
group their 5` end ((Yamasaki et al. 2009), (Honda et al. 2015)). Similar functions were demonstrated for Rny1p, a member of the RNase T, in yeast (Thompson & Parker 2009b) and PrrC, colicin D, and colicin E5 in bacteria, archaea, and plants (reviewed in (Masaki & Ogawa 2002)).

Another group of tRNA-derived fragments (tRFs) are between 13 and 20 nt long and map to the ends of mature tRNA transcripts. 5` tRFs and 3` CAA tRFs exhibit a 5` phosphate and a 3` hydroxyl group at their ends, and several reports suggested that endoribonuclease Dicer is responsible for their generation through cleavage in either the D- or TψC-loop ((Cole et al. 2009), (Maute et al. 2013)) (Figure 1.8). However, (Langenberger et al. 2013) demonstrated in Dicer knocked-down MCF-7 cells that tRFs generation is not based exclusively on Dicer cleavage.

Figure 1.8: Stress-mediated tRNA cleavage. Endoribonuclease Dicer cleaves mature tRNAs in the D- and TψC-loop and generates 5` tRFs and 3` CAA tRFs varying between 13 and 20 nt. Ribonucleases of different species, such as angiogenin in mammals, Rny1 in yeast, and PrrC, colicinc D &E5 in bacteria, cleave mature tRNA in the anticodon loop and generate 5` and 3` tRNAs (adapted from (Raina & Ibba 2014)).
1.5.4 Biological functions of tRNA fragments

1.5.4.1 5` and 3` tiRNA functions

One of the first groups to investigate the function of cleaved tRNA fragments was the Anderson laboratory. (Yamasaki et al. 2009) showed that endogenous 5`tiRNAs but not 3`tiRNAs, purified from angiogenin-treated U2OS cells, repress protein translation. However, the authors identified by northern blot that 5` and 3`tiRNAs halves of Met, Gly$^{\text{GCC}}$ and Pro$^{\text{TGG/AGG}}$, are generated during sodium arsenite stress. Later, the group demonstrated that 5`-TOG-containing tiRNAs, in particular 5`Ala$^{\text{AGC}}$ and 5`Cys$^{\text{GCA}}$, inhibit cap-dependent protein synthesis by displacing eIF4G/eIF4A from the m7G cap and forming stress granules, detailed in section 1.6 (Ivanov et al. 2011). They illustrated that the eIF4G/eIF4A-tiRNA displacement is mediated by RNA-binding protein YB-1 ((Ivanov et al. 2014), (Lyons et al. 2016)).

In recent years, research on cleaved tRNA fragments has increased. (Saikia et al. 2012) showed that angiogenin-cleaved tRNA rapidly accumulated in mouse embryonic fibroblasts (MEFs) exposed to oxidative and hyperosmotic stress, followed by mitochondrial cytochrome c (Cyt c) release. As a follow-up study, (Saikia et al. 2014) investigated whether tiRNA generation and Cyt c release are functionally correlated. The authors treated MEFs with angiogenin during hyperosmotic stress, immunoprecipitate with a Cyt c antibody, and identified 20 tiRNAs in a next-generation sequencing (NGS) approach. NGS data revealed that specific 5` and 3` tiRNAs, especially 5`Cys$^{\text{GCA}}$, 3`Ile$^{\text{UAU}}$, 3`Thr$^{\text{CGU}}$, and 5`Ala$^{\text{AGC}}$, bind Cyt c and inhibit the activation of the intrinsic apoptosis pathway.

Interestingly, binding assays of Cyt c to synthetic tiRNAs revealed that synthetic tiRNAs, which exhibited endogenous similar enrichment values in the NSG data, showed different Cyt c affinity. Furthermore, a selected pool of tiRNAs bound with higher affinity to Cyt c than individual tiRNAs. These finding indicate that secondary structure and modification as well as the composition of tiRNAs are important to protect the cell against specific stress.
Introduction

It is well known that parental environmental exposure and diet-induced metabolic changes can affect the germ line and be passed from father to offspring through epigenetic mechanisms, in particular RNA (reviewed in (Daxinger & Whitelaw 2012), (Ng et al. 2010)). (Chen et al. 2016) reported that sperm isolated from F₀ mice fed a high-fat diet (HFD) produced offspring with impaired glucose tolerance and insulin resistance. To investigate whether a specific RNA population was capable to induce the inherited changes, total sperm RNA, or gel purified RNA fragments (miRNA [15-25 nt], tiRNAs [30-40 nt], and larger fragments [>40 nt]) from HFD and normal diet (ND) were injected into fertilized eggs. The authors illustrated that offspring injected with total RNA content and tiRNA population from HFD F₀ sperm exhibited glucose intolerance, whereas miRNA and larger RNA fragments from HFD F₀ sperm and none of the ND F₀ sperm RNA populations showed abnormalities. Comparative sequence analysis of HFD and ND F₀ sperm RNAs revealed large changes in tiRNA proportion (11.53%), especially 5`Glu^{CTC}, 5`Gly^{GCC}, and 5`Val^{CAC}. Surprisingly, injection of a synthetic tiRNA pool, comprising a combination of the most abundant-identified tiRNAs in the sperm, did not mimic the function of endogenous sperm tiRNAs in the offspring. Mass spectrometry approach revealed that the endogenous tiRNAs were highly modified, especially m₅C and m₂G, in HFD sperm compared with ND sperm. The authors suggested that these modifications might be crucial in mediating native tiRNA functions. Differential RNA-sequence analysis of pancreatic islets in F₁ offspring illustrated down-regulation of genes involved in metabolic regulation, protein transport and localization in the HFD group. Since the DNA-methylation state was not responsible for the transcriptional changes, and the identified tiRNA sequences matched to gene promoter regions of the affected genes, the authors proposed that sperm tiRNAs behave as a type of paternal epigenetic factor.

In a similar approach, (Sharma et al. 2016) revealed that tiRNAs are generated under low protein diet in the epididymis of mice either through fusion of epididymosomes (containing cleaved tiRNAs) with sperm or cleaved during the sperm maturation process. Specific tiRNAs, especially 5`Gly^{CCC/TCC/GCC}, 5`Lys^{CTT}, and 5`His^{GTG}, were up-regulated in sperm RNA from mice on a low-protein diet.
Introduction

compared to normal diet. The authors demonstrated that interference with 5′GlyGCC through antisense oligonucleotides resulted in up-regulation of ~70 genes in two-cell embryos, which were associated with the murine endogenous retrovirus-like (MERVL) element. In a long terminal repeat (LTR) reporter cell line, the authors confirmed the 5′GlyGCC regulation of MERVL LTR-driven transcription. MERVL is a retrotransposon, which is expressed during pre-implantation stages and contributes to the plasticity of mouse embryonic stem cells (reviewed in (Schoorlemmer et al. 2014)). The authors hypothesised that repression of MERVL-regulated genes alter the placenta development of the embryo as well as metabolism pathways.

Although, both reports investigated tiRNA generation in the same cells, it is interesting that the composition of tiRNA alters with the diet type. These results highlight that specific tiRNAs are generated during varying conditions, and fulfil different functions.

(Honda et al. 2015) illustrated that several tiRNAs are generated in estrogen receptor-positive (ER+) breast cancer. The authors demonstrated that these tiRNAs were generated by angiogenin, since angiogenin knock-down showed reduced tiRNA generation. By examining different cell lines for selected tiRNAs, such as 5′AspGUC, 5′HisGUG, and 3′AspGUC, the authors showed that tiRNAs were constitutively produced in ER+ breast cancer and androgen receptor-positive (AR+) prostate cancer cell lines, whereas ER− breast cancer, AR− prostate cancer cell lines, and cell lines from other tissues did not generated tiRNAs. The authors hypothesised that sex hormone sensitivity and hormone receptor expression mediated the production of these tiRNAs. However, neither mRNA or protein level of angiogenin or angiogenin inhibitor RNH1 were influenced by the hormone status in the cell lines. The authors suggested an involvement of either unknown angiogenin cofactors or a regulation through methyltransferase, such as Dnmt2 and NSun2, which may prevent tRNA cleavage in hormone negative cell lines. Interestingly, knockdowns of 5′tiRNAs, in particular 5′AspGUC, 5′HisGUG, and 5′LysCUU, but not 3′tiRNA (3′AspGUC) resulted in reduced cell proliferation. These
findings are consistent with the results from the Anderson laboratory, suggesting an asymmetrical function of 5\textsuperscript{t}iRNAs and 3\textsuperscript{t}iRNAs. Further tiRNA functions, which are associated with dysfunction in the tRNA maturation are summarised in Table 1-4.

1.5.4.2 5\textsuperscript{t}RF and 3\textsuperscript{t} CCA tRF functions
Similar to the 5\textsuperscript{t}iRNA translation repressing properties documented by the Anderson laboratory, (Sobala & Hutvagner 2013) reported that 5\textsuperscript{t}RF Gln\textsuperscript{CTG} repressed the expression of Renilla and firefly luciferases reporter genes in mRNA target-site independent manner. This process required a diguanine sequence at the 3\textsuperscript{-}-end of 5\textsuperscript{t}RFs and was independent from the translation stage. Surprisingly, 5\textsuperscript{t} RF levels were elevated in cells exhibiting a high proliferation rate. The authors suggested that the 5\textsuperscript{t}RF translation reduction might represent a regulation mechanism during enhanced translation activity. (Gebetsberger et al. 2012) showed in halophilic archaeon Haloferax volcanii that 5\textsuperscript{t}RF Val\textsuperscript{GAC} directly binds to 30S ribosomes, the archaeal 40S homolog, during the elongation step and reduces the translational activity by interfering with efficient transpeptidation. This interference was enhanced upon alkaline stress. The authors suggested stress-dependent control mechanism, which regulates protein biosynthesis.

3\textsuperscript{t} CCA tRFs have been also associated with translations repression, however, the mechanisms are different. (Maute et al. 2013) revealed that 3\textsuperscript{t} CCA tRF Gly\textsuperscript{GCC} is derived from mature tRNA by DICER1 cleavage in human B cells. The authors demonstrated that 3\textsuperscript{t} CCA tRF Gly\textsuperscript{GCC} is associated with all Argonaute proteins and represses 3\textsuperscript{U}TR reporter of the RPA1 gene. In agreement of RPA1 functions in replication and DNA repair, 3\textsuperscript{t} CCA tRF Gly\textsuperscript{GCC} reduced the proliferation rate as well as increased sensitivity to DNA damage. Interestingly, 3\textsuperscript{t} CCA tRF Gly\textsuperscript{GCC} was strongly down-regulated in germinal center-derived lymphomas. The authors suggested that the loss of 3\textsuperscript{t} CCA tRF Gly\textsuperscript{GCC} may result in increased tolerance to DNA damage, accumulation of mutations and subsequently tumor development.
(Couvillion et al. 2012) demonstrated that *Tetrahymena thermophila* Ago/Piwi protein Twi12 binds mostly 3` CCA tRF sequences. 3` CCA tRF-bound Twi12 forms a complex with the nuclear exonuclease Xrn2, which is subsequently imported into the nucleus, where it mediates the maturation of rRNA. The authors revealed that 3` CCA tRF binding is essential for the function of Twi12, and depletion of either Twi12 or Xrn2 results in decreased rRNA synthesis.

In line with tRF-mediated gene regulation, (Goodarzi et al. 2015) identified a CU box motif (xCUxxC) in tRFs which interacts with YB-1, a protein involved in pre-mRNA splicing, and competes with binding of YB-1 to 5` and 3` UTRs. As a result mRNA transcripts, which are normally stabilised by YB-1, are destabilised and subsequently degraded. It was shown that these tRFs, in particular Asp\textsubscript{GTC}, Glu\textsubscript{YTC}, and Gly\textsubscript{TCC}, were generated under hypoxic conditions. However, the identified sequences exhibited the anticodon stem, but neither a 5` or 3`-end of mature tRNAs, which might represent another class of tRFs.

Taken together, tRNA-derived fragments represent a new class of non-coding RNA fragments, which have various functions in cell biology (summarised in Table 1-4). tRNA modifications are crucial for biogenesis of tRNA-derived fragments as well as for their function. Furthermore, the stress type determines what fragments are generated and their composition is essential to perform their assigned function.
## Introduction

### Table 1-4: Reported biological functions of tRNA-derived fragments

<table>
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<tr>
<th>Organism</th>
<th>tRF type</th>
<th>Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Human (U2OS)</td>
<td>Different 5<code> and 3</code> tiRNAs (5<code>Ala\textsuperscript{AGC} and 5</code>Cys\textsuperscript{GCA})</td>
<td>Represses cap-dependent protein translation and induces SG formation</td>
<td>(Emara et al. 2010), (Ivanov et al. 2011)</td>
</tr>
<tr>
<td>Mouse (MEF)</td>
<td>Different 5<code> and 3</code> tiRNAs</td>
<td>Pool of tiRNAs competitively bind to Cyt c and inhibit intrinsic apoptosis pathway during osmotic stress</td>
<td>(Saikia et al. 2014)</td>
</tr>
<tr>
<td>Mouse (HSCs and MyePro cells)</td>
<td>Different 5<code> and 3</code> tiRNAs (5`Gly\textsuperscript{CCC})</td>
<td>5`Gly\textsuperscript{CCC} is generated in hematopoietic stem cells (HSC) by angiogenin and promotes quiescence by repressing translation</td>
<td>(Goncalves et al. 2016)</td>
</tr>
<tr>
<td>Human and Mouse</td>
<td>Different 5` tiRNAs</td>
<td>Accumulation of 5` tiRNAs in the absence of methyltransferase NSSun2 reduce protein translation and activates stress pathways, which leads to decreased cell size and enhanced apoptosis of cortical, hippocampal and striatal neurons</td>
<td>(Blanco et al. 2014)</td>
</tr>
<tr>
<td>Human (ER+ and AR+ cell lines)</td>
<td>Different 5<code> tiRNAs (5</code>Asp\textsuperscript{GUC}, 5<code>His\textsuperscript{GUG}, and 5</code>Lys\textsuperscript{CUU})</td>
<td>Enhance cell proliferation</td>
<td>(Honda et al. 2015)</td>
</tr>
<tr>
<td>Mouse (sperm)</td>
<td>Different 5<code> tiRNAs (5</code>Glu\textsuperscript{CTC}, 5<code>Gly\textsuperscript{GCC}, and 5</code>Val\textsuperscript{CAC})</td>
<td>5`tiRNAs mediate intergenerational inheritance; modulate gene expression by binding gene regions</td>
<td>(Chen et al. 2016), (Sharma et al. 2016)</td>
</tr>
<tr>
<td>Human (CLP1\textsuperscript{R140H} patient derived cultures)</td>
<td>Different 3<code> tiRNAs (3</code>Tyr\textsuperscript{GTA})</td>
<td>3<code>tiRNAs with 5</code>'-OH generated by impaired pre-tRNA splicing are toxic to patient derived cultures</td>
<td>(Schaffer et al. 2014)</td>
</tr>
<tr>
<td>Organism</td>
<td>tRF type</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
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<td>-----------</td>
</tr>
<tr>
<td>Human (HeLa)</td>
<td>5’ tRF (Gln&lt;sup&gt;CTG&lt;/sup&gt;)</td>
<td>Translation repression in <em>Renilla</em> and firefly luciferases reporter genes</td>
<td>(Sobala &amp; Hutvagner 2013)</td>
</tr>
<tr>
<td><em>Halofexa volcanii</em></td>
<td>Different 5’tRFs and 5’tiRNAs (5’tRF Val&lt;sup&gt;GAC&lt;/sup&gt;)</td>
<td>Binds 30S ribosomes during the elongation step and reduces the translational activity by interfering with efficient transpeptidation</td>
<td>(Gebetsberger et al. 2012)</td>
</tr>
<tr>
<td>Human (HEp-2)</td>
<td>Different 5’tRFs (Glu&lt;sup&gt;CTC&lt;/sup&gt;)</td>
<td>Respiratory syncytiatal virus (RSV) infection induces abundant production of 5’tRFs, which silence mRNA targets and promote RSV replication</td>
<td>(Wang et al. 2013), (Deng et al. 2015)</td>
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<tr>
<td>Human (purified CD14+ monocytes)</td>
<td>Different 5’tRFs (Glu&lt;sup&gt;CTC&lt;/sup&gt;)</td>
<td>5’tRF Glu&lt;sup&gt;CTC&lt;/sup&gt;/PIWIL4 complex recruited SETDB1, SUV39H1, and heterochromatin protein 1β to the <em>CD1A</em> promoter region and facilitated H3K9 methylation; resulting in decreased <em>CD1A</em> transcription</td>
<td>(X. Zhang et al. 2016)</td>
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<td>Human (B cells)</td>
<td>5’ tRFs and 3’CCA tRFs (3’CCA tRF Gly&lt;sup&gt;GCC&lt;/sup&gt;)</td>
<td>Associates with Argonaute proteins and represses 3’UTR reporter of the <em>RPA1</em> gene; reduces the proliferation as well as increases sensitivity to DNA damage</td>
<td>(Maute et al. 2013)</td>
</tr>
<tr>
<td>Human (MT4 T-cells)</td>
<td>Different RNA fragments (3’CCA tRF Gly&lt;sup&gt;TTT&lt;/sup&gt;)</td>
<td>HIV-1 infected cells produced 3’CCA tRF Gly&lt;sup&gt;TTT&lt;/sup&gt; exhibiting an antisense sequence to the HIV-1 primer-binding site, which served as reverse transcription primer and was associated with enhanced HIV-1 replication</td>
<td>(Yeung et al. 2009)</td>
</tr>
<tr>
<td><em>Tetrahymena thermophila</em></td>
<td>3’CCA tRFs</td>
<td>3’CCA tRF-bound Twi12 binds Xrn2 and are imported into the nucleus, where they mediate the maturation or rRNA</td>
<td>(Couvillion et al. 2012)</td>
</tr>
<tr>
<td>Organism</td>
<td>tRF type</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
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</tr>
<tr>
<td>Human (breast cancer cells)</td>
<td>CU box motif containing tRFs</td>
<td>Enrichment of tRFs under hypoxic conditions; CU box motif interacts with YB-1 and competes with 5<code> and 3</code> UTRs binding; resulting in destabilisation and degradation of mRNA transcripts</td>
<td>(Goodarzi et al. 2015)</td>
</tr>
</tbody>
</table>

### 1.6 Stress granules and processing bodies

In a traditional view functional units are divided by membranes into separated organelles in the cell. However, findings indicate that non-membrane cellular compartments also exist. Proteins and functional molecules such as RNA form liquid-liquid phase separation reduce interference with the surrounding and create distinct chemical environment (reviewed in (Hyman et al. 2014)). Well-known examples in the nucleus are nucleoli, where ribosomes are generated (reviewed in (Boisvert et al. 2007)); centrosomes (reviewed in (Mahen & Venkitaraman 2012)), which nucleate microtubules; Cajal bodies, which contribute to genome organization and RNA splicing (reviewed in (Gall 2003)), and paraspeckles, which store highly edited mRNA molecules that are rapidly released upon physiologic stress (Prasanth et al. 2005). Stress granules (SGs) and processing bodies (PBs) are cytoplasmic located compartments, which are associated with mRNA metabolism.

SGs are cytoplasmic foci formed upon viral infection ((McInerney et al. 2005), (White et al. 2007)) or environmental stress such as oxidation, heat, and starvation (reviewed in (Kedersha & Anderson 2007)). Furthermore, they have been implicated in the pathogenesis of many diseases including inflammatory disorders, cancer, and neurodegeneration (reviewed in (Wolozin 2012), (Buchan 2014), (Panas et al. 2016)). SG formation is strongly associated with protein translation initiation, since accumulation of untranslated messenger ribonucleoprotein (mRNP) and dissociation of polysomes support SG assembly (Kedersha et al. 2000).
SG formation can be achieved in an eIF2-dependent or independent manner. Phosphorylation of eIF2 is a well-known method to reduce GDP-GTP exchange efficiency of eIF2. GDP-bound eIF2 is unable to form the ternary complex (eIF2-GTP-tRNA\textsuperscript{Met}) and consequently abolishes 43S complex formation. This inhibition results in the accumulation of eIF2-deficient 48S preinitiation complexes, which favours SG formation (Kedersha et al. 2002) (Figure 1.9-1). There are several eIF2α kinases known such as heme-regulated eIF2α kinase (HRI), general control nonderepressible 2 (GCN2), protein kinase R (PKR), and PKR-like ER kinase (PERK) ((Wek et al. 1995), (Srivastava et al. 1998), (Harding et al. 2000), (McEwen et al. 2005)).

Various stress treatments have been shown to induce SG formation by inhibiting translation initiation in an eIF2-independent manner. Sodium selenite, amino acid starvation, rapamycin, and H\textsubscript{2}O\textsubscript{2} inhibit serine/threonine-protein kinase (mTOR) to phosphorylate eIF4E-binding protein (4E-BP), which results in binding of 4E-BP to eIF4E and consequently inhibiting mRNA activation (reviewed in (Sonenberg & Hinnebusch 2009)) (Figure 1.9-2). Another eIF2-independent mechanism is also associated with mRNA activation and can be divided into two classes. One class is mediated by inhibition of the RNA helicase eIF4A function. Several compounds block either directly the RNA-eIF4A interaction or inhibit the helicase function of eIF4A, such as 15-deoxy-Δ(12,14)-prostaglandin J2 (15d PGJ2 - (Kim et al. 2007)), pateamine A ((Bordeleau, Cencic, et al. 2006), (Dang et al. 2006)), silvestrol (Bordeleau et al. 2008), or hippocristanol ((Bordeleau, Mori, et al. 2006), (Cencic et al. 2013)). The other class disrupts the eIF4E-eIF4G/eIF4A interaction. ((Emara et al. 2010), (Ivanov et al. 2011)) demonstrating that tiRNAs, produced by angiogenin under stress conditions, displace eIF4G/eIF4A from cap-bound eIF4E and repress specifically the cap-dependent protein translation (Figure 1.9-3).
Figure 1.9: Models for SG formation initiation under different conditions. (1) Phosphorylation of eIF2α reduced GDP-GTP exchange efficiency of eIF2, which results in depletion of ternary complex from 48S preinitiation complexes. (2) Different stress conditions inhibit mTOR-mediated 4E-BP phosphorylation, which prevents the dissociation of 4E-BP from eIF4E. eIF4E:4E-BP complex blocks the eIF4F complex formation and subsequently inhibits translation initiation. (3) Chemical compounds such as pateamine A, 15-deoxy-Δ(12,14)-prostaglandin J2 (15d PGJ2), silvestrol, and hippuristanol block either directly the RNA-eIF4A interaction or inhibit the helicase function of eIF4A. Specific tiRNAs prevent the formation of eIF4F complexes by displacing eIF4G/eIF4A subunits. Both mechanisms prevent the formation of 48S preinitiation complexes (adapted from (Panas et al. 2016)).

Since several cellular conditions are able to induce SG formation, it is not surprising that SG composition can vary. Immunocytochemistry (ICC) established SG markers are G3BP, PABP1, FXR1, and several eIFs (p-eIF2α, eIF3, eIF4A1, eIF4B, eIF4G) (Kedersha & Anderson 2007). Surprisingly, recent proteomic analysis of SGs revealed slightly different proteins than ICC established SG markers (Table 1-5).
**Table 1-5: Stress granule core proteins identified by mass spectrometry** (adapted from (Jain et al. 2016)). Red box illustrates proteins identified only in the yeast granule proteome, blue illustrates proteins identified only in the mammalian granule proteome, and yellow illustrates proteins identified in both proteomes.

Mass spectrometry identified that, apart from RNA binding proteins, the stress granule proteome consists of proteins involved in post-translational modification, metabolism, and protein or RNA folding (Jain et al. 2016). The authors suggested that SG consist of a stable “cores” surrounded by a dynamic shell. (Protter & Parker 2016) proposed two models for discrete phases of stress granule assembly. The “cores first” model suggests that untranslated mRNPs oligomerise and assemble with increasing concentration of translationally repressed mRNPs to SG cores. In the next phase SG cores recruit a dynamic shell through protein-protein interaction also with other proteins (Figure 1.10A). The “liquid–liquid phase separation (LLPS) first” model suggests that the increasing concentration of mRNPs leads to growing liquid droplets structure held together by weak interactions. Once the liquid droplets structure reaches a critical mass, SG cores are formed within the liquid droplets structure due to the high local concentration of proteins (Figure 1.10B).
Figure 1.10: Models for SG assembly consisting of a stable “cores” surrounded by a dynamic shell. (A) “Core first” model suggests untranslated messenger ribonucleoproteins (mRNPs) oligomerise with increasing concentration to SG cores. After SG cores are formed, they fuse by recruiting a dynamic shell through protein-protein interaction also with other proteins. (B) “Liquid–liquid phase separation (LLPS) first” model suggests untranslated mRNPs are continuously accumulate in a liquid droplets structure. Once the liquid droplets structure reaches a critical mass, SG core are formed within due to the high local concentration of proteins (Protter & Parker 2016).

PBs contain proteins involved in mRNA degradation, mRNA surveillance, translational repression and RNA-mediated gene silencing (reviewed in (Anderson et al. 2015)). One proposed function of PBs is the degradation of mRNA. Studies have shown that PBs dissociate when protein translation is inhibited ((Cougot et al. 2004), (Teixeira et al. 2005)), or mRNA decay is blocked at an early stage such as preventing deadenylation (Sheth & Parker 2003). In contrast, blocking mRNA decay at later stages by exoribonuclease or decapping co-activator depletion leads to an increase in size and number of PBs ((Sheth & Parker 2003), (Cougot et al. 2004), (Teixeira et al. 2005), (Andrei et al. 2005)). Another interesting aspect
associated with siRNAs- and miRNAs-mediated decay is translational repression of mRNAs. It has been shown that miRNAs and miRNA targets are detected in PBs ((Liu et al. 2005), (Pauley et al. 2006)). Furthermore, studies revealed that Argonaute proteins, which are required for regulatory functions of siRNA and miRNA, interact with PBs enriched proteins such as GW182, DCP1, DCP2 and RCK/p54 ((Chu & Rana 2006), (Behm-Ansmant et al. 2006), (Sen & Blau 2005)). Although, this evidence indicates PBs are involved in mRNA decay, it has been reported that these processes occur also in the absence of PBs ((Stoecklin et al. 2006), (Eulalio, Behm-Ansmant, Schweizer, et al. 2007)). The fact that many PBs proteins exchange rapidly with the cytoplasmic pool and others seem to be very stable PB components, such as Dcp2 (Kedersha et al. 2005), suggests that PBs might be mRNA and protein storage sites ((Aizer et al. 2008), (Aizer et al. 2014)).
1.7 Aims of this study

It is known that mutations affecting the ribonucleolytic activity or the nuclear translocation of angiogenin may cause ALS. However, the function of angiogenin in both angiogenin expressing and endocytosing cells remains to be elucidated. The objective of this study was to characterise the role of angiogenin in the control of motoneuron-astrocyte interaction. The particular focus of this study was to investigate whether angiogenin mediated protection is due to tiRNA generation.

The aims of the study were:

- To create stable SH-SY5Y lines overexpressing wild-type angiogenin and two different ALS-associated mutants (K40I, R31K) and explore the effect of ANG WT and mutants K40I and R31K on angiogenin function in order to:
  - Investigate how the ribonucleolytic inactive K40I mutant and mutation in the nuclear localisation sequence of R31K affecting RNA cleavage and whether ANG mutations alternate tiRNA generation
  - Determine the paracrine activity of stable SH-SY5Y cell lines with the focus on secretion of ANG and different tiRNAs
- To characterise the function of tiRNAs with the focus on SG formation in angiogenin expressing and endocytosing cells and whether tiRNAs protect SH-SY5Y cells against ALS pathology related stress
- To explore whether the protection of angiogenin against hypoxia in primary motoneurons is mediated by specific tiRNAs
- To examine whether angiogenin alters the protein translation in primary astrocytes and whether this alteration is mediated by specific tiRNAs
# Material and Methods

## 2. Chapter 2: Materials and Methods

### 2.1 Materials

#### 2.1.1 Reagents

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<th>Manufacturer</th>
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<td>10 mM dNTP mix</td>
<td>R0192</td>
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<td>10X DNase I Reaction Buffer</td>
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<td>Invitrogen</td>
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### Material and Methods

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#### 2.1.2 Equipment

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## Material and Methods

### Consumable

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2.1.4 Plasmid vector

ANG WT and ALS associated mutants (R31K and K40I) were cloned into pcDNA3.1 (+) vector by Dr. Marion C. Hogg. NheI and EcoRI were used as restriction sites.

Figure 2.1: pcDNA3.1 (+) backbone vector for ANG WT and ALS associated mutants (R31K and K40I) (image source: www.addgene.org/vector-database/).

2.2 Cell culture

2.2.1 Eukaryotic cell lines

2.2.1.1 SH-SY5Y

The SH-SY5Y cell line is a subline of the human neuroblastoma cell line SK-N-SH. This cell line was first established in culture from a bone marrow biopsy by (Biedler et al. 1973). SH-SY5Y cells were reported to have moderate levels of dopamine beta hydroxylase activity (Biedler et al. 1978). SH-SY5Y cells normally grow as a mixture of floating and adherent cells in a 1:1 mixture of DMEM and F-
Material and Methods

12 Medium (Thermo Fischer Scientific) in a humidified incubator at 37 °C and 5% CO₂. The following components were added to the base medium to make the complete growth DMEM/F-12 medium:

- 10 % (v/v) fetal bovine serum (FBS, Sigma)
- 1 % (v/v) Penicillin/Streptomycin (P/S) (100 units penicillin and 0.1 mg streptomycin per ml [Sigma])
- 2 mM L-Glutamine (Sigma)

SH-SY5Y cells grow as clusters of neuroblastic cells with multiple, short, fine cell processes (neurites). Cells were sub-cultured at a confluence of approx. 90 % by first aspirating the old medium. The adherent cells were then rinsed with warmed HBSS (Sigma), before adding 3 ml Trypsin/EDTA solution (Sigma) and incubating the culture at 37°C until the cells detached (~5 min). This trypsinisation was stopped by adding fresh medium, centrifuging the cell suspension at 200 X g. The cell pellet was resuspended in complete growth medium and dispensed into new flasks.

2.2.1.2 MZ-294

The MZ-294 line is an established cell line which was derived from patient primary Glioblastoma (Hetschko et al. 2008). MZ-294 cells grow as adherent cells in DMEM Medium (Lonza) in a humidified incubator at 37 °C and 5% CO₂. The following components were added to the base medium to make the complete growth DMEM medium:

- 10 % (v/v) FBS
- 1 % (v/v) P/S
- 2 mM L-Glutamine

MZ-294 cells were sub-cultured as described previously for SH-SY5Y cells.
2.2.2 Preparation of primary motoneuron cultures

2.2.2.1 Precoating of glass coverslips

Glass cover slips (VWR International) were coated to aid adhesion of primary motoneurons cultures to the glass surface. Glass cover slips were stored in 70% EtOH to ensure sterility. Glass cover slips were placed into wells of 24-well plate and rinsed once with 1x filter sterilised (0.22 µm filer) PBS (Lonza). Poly-L-Ornithine (Sigma) was diluted in 1x filter sterilised PBS to a concentration of 10 µg/ml and ~0.5 ml were added to each well at least over night at RT. The solution was aspirated at an angle to ensure the tip does not scratch any coating form the surface of the cover slip. Laminin (Sigma) was diluted in DMEM (Lonza) with 1% (v/v) P/S (Sigma) to a final concentration of 1.5 µg/ml, ~0.5 ml were added to each well, and incubate at 37 °C until motoneuron cultures were ready to seed.

2.2.2.2 Ventral horn dissection from E12-14 embryos from C57Bl6 mice

Aseptic techniques were used for entire process to prevent contamination and infection of the cultured cells. All dissection instruments used were autoclaved prior to use. Animals were anaesthetised with 100 µl 20% sodium pentobarbitone (Vetoquinol), sedation was assessed by pinch-reflex, and mouse was decapitated. Abdomen was sprayed with 70% EtOH and skin was cut away along ventral midline of abdomen with scissors. Scissors were changed and the underlying abdominal muscle was cut through to expose cavity. Embryonic sac was removed and stored in sterile PBS on ice. Sedation and removal of the embryonic sac was carried out by a colleague who completed Laboratory Animal Science and Training animal handing course. To assure sterility sterile 6-well plate was prepared with two well of 70% EtOH, two wells of sterile PBS and one well of sterile PBS containing 0.1% (v/v) Fungizone (Thermo Fisher Scientific). Embryonic sac was washed in a 70% EtOH well, placed in the other 70% EtOH well, and embryos were removed one-by-one. Embryos were washed in a sterile PBS well, washed again in the well containing PBS with Fungizone, and placed in a well with sterile PBS on ice.
The dissection steps were executed by employing dissecting microscope SZ51 (Olympus). Embryos were placed into a dish with in DMEM/F12 medium containing 1% (v/v) P/S and decapitated with a scalpel. Skin was removed from the dorsal surface of the neural plate. The lumbar part of neural plate was removed with a pair of tweezers by saw-like movements on both sides below the neural plate. The meninges was gently removed in rostrocaudal direction from the neural plate. Neural plate was flattened on the dish bottom and dorsal horns were dissected away from both sides along the length of the neural plate. The ventral horns were transferred into sterile PBS and stored on ice.

2.2.2.3 Motoneuron purification

All further steps were carried out in a laminar flow hood. Excess PBS was aspirated from ventral horns and replaced by fresh 1 ml sterile PBS. 10 µl bovine pancreatic trypsin (Sigma) was added and solution was incubate at 37 °C for 10 min in water bath. Complete neurobasal medium was freshly prepared to ensure optimal preparation and growth conditions for motoneurons.

Table 2-1: Recipe for 50 ml complete neurobasal medium (cNB)

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<td>50x B27 supplement (Thermo Fisher Scientific)</td>
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<td>Penicillin/Streptomycin (Sigma)</td>
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<td>L-Glutamine (Sigma)</td>
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</tr>
<tr>
<td>5 µg/ml CNTF (R&amp;D Systems)</td>
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Cells were transferred with as less as possible liquid to 0.8 ml cNB with 30 µl of bovine pancreatic DNase I (Sigma). Solution was triturated with a 1 ml pipette, allowed settling for 5 min, and supernatant was transferred into a new tube. 0.9 ml cNB with 20 µl of bovine pancreatic DNase I was added to the cell pellet, triturated, allowed settling for 5 min, and supernatant was transferred to the other
supernatant. Supernatant was centrifuged at 350 X g for 3 min and supernatant was aspirated. Cell pellet was resuspended in 3-4 ml cNB and cells were counted. Laminin solution was aspirated from one plate at a time to prevent coverslips from drying. 35 x 10⁴ cells were seeded per well with a density of 10% motoneurons and cells were cultured at 37 °C and 5% CO₂. To prevent cell stress during culturing conditions, the medium was very carefully replaced by removing 0.2 ml and adding 0.3 ml cNB. Media was changed 24 h after seeding (day in vitro[DIV] 1), and at DIV 4 and 7.

2.2.3 Preparation of primary astrocyte cultures

Primary astrocytes were prepared from cortices of 1 to 2 day old C57 Black 6 mouse pups of either sex. Aseptic techniques were used for the entire process to prevent contamination and infection of the cultured cells. All dissection instruments used were autoclaved prior to use. Mouse pups were decapitated, the skin and skull were cut using a small scissors and peeled back with forceps. The total brain was removed and stored for further dissection in DMEM (Lonza) containing 100 units penicillin and 0.1 mg streptomycin per ml (Sigma). Then cortices of all brains were dissected and the meninges were removed. All further steps were carried out in a laminar flow hood. The supernatant of prepared cortices were discarded and a few ml Trypsin-EDTA solution (Sigma) added. The tissue was roughly triturated, and incubated for 5-10 min at 37 °C to dissociate cells. Trypsin was inactivated by adding full DMEM containing 100 U/ml penicillin and 0.1 mg/ml streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum (FBS; Sigma), and triturated again using a sterile serological pipette. The cell suspension was passed through a cell strainer (40 µm Nylon, Falcon) and centrifuged for 3 min at 600 X g and RT. The pellet was resuspended in complete growth DMEM medium (compare 2.2.1.2), with 2 cortices or 50% confluency per T75 flask, and cultured at 37 °C and 5% CO₂. The following day, the media was replaced by washing the cells with warm HBSS and fresh full DMEM was added. The media change was performed every 2-3 day for 14 days until the flask was confluent. To minimise contamination with microglia and oligodendrocyte precursor cells, the culture flask
was shaken vigorously by hand, and supernatant was discarded. Astrocytes were then trypsinised, and passaged in new flasks. The cells were passaged twice with a total of approximately 21 DIV to enrich astrocytes in the culture. After the primary astrocyte cultures reached confluency for a 3rd time the cells were seeded
2x10^4 cells/well on cover slips in 24-well plate with 70% confluency. The medium was changed 48 h later (3rd day) and on the 5th day cells were employed for further studies.

2.3 Transfection

2.3.1 Plasmid transfection and generation of stable cell lines

SH-SY5Y cells were seeded 24 h prior to transfection in a 12-well plate at a density of 1x10^5 cells/well in a volume of 1 ml/well. To transfect 1 well of a 12 well plate, 400 ng plasmid DNA and 1.6 µl Metafectene (Biontex Laboratories GmbH) were first added to 200 µl OptiMEM (Gibco). This solution was then vortexed for 5 sec, briefly centrifuged and incubated for 20 min. During this incubation, the cells were washed with warmed HBSS, before adding 800 µl OptiMEM. The medium containing the transfection mix was then added for 5 h and mixed gently by rocking the plate back and forth. After this incubation, medium was replaced with complete DMEM/F-12 medium, and the cells were allowed to recover overnight at 37 °C and 5 % CO₂. Following the overnight incubation, in order to select for cell lines stably expressing a plasmid, the cells were washed with warmed HBSS and the medium was changed to complete DMEM/F-12 medium containing 0.5 mg/ml (w/v) neomycin (G418 - Sigma). Cells were cultured in 0.5 mg/ml G418 containing medium until all control cells had died (approx. 27 days). The surviving stable transfected cells were finally cultivated in complete DMEM/F-12 medium containing 0.1 mg/ml (w/v) G418.

2.3.2 tiRNA transfection in SH-SY5Y and MZ-294 cell lines

1x10^5 SH-SY5Y cells/well and 2.5x10^4 MZ-294 cells/well were seeded on cover slips in 24 well plates at a working volume of 0.5 ml/well and incubated overnight
Material and Methods

at 37 °C and 5 % CO₂. tiRNA concentration (as reported in the result sections) were denatured/restructured at 90°C for 2 min and added to 50 µl OptiMEM. For each well a separated tiRNA tube was prepared. Lipofectamine2000® (Invitrogen) stock solution was prepared by adding 2.5 µl Lipofectamine to 50 µl OptiMEM for each well which has been transfected. The Lipofectamine stock solution was vortexed for 5 sec and 52.5 µl was added to each tiRNA tube. The solution was vortexed for 5 sec, span briefly down and incubated for 20 min at RT. In-between, wells were washed with warmed HBSS for 5 min and 400 µl OptiMEM were added to each well. tiRNA solution were added dropwise to each well and mixed gently by rocking the plate back and forth. The plate was incubated at 37 °C and 5 % CO₂ for 5 h.

Table 2-2: Purchased tiRNAs (Integrated DNA Technologies)

<table>
<thead>
<tr>
<th>ID/Label</th>
<th>Locus on Chromosome</th>
<th>Codon</th>
<th>Length [nt]</th>
<th>Sequence (5’ to 3’)</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>5’</td>
</tr>
<tr>
<td>5’Ala</td>
<td>chr6.trna102</td>
<td>AGC</td>
<td>29</td>
<td>GGGGGUGUAGCUCAGUGG UAGAGCGCGUG</td>
<td>phosphor ylated fluorophore (ROX)</td>
</tr>
<tr>
<td>5’Arg</td>
<td>chr17.trna19</td>
<td>TCG</td>
<td>44</td>
<td>GACCGCGUGGCCUAAUGG AUAGGCUGACUCUG GAUCAGA</td>
<td>phosphor ylated fluorophore (ROX)</td>
</tr>
<tr>
<td>3’Arg</td>
<td>chr17.trna19</td>
<td>TCG</td>
<td>33</td>
<td>AGAUUGAGGGUUCGAGUC CCUUCUGUGGUGCCA</td>
<td>fluorophore (ROX)</td>
</tr>
<tr>
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<td>GUC</td>
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</tr>
<tr>
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<td>chr6.trna48</td>
<td>GUC</td>
<td>43</td>
<td>GCCUGUCACGCAGGAGAC CGGGGUUCGAGAUUCCCCGA CGGGGAG</td>
<td>fluorophore (ROX)</td>
</tr>
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### Material and Methods

<table>
<thead>
<tr>
<th>ID/Label</th>
<th>Locus on Chromosome</th>
<th>Codon</th>
<th>Length [nt]</th>
<th>Sequence (5' to 3')</th>
<th>Modifications</th>
</tr>
</thead>
<tbody>
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<td>GCC</td>
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<td>GCAUUGGUGGUUCAGUGG UAGAAUUCUCG</td>
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</tr>
<tr>
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<tr>
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<td>42</td>
<td>ACACGCGAAAGGUCCCCGG UUCGAAACUGGGCGGAAAC ACCA</td>
<td>-</td>
</tr>
<tr>
<td>Ctrl tiRNA</td>
<td>-</td>
<td>-</td>
<td>29</td>
<td>UGUGAGUCACGUGAGG GC AGAAUCUCGCUC</td>
<td>phosphor ylated, fluorophore (ROX)</td>
</tr>
</tbody>
</table>

#### 2.3.3 tiRNA transfection in primary motoneuron cultures

Primary motoneuron cultures were transfected with 125 pmol 5`Asp tiRNA or 2.15x10^8 copies/cell on DIV 8. NeuroMag® (OZ Bioscience), Lipofectamine2000® (Invitrogen), Viromer® BLACK (Lipocalyx), and calcium phosphate (CaPO₄) precipitation were employed as transfection reagents/methods to select the optimal transfection technique with the lowest toxic effects. NeuroMag®, Lipofectamine2000® (Lipofectamine), and Viromer® BLACK were performed following individual manufacturer’s instructions. Calcium phosphate precipitation protocol was modified after (Goetze et al. 2004). Experiments were performed for each condition in triplicates.
2.3.3.1 NeuroMag

1 µl and 3 µl of NeuroMag reagent were examined. Therefore, both NeuroMag reagent volumes were mixed with tiRNA in 100 µl Neurobasal® medium and incubated for 15-20 min. 100 µl conditioned media was transferred in a different tube and NeuroMag-tiRNA solution was added dropwise to cultured motoneurons. Plate was placed on a specific magnetic plate (OZ Bioscience) for 15 min. Then, plate was incubated for 24 h at 37 °C and 5% CO₂.

2.3.3.2 Lipofectamine2000

1.5 µl Lipofectamine were mixed with tiRNA in 100 µl OptiMEM and incubated for 15-20 min. Two different transfection media were examined: (i) 400 µl conditioned media; and (ii) 400 µl OptiMEM. 100 µl conditioned media was transferred in a different tube for the former. Whereas for the 400 µl OptiMEM, the entire conditioned medium was transferred into a different tube, motoneurons were gently rinsed with warm Neurobasal® medium and 400 µl warm OptiMEM was added to the motoneurons. Lipofectamine-tiRNA solution was added dropwise to cultured motoneurons and incubated for 1 h at 37 °C and 5% CO₂. Afterwards, transfection medium was replaced by 500 µl warm conditioned medium and motoneurons were incubated for 23 h at 37 °C and 5% CO₂.

2.3.3.3 Viromer BLACK

1 µl Viromer BLACK reagent was mixed with tiRNA in 100 µl OptiMEM and incubated for 10 min. 100 µl conditioned media was transferred in a different tube and Viromer BLACK-tiRNA solution was added dropwise to cultured motoneurons. Then, plate was incubated for 24 h at 37 °C and 5% CO₂.

2.3.3.4 Calciumphosphate-precipitation

CaPO₄-precipitation was performed following (Goetze et al. 2004). 3 µl 2.5 M CaCl₂ was gently mixed with tiRNA in 60 µl RNase free H₂O. 60 µl 2x BBS-buffer was added and incubated for 20 min.
Material and Methods

2x BBS-Buffer (pH 7.1)
280 mM NaCl
50 mM BES (N,N-Bis(2-hydroxyethyl)-2-aminoethanesulfonic acid) (Sigma)
1.5 mM Na$_2$HPO$_4$ (Sigma)

Entire conditioned medium was transferred into a different tube, motoneurons were gently rinsed with warm HBSS washing buffer, and 380 µl Ca-transfection medium was added. Ca-tiRNA solution was dropwise added to the motoneurons and plate was incubated for 2 h at 37 °C in a CO$_2$-free incubator. Moreover, CaCl$_2$ solution without tiRNA was used as additional control for the CaPO$_4$ transfection method.

HBSS washing buffer
1x HBSS (with calcium and magnesium) (Thermo Fisher Scientific)
10 mM D-(+)-Glucose (Sigma)
20 mM HEPES (Thermo Fisher Scientific)
1 mM Sodium Pyruvate (Sigma)

Ca-transfection medium
1x MEM (Thermo Fisher Scientific)
1 mM Sodium Pyruvate
15 mM HEPES
200 mM Glutamax (Thermo Fisher Scientific)
33 mM D-(+)-Glucose
1x B27 supplement

Afterwards, transfection medium was replaced by 500 µl warm conditioned medium and motoneurons were incubated for 24 h at 37 °C and 5% CO$_2$.

2.3.3.5 Endocytosis of tiRNA

To examine whether tiRNA fragments were endocytosed by primary motoneuron without transfection reagents, tiRNA was added without any transfection reagent to changed culture medium at DIV 7 for 72 h.

Transfected cells were washed with 0.5 ml warm HBSS and incubated with 0.5 ml Trypan Blue (Sigma) for 5 min. Cells were washed with 0.5 ml warm HBSS and ICC steps (as described in 2.7.8) were performed. Trypan blue penetrates dying
cells with compromised plasma membranes and stains the cytoplasm of nonviable cells blue (Strober 2001). It was employed to determine healthy motoneurons.

2.3.3.6 tiRNA transfection analysis in primary motoneurons

Transfected motoneurons were measured on the confocal microscope LSM 7.10 (Zeiss, settings listed in 2.9.2). Images were analysed by employing the software ImageJ. Motoneurons were defined as SMI-32 positive cells with distinct neurites and Trypan Blue negative staining. The cell outlines were defined in the SMI-32 channel with particles bigger 100 µm$^2$ and intensity higher 200 AU. The cell outlines were employed to measure the median ROX intensity.

Transfection efficiency of different transfection reagents (Figure 4.15) was determined in 17-20 motoneurons. Channel crosstalk intensity was calculated from untreated control motoneuron cultures. tiRNA levels in hypoxia-treated primary motoneuron cultures (Figure 4.16) were quantified from 20-30 motoneurons of a single culture. Channel crosstalk intensity was calculated from MOCK transfected motoneuron.

Channel crosstalk was subtracted from ROX intensities in SMI-32 defined cell area of tiRNA transfected motoneurons. Corrected median ROX intensities were employed to calculate the SEM value.

2.3.4 tiRNA transfection in primary astrocytes

To investigate tiRNA transfection in primary astrocytes, 2x10$^4$ cells/well were seeded on cover slips in 24-well plate. The medium was changed 48 h later (3rd day) and on the 5th day cells were transfected. Two different volumes of Lipofectamine were examined, whereas the transfection duration was reduced to 1 h to minimise Lipofectamine toxicity. 1.5 µl Lipofectamine was added to 50 µl OptiMEM and mixed with 250 nM (37.5x10$^8$ copies/cell), 500 nM (75x10$^8$ copies/cell), or 750 nM (112.5x10$^8$ copies/cell) tiRNA in 50 µl OptiMEM.

2.5 µl Lipofectamine was added to 50 µl OptiMEM and mixed with 500 nM (75x10$^8$ copies/cell), or 750 nM (112.5x10$^8$ copies/cell) tiRNA in 50 µl OptiMEM. tiRNA-OptiMEM solution were vortexed for 5 sec, span briefly down and incubated
for 20 min at RT. Then 100 µl culture medium was discarded from each well, tiRNA-OptiMEM solution was added dropwise and mixed gently by rocking the plate back and forth. The plate was incubated at 37 °C and 5 % CO₂ for 1 h. The ICC steps were performed as described in 2.7.8 and imaged on the confocal microscope LSM 710 (settings in Table 2-13).

2.4 SG formation

2.4.1 SH-SY5Y and MZ-294 cells

We investigated whether tiRNA and angiogenin induced SG formation or whether the treatment enhanced SG formation upon sodium arsenite stress. The experimental setup was adopted from The Anderson laboratory reports ((Emara et al. 2010), (Ivanov et al. 2011)).

To investigate the direct influence of tiRNA, we seeded 1x10⁵ SH-SY5Y cells/well and 2.5x10⁴ MZ-294 cells/well on cover slips in 24 well plates and transfected the cell with indicated tiRNA amount on the next day. Cells were gently washed with 0.5 ml warm HBSS, and ICC steps were performed. Cells were imaged on the confocal microscope LSM 710 (compare Table 2-13) and analysed by either employing the CellProfiler pipeline or counting blinded each image. During manual counting only cells with >1 SG were assumed as SG positive cells.

To examine whether tiRNA enhanced SG formation upon sodium arsenite stress in SH-SY5Y cells, transfected cells were washed 3x with 0.5 ml warm HBSS and treated with 20 µl sodium arsenite in complete growth DMEM/F-12 medium for 1 h at 37 °C and 5% CO₂. The following steps were the same as previously described.

To determine whether angiogenin enhance SG formation upon sodium arsenite stress and whether missense mutations altered the effect, stable SH-SY5Y and control cell lines were seeded at 1x10⁵ cells/well on cover slips in 24 well plates. Next day, cells were gently washed with 0.5 ml warm HBSS, complete growth DMEM/F-12 medium with indicated sodium arsenite concentration (0 µM, 20 µM,
25 µM, and 30 µM) were added and incubated for 1 h at 37 °C and 5% CO₂. The following steps were the same as previously described.

2.4.2 Primary astrocyte cultures

SG formation was examined in primary astrocyte cultures under established SG-inducing conditions (reviewed in (Kedersha & Anderson 2007)). Primary astrocytes were seeded 2x10⁴ cells/well on cover slips in 24-well plate. Medium was changed 48 h later, and on the 5th day primary astrocytes were treated. Primary astrocytes were gently washed with 0.5 ml warm HBSS and treated with either: (i) different sodium arsenite concentration (0 µM, 50 µM, 300 µM, and 500 µM) for 1 h, (ii) 1 M sorbitol (Sigma) for 0.5 h, 1 h, and 2 h, or (iii) cultured for 1 h at 43 °C. Cells were gently washed with 0.5 ml warm HBSS, and ICC steps were performed (compare 2.7.8). Cells were imaged on the confocal microscope LSM 710 (compare Table 2-13). MZ-294 cells were used as a control cell line. MZ-294 cells were seeded and 2.5x10⁴ MZ-294 cells/well on cover slips in 24 well plates and treated with sodium arsenite (0 µM, 100 µM, and 200 µM) for 1 h on the next day. The following steps were the same as previously described.

2.5 Hypoxia assessment in primary motoneuron cultures

HBSS and serum-free supplemented culture medium, in the presents and the absence of ANG/BSA, were equilibrated for 4 h in hypoxic chamber (1% O₂; 5% CO₂; and 37 °C).

2.5.1 Angiogenin treated primary motoneurons

Motoneurons were exposed to hypoxic stress (1% O₂ for 24 h) and ANG protection was assessed at DIV 9. Therefore, motoneurons were gently rinsed with 0.5 ml warm HBSS and incubated with 0.5 ml warm serum-free cNB in the presence or absence of ANG (100 ng/ml and 500 ng/ml), and BSA (25 mg/ml). Motoneurons were exposed to hypoxic conditions (1% O₂) for 24 h. Control cultures were treated the same way, however, they were cultured under normal conditions for 24 h at 37 °C and 5% CO₂.
2.5.2 tiRNA transfected primary motoneurons

Primary motoneurons were transfected with 3 µl NeuroMag and 125 pmol tiRNAs or 2.15×10⁸ copies/cell at DIV 8 as described above. Transfected motoneurons were transferred at DIV 9 to the hypoxic chamber. Medium was discarded and motoneurons were gently rinsed with 0.5 ml equilibrated HBSS. Serum-free supplemented culture medium was added and exposed to hypoxic conditions (1% O₂) for 24 h.

Additionally, transfected motoneurons were separately examined under normal culture conditions (normoxia). Therefore, medium was discarded and transfected motoneurons were gently rinsed with warm HBSS. 0.5 ml warm serum-free cNB was added and cultured for 24 h at 37 °C and 5% CO₂.

2.5.3 Motoneuron protection analysis

Treated motoneurons were washed with 0.5 ml warm HBSS and incubated with 0.5 ml Trypan Blue for 5 min. Cells were washed with 0.5 ml warm HBSS and ICC steps (as described in 2.7.8) were performed.

Five different fields of views per well were imaged on the confocal microscope LSM 710 (settings listed in 2.9.2). Motoneurons, SMI-32 positive cells with distinct neurites and trypan blue negative staining, were counted blinded and normalised to the average motoneuron count (~100 cells/well) of untreated normoxia control of each experiment. SEM was calculated from separated wells of the independent experiments.

2.6 tiRNA assessment on protein translation in primary astrocytes

Protein translation was determined by employing Click-iT® labelling technology (Thermo Fisher Scientific) following manufacturer's instructions. Click-iT® labelling has three essential steps: (i) amino acids (AA) depletion from cells, (ii) incubation with the alanine analog containing an azide modification (L-Azidohomoalanine – AHA), and (iii) insertion of alkyne modified fluorophores (Alexa Fluor® 488) into nascent synthesised proteins containing the azide modification through a
chemoselective reaction (“click” reaction - copper(I)-catalysed azide alkyne cycloaddition (Meldal & Tornøe 2008)).

2.6.1 Click-iT® protocol validation in MZ-294 cells

MZ-294 cells were employed to validate the Click-iT® protocol. Therefore, cells were seeded at 2x10^4 cells/well on cover slips in 24-well plate. The following day cells were washed with 0.5 ml warm HBSS and cultured in AA-free DMEM (Thermo Fisher Scientific) for 1 h to deplete AA reserves. The cells were incubated with 0.5 ml AA-free DMEM containing different AHA (Thermo Fisher Scientific) concentration (0-500 µM) for 6 h. To examine translation repression different cycloheximide (CHX, Sigma) concentrations (0-500 µM) were added to AHA containing medium. The cells were washed with 0.5 ml warm HBSS and fixed with 0.4 ml 4% PFA for 10 min. After two washes with 0.5 ml PBS, cells were permeabilised with -20 °C 95% ethanol-5% glacial acetic acid solution for 10 min. The cells were washes twice with 0.5 ml PBS and incubated with 0.4 ml/well 3 % (w/v) BSA in PBS for 30 min at 37 °C. 0.5 ml Click-iT reaction solution was added and incubated for 30 min protected from light at RT. The cells were washed with 3 % (w/v) BSA and incubated with 250 µl of the primary AB (Table 2-4) in 5 % (w/v) BSA in PBS at 4 °C over night. The next ICC steps were the same as described in section 2.7.8.

Table 2-3: Click-iT reaction solution recipe

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x Click-iT® cell reaction buffer (Click-iT® Cell Reaction Buffer Kit)</td>
<td>440 µl</td>
</tr>
<tr>
<td>CuSO₄-solution (Click-iT® Cell Reaction Buffer Kit)</td>
<td>10 µl</td>
</tr>
<tr>
<td>1x Click-iT® cell buffer additive (Click-iT® Cell Reaction Buffer Kit)</td>
<td>50 µl</td>
</tr>
<tr>
<td>1.25 mM Alexa Fluor® 488 alkyne (Thermo Fisher Scientific)</td>
<td>1 µl</td>
</tr>
</tbody>
</table>

Five different fields of views per well were imaged on the confocal microscope LSM 7.10 (settings listed in Table 2-13). Fluorescence intensities of nascent synthesised proteins were analysed by employing the program ImageJ. The cell outlines were defined in the G3BP channel with particles bigger 200 µm^2 and intensity higher 200 AU. The cell outlines were employed to measure the median
fluorescence intensities of nascent synthesised proteins. SEM was calculated from a single well with 100-150 cells/cover slip.

2.6.2 Protein translation in primary astrocytes

To investigate the influence of angiogenin and tiRNAs on protein translation primary astrocytes were seeded 2x10^4 cells/well on cover slips in 24-well plate, medium was changed 48 h later (3rd day) and on the 5th day cells were treated.

2.6.2.1 Angiogenin influence on protein translation

Primary astrocytes were washed with 0.5 ml warm HBSS, and cultured for 1 h in AA-free DMEM. After depletion of AA reserves, primary astrocytes were incubated with 0.5 ml AA-free DMEM containing 50 μM AHA in the presence or absence of ANG (100 ng/ml and 500 ng/ml), and controls BSA (25 mg/ml), and CHX (0.5 μM and 5 μM). The chemoselective cycloaddition and ICC steps were performed as described in section 2.6.1. Cells were co-stained with antibodies against GFAP and S100b to ensure full astrocyte spectrum. Images were taken on the confocal microscope LSM 710 (settings listed in Table 2-13) and analysed by employing a CellProfiler pipeline (described in 2.11.2).

2.6.2.2 tiRNA influence on protein translation

Primary astrocytes were washed with 0.5 ml warm HBSS, and transfected with 750 nM synthetic tiRNAs (112.5x10^8 copies/cell) using 2.5 μl Lipofectamine for 1 h (as described in 2.3.4). Transfected cells were washed with 0.5 ml warm HBSS and cultured in AA-free DMEM for 1 h. Then primary astrocytes were incubated with 0.5 ml AA-free DMEM containing 50 μM AHA for 6 h and further processed as in section 2.6.2.1.

2.7 Molecular biological methods

2.7.1 DNA extraction

DNA was extracted from cultured stable SH-SY5Y cells using the High Pure PCR Template Preparation Kit (Roche) following manufacturer’s instructions. Therefore,
confluent T75 flask (~3*10^6 cells) were trypsinised as described previously in chapter (2.2.1.1), cell pellet was resuspended in 5 ml PBS, and centrifuged again at 200 X g for 3 min. Washed cell pellet was resuspended in 200 µl PBS, and transferred in to 1.5 ml tube. 200 µl Binding Buffer and 40 µl reconstituted Proteinase K was added to the cells which were mixed immediately and lysed at 70 °C for 10 min. 100 µl isopropanol (Sigma) was added and mixed by inverting the tube. The cell lysate solution was loaded on the column, centrifuged at 8,000 X g for 1 min, and the flow through liquid was discarded. 500 µl Removal Buffer was added to the tube, centrifuged at 8,000 X g for 1 min, and the flow through liquid was discarded. The column was washed twice, and each step involved: adding 500 µl Wash Buffer to the tube, centrifugation at 8,000 X g for 1 min, and the flow through liquid was discarded. The column was centrifuged at maximum speed (16.1 X kg) for 10 sec to entirely remove EtOH from the Washing Buffer. The DNA was eluted twice by incubating the column with 25 µl 65 °C distilled H₂O (Thermo Fisher Scientific) for 1 min followed by centrifugation at 8,000 X g for 1 min. The eluted DNA was stored at -20 °C for later analysis.

2.7.2 Conditioned medium generation

Stable SH-SY5Y cell lines were seeded in petri dishes (Ø 10 cm) at 3x10^6 cells/dish and 10 ml working volume. After 24 h incubation at 37 °C and 5 % CO₂, the cells were washed 3x with warmed HBSS and the medium was changed to Neurobasal® Medium (Gibco) containing 1% P/S and 1% L-Gln. After 24 h the conditioned medium (CM) was centrifuged for 5 min at 3000 X g and transferred into a new tube. CM was stored at -80 °C. 2-3 dishes were employed for each cell line in order to extract protein and RNA separately of the same cell line passage.

2.7.3 Protein extraction

To examine angiogenin production in stable SH-SY5Y cell lines, stable and control cell lines were cultured under normal (control - 2.2.1.1; stable cells - 2.3.1) or CM conditions (2.7.2). The cells were washed with ice-cold PBS and lysed in 75 µl
Material and Methods

RIPA-buffer containing phosphatase inhibitor cocktail 2 and 3 (Sigma) and protease inhibitor cocktail (Sigma).

RIPA-buffer
50 mM Tris (biomol) pH 7.8 (with HCl)
150 mM NaCl (Sigma)
0.1% (w/v) SDS (Sigma)
0.5% (w/v) Na Deoxycholate (Sigma)
1% (w/v) Triton X 100 (Sigma)

The cells were scraped to a rim with a sterile cell scraper (VWR International), transfer to a tube and triturated with P200 pipette. The cell suspension was incubated on mixer for 30 min at 4 °C and centrifuged for 30 min at 4 °C and 16,300 X g. The supernatant was transferred into a new tube and stored at -20 °C.

2.7.4 Angiogenin treatment in MZ-294 cells
MZ-294 cells were seeded 24 h prior treatment at a density of 2x10^5 cells/well of a 6-well plate or 2x10^6 cells/T75 with a confluency of 80% and working volume of 12 ml/T75 or 2 ml/well of a 6-well plate. On the treatment day, the supernatant was discarded and the cells were washed thrice with 8 ml/T75 or 1 ml/well of a 6-well plate of warmed HBSS for approx. 5 min by putting into incubator. MZ-294 cells were treated for 6 h with serum-free neurobasal medium, in the presence and the absence of rhANG/BSA.

For angiogenin uptake experiments, cells in 6-well plates were washed twice with ice-cold PBS and lysed in 100 μl RIPA-buffer containing phosphatase inhibitor cocktail 2 and 3 and protease inhibitor cocktail. The further steps have been performed as described in paragraph 2.7.3.

For angiogenin mediated RNA cleavage assessment, RNA was extracted from cells of T75 flasks. The protocol was identical to the steps described in paragraph 2.7.5.

2.7.5 RNA extraction from cells
TRIzol® (Invitrogen) was used to extract RNA following manufacturer’s instructions from cells which were used to generate CM. Therefore, cells were washed twice
with 8 ml/dish ice cold PBS and aspirated off. Then 1 ml/dish TRIZOL® were added underneath the fume hood, shook gently and incubated for 5 min on ice. Cells were scraped to one rim with a sterile cell scraper, cell suspension was transferred in 1.5 ml RNAse free tube and triturated with P200 (20x up&down). After incubation for 5 min at RT, cell suspension were centrifuged for 10 min at 4 °C and 12,000 X g and the supernatant were transferred in a new 1.5 ml RNAse free tubes. 250 µl Chloroform (Sigma)/1 ml TRIZOL® were added and the tubes were shook vigorously by hand for 15 sec. After centrifugation for 15 min at 4 °C and 12,000 X g, the mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. The aqueous phase was transferred by angling the tube at 45° to a new 1.5 ml RNAse free tube. 0.5 ml isopropanol (Sigma)/1 ml TRIzol® was added to the aqueous phase and incubated at −20 °C for 1-2 h. The suspension was centrifuged for 10 min at 4 °C and 12,000 X g. The supernatant was discarded and 1 ml 75 % ethanol/1 ml TRIzol® was added to the pellet. The sample was vortexed and centrifuged for 5 min at 4 °C and 7,500 X g. Supernatant was discarded and the RNA pellet was air dried till the pellet got from milky to clear. 10 µl RNAse free Water (Invitrogen) with 1 µl RNAse OUT (Invirtogen) was added and resolved by incubating for 10 min at 55 °C. The samples were cooled on ice and quantified by Nanodrop (Thermo Fisher Scientific). The samples were stored at -80 °C.

### 2.7.6 TCA precipitation

To examine whether the generated stable SH-SY5Y cell showed secretion of angiogenin, and determine how the ALS associated ANG mutations differ from wild-type ANG, proteins were precipitated from conditioned medium of stable and control SH-SY5Y cells. TCA (Sigma) was dissolved in H2O (1 g/ml or 100 % (w/v)) and added to the supernatant to a final concentration of 20 % (v/v) TCA. After brief vortexing, 1 µl sterile filtered 1 % (w/v) BSA (Thermo Fisher Scientific) in PBS were mixed, and incubated on ice for 1 h. Then the solution was centrifuged at 4 °C and 16,100 X g for 30 min and the pellet was air dried overnight. The dried
pellet was then resuspended in 20 µl 62.5 mM Tris (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) Glycerol (Sigma) and stored at -20 °C till it was analysed by 1D-PAGE.

2.7.7 RNA extraction from conditioned medium

RNA was extracted from CM using the miRCURY™ RNA Isolation Kit – Biofluids (Exiqon) following manufacturer’s instructions. Therefore, 150 µl Lysis solution BF was added to 0.5 ml sample, and vortexed for 5 sec. After 3 min incubation at RT, 50 µl of Protein Precipitation Solution BF was added, vortexed for 5 sec, and incubated for 1 min at RT. Solution was centrifuged for 3 min at 11,000 x g and RT, and supernatant was transferred into a new 1.5 ml RNAse free tube. 675 µl isopropanol was added to the supernatant, vortexed for 5 sec, and loaded on a microRNA Mini Spin Column BF. Column was incubated for 2 min at RT, centrifuged for 30 sec at 11,000 x g and RT, and the flow through liquid was discarded. Sample volume >700 µl was loaded in additional steps on the same column. 100 µl Wash Solution 1 BF was added to the column, centrifuged for 30 sec at 11,000 x g and RT, and the flow through liquid was discarded. 700 µl Wash Solution 2 BF was added to the column, centrifuged for 30 sec at 11,000 x g and RT, and the flow through liquid was discarded. In the last washing step, 250 µl Wash Solution 2 BF was added to the column, centrifuged for 2 min at 11,000 x g and RT, and the flow through liquid was discarded. RNA was eluted twice by incubating the column with 25 µl distilled H₂O (Thermo Fisher Scientific) for 1 min followed by centrifugation at 11,000 X g for 1 min. Eluted RNA was stored at -80 °C for later analysis.

2.7.8 Immunocytochemistry (ICC)

Sterile glass cover slips (VWR International) were added in 24-well plate and washed twice with HBSS. SH-SY5Y/MZ-294 cells were seeded at a density of 1x10⁵ SH-SY5Y cells/well and 2x10⁴ MZ-294 cells/well and incubated overnight at 37 °C and 5 % CO₂. Cells were washed with 0.5 ml warmed PBS (Lonza) and fixed by adding warmed 0.4 ml/well 4 % PFA for 10 min at RT. After two washes with 0.5 ml PBS, cells
were permeabilised with -20 °C 95% ethanol-5% glacial acetic acid solution for 10 min. Cells were washed twice with 0.5 ml PBS and incubated with 0.4 ml/well 5 % (w/v) BSA (Thermo Fisher Scientific) in PBS for 30 min at 37 °C. Afterwards cells were incubated with 250 µl of the primary AB in 5 % (w/v) BSA in PBS at 4 °C over night. Cells were washed twice with 0.5 ml PBS and incubated with 250 µl of the secondary AB in 5 % (w/v) BSA in PBS at RT for 2 h in the dark. Cells were washed twice with 0.5 ml PBS, and were mounted with Prolong® Gold antifade with DAPI reagent (Invitrogen) on glass slides (VWR International).

**Table 2-4: Primary antibodies for Immunocytochemistry**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Catalogue no</th>
<th>Origin</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti angiogenin</td>
<td>AB-265-NA</td>
<td>goat</td>
<td>R&amp;D Systems</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti G3BP</td>
<td>611126</td>
<td>mouse</td>
<td>BD Biosciences</td>
<td>1:800</td>
</tr>
<tr>
<td>Anti YB-1</td>
<td>ab12148</td>
<td>rabbit</td>
<td>Abcam</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti α-tubulin</td>
<td>T6199</td>
<td>mouse</td>
<td>Sigma</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti Fibrillarin</td>
<td>#2639</td>
<td>rabbit</td>
<td>CST</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti SMI-32</td>
<td>#801701</td>
<td>mouse</td>
<td>BioLedend</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti GFAP</td>
<td>G9269</td>
<td>mouse</td>
<td>Sigma</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti S100b</td>
<td>S2532</td>
<td>rabbit</td>
<td>Sigma</td>
<td>1:500</td>
</tr>
</tbody>
</table>

**Table 2-5: Secondary antibodies for Immunocytochemistry**

<table>
<thead>
<tr>
<th>Anti-serum</th>
<th>Catalogue no</th>
<th>Origin</th>
<th>Conjugate</th>
<th>Manufacturer</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti mouse</td>
<td>A-21202</td>
<td>donkey</td>
<td>Alexa Fluor® 488</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti rabbit</td>
<td>A-21206</td>
<td>donkey</td>
<td>Alexa Fluor® 488</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti mouse</td>
<td>A-10037</td>
<td>donkey</td>
<td>Alexa Fluor® 568</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti rabbit</td>
<td>A-10042</td>
<td>donkey</td>
<td>Alexa Fluor® 568</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti goat</td>
<td>A-11057</td>
<td>donkey</td>
<td>Alexa Fluor® 568</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti rabbit</td>
<td>A-31573</td>
<td>donkey</td>
<td>Alexa Fluor® 647</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti mouse</td>
<td>A-31571</td>
<td>donkey</td>
<td>Alexa Fluor® 647</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
<tr>
<td>Anti goat</td>
<td>A-21447</td>
<td>donkey</td>
<td>Alexa Fluor® 647</td>
<td>Thermo Fisher Scientific</td>
<td>1:500</td>
</tr>
</tbody>
</table>
2.8 Analytic methods

2.8.1 Polymerase chain reaction (PCR)

PCR reaction components were stored and assembled on ice. 3 ng template DNA was added to 25 µl PCR solution.

PCR solution
1x reaction buffer without MgCl₂ (content of Taq DNA Pol)
1.5 mM MgCl₂ (content of Taq DNA Pol)
0.2 mM dNTPs (Thermo Fisher Scientific)
0.5 µM of each primer (Table 2-6)
1.5 U Taq DNA Pol (Invitrogen)

Table 2-6: pcDNA3.1 (+) vector primer

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' - 3')</th>
<th>T_M</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV-fwd</td>
<td>CGC AAA TGG GCG GTA GGC GTG</td>
<td>76.9 °C</td>
<td>Sigma</td>
</tr>
<tr>
<td>pcDNA3_rev</td>
<td>GGC AAC TAG AAG GCA CAG TC</td>
<td>61.5 °C</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

After all PCR components were assembled, the following thermocycling conditions were employed at the Thermalcycler (MJ Research):

- Initial denaturation: 95 °C 30 sec
  - Denaturation: 95 °C 30 sec
  - Annealing: 57 °C 30 sec
  - Extension: 72 °C 60 sec
- Final extension: 72 °C 5 min

40 cycles

PCR products were stored at -20 °C till further usage.

2.8.2 Reverse transcription quantitative real-time PCR (RT-qPCR)

RT-qPCR is a two-step process. In the first step “reverse transcription–PCR” (RT-PCR), complementary DNA (cDNA) is generated from RNA template by reverse transcriptase. In the second step “quantitative real-time PCR” (qPCR), cDNA is amplified in several PCR cycles, and the amount of generated PCR products are simultaneously measured. It is possible to determine the starting quantity of the
Material and Methods

target sequence contained in the sample by quantifying the PCR product amount in the exponential phase of the reaction ((Lee et al. 1993), (Huang et al. 2011)). To determine the hANG mRNA levels in stable SH-SY5Y and control cell lines we employed fluorescence DNA-intercalating dye SYBR Green I (2.8.2.2). This method was firstly described by (Higuchi et al. 1993), and is based on the fluorescence accumulation of the DNA-intercalating dye during thermocycling steps in the amplified PCR products.

TaqMan probe-based assay (2.8.2.3) was applied to quantify tiRNA levels in cellular RNA (2.7.5) and in the RNA content extracted from the supernatant (2.7.7). (Heid et al. 1996) developed this method based on the usage of a dual-labelled fluorogenic probe (TaqMan probe). TaqMan probe is a primer-like sequence, which cannot be extended by polymerases since it lacks a free hydroxyl group at the 3´end. TaqMan probe sequence is identical to the sample-of-interest middle region. Furthermore, it exhibited at the 5´end a fluorophore (FAM™) and at the 3´end a quencher (TAMRA™). The method employs the 5´ exonuclease activity of the polymerase to cleave the TaqMan probe during the extension step. This results in the release of the fluorophore from the close proximity to the quencher, and leads to increased fluorescence intensity.

In both methods fluorescence intensities were measured after each cycle, which permitted an accurate definition of the exponential phase and consequently quantifying the starting concentration of the target sequence.

2.8.2.1 DNase I step

A DNase I treatment was performed to prevent carryover of genomic DNA from RNA isolation. 2 µg RNA was added to 1 unit DNase I (Invitrogen) in 10 µl 1X DNase I Reaction Buffer (Invitrogen). Supernatant extracted RNA concentrations were low (<5 ng/µl). Therefore, 44 µl supernatant extracted RNA was mixed with 5 µl 10X DNase I Reaction Buffer and 1 unit DNase I. Solution was mixed and incubated for 15 min at RT. Reaction was terminated by adding 1 µl EDTA 25 mM (Invitrogen) and 10 min incubation at 65 °C. Samples concentration was measured again by Nanodrop and stored till further usage on ice.
Material and Methods

2.8.2.2 SYBR Green I assay

Initially, cDNA was generated from DNase I treated RNA samples. Therefore, 1 µg sample RNA was resuspended in 11 µl RNase free H₂O and the following reagents added:

- 1 µl 100 µM random hexamer primers (Fermentas)
- 1 µl 10 mM dNTP mix (Thermo Fisher Scientific)

RNA samples were denatured for 5 min at 65 °C and the following reagents added:

- 4 µl 5x First-Strand Buffer (content of SuperScript™ III RT)
- 1 µl 0.1 M DTT (content of SuperScript™ III RT)
- 1 µl SuperScript™ III RT (200 units/µl) (Thermo Fisher Scientific)
- 1 µl RNaseOut Ribonuclease Inhibitor (Invitrogen)

In case of reverse transcriptase-negative control, SuperScript™ III RT was replaced by 1 µl RNase free H₂O. Solution was mixed gently and placed in the thermal cycler with the following program:

- 25 °C 5 min
- 50 °C 50 min
- 70 °C 15 min

Amplified cDNA was quantified on StepOnePlus™ (Thermo Fisher Scientific) by using Quantitect SYBR Green PCR Kit (Qiagen Ltd.) following manufacturer’s instructions. Therefore, SYBR Green master mix was prepared containing:

- 1 µl 10 µM of forward and reverse primer (Table 2-7)
- 10 µl 2x QuantiTect SYBR Green PCR Master Mix (Qiagen Ltd.)
- 7 µl RNase free H₂O

Table 2-7: ANG and GAPDH primer

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' - 3')</th>
<th>T_M</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>hANG_for</td>
<td>CAC ACT TCC TGA CCC AGC ACT A</td>
<td>66.4 °C</td>
<td>Sigma</td>
</tr>
<tr>
<td>hANG_rev</td>
<td>TTC TCT GTG AGG GTT TCC ATT A</td>
<td>64.2 °C</td>
<td>Sigma</td>
</tr>
<tr>
<td>GAPDH_for</td>
<td>AAC TTT GGC ATT GTG GAA GG</td>
<td>58.5 °C</td>
<td>Sigma</td>
</tr>
<tr>
<td>GAPDH_rev</td>
<td>ACA CAT TGG GGG TAG GAA CA</td>
<td>58.6 °C</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
Material and Methods

2 µl amplified template cDNA (~100 ng) was added directly into separated wells of the MicroAmp® Fast Optical 96-Well Reaction Plate (Thermo Fisher Scientific). 18 µl SYBR Green master mix was added to each well, and sealed with MicroAmp® 96-Well Optical Adhesive Film (Thermo Fisher Scientific). Plate was vortexed, span down for 3 min at 2,000 X g, and placed into StepOnePlus™. The following program was set-up:

- Initial denaturation: 95 °C 15 sec
  - Denaturation: 94 °C 15 sec
  - Annealing: 56 °C 30 sec
  - Extension: 72 °C 30 sec

- Melting curve

The Melting curve step increases the temperature slowly from 60 °C to 95 °C, and SYBR Green intensities are measured in 0.3 °C-steps. Samples with a single PCR product exhibit only one peak at a temperature specific to the length and GC-content of the product. Samples with several peaks indicate different PCR products. In the analysis only results with a single melting curve peak were employed. Each condition was measured in three separate wells (technical replicates).

2.8.2.3 TaqMan assay

In the initial step, cDNA was generated from DNase I treated RNA samples by using the Taqman microRNA reverse Transcription kit (Thermo Fisher Scientific) following manufacturer’s instructions. Therefore, 100 ng sample RNA was resuspended in 5 µl RNase free H₂O and the following reagents were added:

- 0.15 µl 100 mM dNTPs (from the kit)
- 1.5 µl 10x Reverse Transcription Buffer (from the kit)
- 1 µl MultiScribe™ Reverse Transcriptase, 50 U/µl (from the kit)
- 0.19 µl RNase Inhibitor, 20 U/µl (from the kit)
- 4.16 µl RNase free H₂O
- 3 µl 5x RT primer
In the case of supernatant extracted RNA, 5 µl DNase I treated sample was mixed with the above mentioned reagents. The TaqMan primer and probes are copyright protected by Thermo Fisher Scientific. During the ordering process, tRNA sequences (Table 2-2) were sent to Thermo Fisher Scientific and they designed the TaqMan primer and probes. For the reverse transcriptase-negative control, MultiScribe™ Reverse Transcriptase was replaced by 1 µl RNase free H₂O. Solution was mixed gently and placed in the thermal cycler with the following program:

- 16 °C 30 min
- 42 °C 30 min
- 85 °C 5 min

cDNA products were diluted 1:10 with RNase free H₂O, and 1 µl was added into wells of the MicroAmp® Fast Optical 96-Well Reaction Plate. 9 µl of the following solution was added to each well:

- 3.5 µl RNase free H₂O
- 5 µl 2x TaqMan® Universal PCR Master Mix, No AmpErase® UNG
- 0.5 µl 20x TaqMan® Small RNA Assay primer and probe

Plate was sealed with MicroAmp® 96-Well Optical Adhesive Film, and vortexed. Samples were span down for 3 min at 2,000 X g, and placed into StepOnePlus™. The following program was set-up:

- Initial denaturation: 95 °C 10 min
  - Denaturation: 95 °C 15 sec
  - Annealing/ Extension: 60 °C 60 sec

Melting curve quality control is not required, since TaqMan probes are sequence specific. Each condition was measured in three separate wells (technical replicates).
2.8.2.4 Analysis of quantitative real-time PCR

Quantitative real-time PCR readout is a threshold cycle (C<sub>T</sub>) when PCR product fluorescence is higher than the background. At this cycle PCR product amplification exhibited an exponential phase of the reaction. In the exponential phase each further cycle duplicates the PCR product amount. Therefore, C<sub>T</sub> value is employed to determine the initial quantity of the product. Lower C<sub>T</sub> values indicate high initial sample concentration, whereas higher C<sub>T</sub> values mean lower.

Two different quantification methods exist: (i) relative quantification, and (ii) absolute quantification.

Relative quantification compares the target expression relative to a reference group such as an untreated control. 2^(-ΔΔC<sub>T</sub>)-method (described by (Livak & Schmittgen 2001)) was employed to quantify target expression in generated stable SH-SY5Y cells (compare Table 2-8). The method normalises on two levels: (i) target expression relative to a housekeeping gene, such as GAPDH and U6 snRNA. (ii) Treated cell line relative to control cell line. Here is an example for ANG expression in wild-type overexpressing cells compared to control cell line exhibiting the empty vector. ΔΔCT = \{ (C<sub>T,ANG(WT<sub>1</sub>) - C<sub>T,GAPDH(WT<sub>1</sub>) \) -
AVERAGE((C<sub>T,ANG(pcDNA<sub>1</sub>) - C<sub>T,GAPDH(pcDNA<sub>1</sub>)), (C<sub>T,ANG(pcDNA<sub>2</sub>) - C<sub>T,GAPDH(pcDNA<sub>2</sub>)), (C<sub>T,ANG(pcDNA<sub>3</sub>) - C<sub>T,GAPDH(pcDNA<sub>3</sub>) ))\}. ΔΔCT values <0 mean higher target expression.

**Table 2-8: Relative quantified qPCR data**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>qPCR method</th>
<th>Target expression</th>
<th>Normalisation 1 (housekeeping gene)</th>
<th>Normalisation 2 (control cell line)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANG fold change in stable SH-SY5Y</td>
<td>SYBR Green</td>
<td>ANG</td>
<td>GAPDH</td>
<td>pcDNA</td>
</tr>
<tr>
<td>tiRNA generation in stable SH-SY5Y</td>
<td>Custom designed TaqMan</td>
<td>5<code>Val, 5</code>Gly, 3<code>Arg, 5</code>Ala</td>
<td>U6 snRNA</td>
<td>pcDNA</td>
</tr>
</tbody>
</table>
Material and Methods

Absolute quantification determines the initial sample concentration relating to a standard curve. This method was employed for the quantification of specific tiRNAs in the supernatant of stable SH-SY5Y cell lines. cDNA of a known synthetic tiRNA copy number was generated and a serial dilution for each tiRNA quantification was run on the same plate. The resulting linear regression (trendline) of the serial dilution was employed to calculate the tiRNA concentration in the stable SH-SY5Y cell lines (compare Figure 2.2).

Figure 2.2: Absolute quantification of 5`Val tiRNA secretion in stable SH-SY5Y cell lines. RNA extracted from supernatants of stable SH-SY5Y cell lines were examined for 5`Val secretion by using custom designed TaqMan assay. Serial dilution of 5`Val cDNA (visualised as cross) with a known copy number was employed to generate linear regression. The function of the linear regression was used to determine the copy number of the secreted 5`Val tiRNA in the supernatant of stable SH-SY5Y cell line overexpressing wild-type angiogenin (Ang WT, red diamond), ANG K40I (Ang K40I, green triangle), ANG R31K (Ang R31K, purple circle), and control cell line exhibiting empty vector (pcDNA, cyan square).
2.8.3 Micro BCA protein assay

Micro BCA protein assay (Thermo Fisher Scientific) was performed to quantify the protein concentration by following manufacturer’s instructions. Therefore, at first the working solution (WK) was prepared:

- 75 µl Solution A
- 72 µl Solution B
- 3 µl Solution C

The WK was stored at RT in dark till its usage. 150 µl 0.9 % NaCl was added to each well in a flat bottom 96 well plate. The standard curve was set by adding manufacturer’s 2 mg/ml BSA to 0.9 % NaCl (1 µg, 1.5 µg, 2 µg, 2.5 µg, 3 µg, 3.5 µg, 4 µg, 5 µg). Then samples and the same volume of sample buffer were added to 0.9 % NaCl. All quantifications were performed in triplicate to reduce pipetting errors. After adding 150 µl WK to wells, the plate was incubated for 30 min at 37 ºC in dark and the absorbance was measured at 570 nm. Protein concentration was quantified by subtracting the buffer absorbance and using a standard curve equation, which was generated for each quantification.

2.8.4 1D-PAGE & Western Blotting

2.8.4.1 Casting gel

After cleaning glass plates (Bio-Rad), glass plates were fixed in the gel casting rack and 12 % separation gel solution was prepared. The 12 % separation gel solution was added between the glass plates and overlaid with a few ml of isopropanol (Sigma). After gel solution was polymerised, isopropanol was discarded, stacking gel solution was added and a gel comb was inserted. If casted gels were not used on the same day, gels were stored in running buffer wet paper tissues at 4 ºC for few days.
Table 2-9: Recipe for 10 ml 12 % separation gel

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>3.75 ml</td>
<td>37.5% (v/v)</td>
</tr>
<tr>
<td>40 % Acrylamide/bis-Acrylamide</td>
<td>3.0 ml</td>
<td>12% (v/v)</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>3.25 ml</td>
<td>487.5 mM</td>
</tr>
<tr>
<td>10 % (w/v) SDS</td>
<td>100 µl</td>
<td>0.001% (w/v)</td>
</tr>
<tr>
<td>10 % (w/v) APS</td>
<td>100 µl</td>
<td>0.001% (w/v)</td>
</tr>
<tr>
<td>TEMED (Sigma)</td>
<td>5 µl</td>
<td>0.0005% (v/v)</td>
</tr>
</tbody>
</table>

Table 2-10: Recipe for 5 ml Stacking gel

<table>
<thead>
<tr>
<th></th>
<th>Amount</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH2O</td>
<td>3.725 ml</td>
<td>74.5% (v/v)</td>
</tr>
<tr>
<td>40 % Acrylamide/bis-Acrylamide</td>
<td>0.625 ml</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td>1.5 M Tris pH 6.8</td>
<td>0.6 ml</td>
<td>180 mM</td>
</tr>
<tr>
<td>10 % (w/v) SDS</td>
<td>50 µl</td>
<td>0.001% (w/v)</td>
</tr>
<tr>
<td>10 % (v/v) APS</td>
<td>50 µl</td>
<td>0.001% (w/v)</td>
</tr>
<tr>
<td>TEMED</td>
<td>2.5 µl</td>
<td>0.0005% (v/v)</td>
</tr>
</tbody>
</table>

2.8.4.2 Sample preparation

6x Laemmli-buffer was added to samples and denaturated at 95 °C and 800 rpm for 5 min. Then samples were cooled down to RT and centrifuged for few sec.

6x Laemmli-buffer
- 1.5% (w/v) SDS
- 10% (v/v) Glycerol
- 62.5 mM Tris (pH 6.8)
- 0.0025 % (w/v) Bromophenol blue
- 2% (v/v) β-mercaptoethanol

2.8.4.3 Sample loading and gel running

Cast gels were rinsed with dH2O, and were inserted into the gel chamber (Bio-Rad). Gel chamber was filled up with running buffer, and comb was removed. Each well was rinsed with running buffer, before samples were loaded. Samples were prerun at constant current for 30 min at 60 V, before increasing the current to 120 V and running additionally 90 min.
Material and Methods

5x Running buffer
12.8 mM Tris Base (pH 8.3)
1.25 M Glycine
0.5% (w/v) SDS

2.8.4.4 Semi-dry protein transfer to membrane

At first the plates of transfer machine (Bio-Rad) were cleaned with H₂O and rinsed with semi-wet transfer buffer. All parts of the western blot transfer sandwich were wetted in semi-wet transfer buffer and assembled air bubble-free as followed (button to top):

1. Two blotting paper sheets (850 g/m² - Thermo Fisher Scientific) on button
2. Nitrocellulose-membrane (0.2 µm - Thermo Fisher Scientific)
3. Gel
4. Two blotting paper sheets (850 g/m²) on top

At the end air bubbles and redundant liquid were removed with a roller. Proteins were transfered at constant current at 18 V for 90 min.

Semi-wet transfer buffer
25 mM Tris-Base
192 mM Glycine
20% (v/v) MeOH

2.8.4.5 Western blot analysis

The membrane was washed with H₂O, stained with ponceau solution (Sigma) and washed with dH₂O to remove superfluous ponceau. Ponceau staining was documented by LAS 4000 (FujiFilm). The blot was blocked with 5 % (w/v) dried milk in TBS-T (milk TBS-T) for 1 h at RT on shaker, and incubated with first antibody (AB) in 4 ml milk TBS-T with 0.01 % (v/v) Na-Azide (Sigma) overnight at 4 °C on roller mixer. Then the blot was washed 3x with TBS-T each wash for 5 min at RT on shaker, and incubated with second AB in 15 ml milk TBS-T for 3 h at RT on shaker. After the blot was washed 3x with TBS-T each wash for 5 min at RT on shaker, 0.5 ml Peroxid Solution and 0.5 ml Luminol Reagent (chemiluminescent
working solution – Millipore) were added and incubated for 2 min at RT and in dark. The chemiluminescent reaction was recorded by LAS 4000. The blot was briefly washed in TBS-T and either incubated with next first AB or stored at -20 °C.

TBS-T
130 mM Tris-Base (pH 6.8)
1.5 M NaCl
0.05 % (v/v) Tween 20 (Sigma)

Table 2-11: Primary antibodies for Western Blotting

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Catalogue no</th>
<th>Origin</th>
<th>Manufacturer</th>
<th>Dilution</th>
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<tr>
<td>Anti actin</td>
<td>A5441</td>
<td>mouse</td>
<td>Sigma</td>
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<tr>
<td>Anti angiogenin</td>
<td>AB-265-NA</td>
<td>goat</td>
<td>R&amp;D Systems</td>
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<td>Anti p-Akt</td>
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<td>1:500</td>
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<td>Anti Mcl-1</td>
<td>559027</td>
<td>mouse</td>
<td>BD Biosciences</td>
<td>1:1000</td>
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<td>Anti Bak</td>
<td>sc-832</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>1:100</td>
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<td>Anti Bim</td>
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<td>rabbit</td>
<td>Epitomics</td>
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<td>Anti Bcl-XL</td>
<td>#2762</td>
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<td>Anti Bcl-2</td>
<td>sc-509</td>
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<td>Anti Col4a</td>
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Table 2-12: Secondary antibodies for Western Blotting

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<th>Anti-serum</th>
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<td>goat</td>
<td>HRP</td>
<td>Merck Millipore</td>
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<td>AP132P</td>
<td>goat</td>
<td>HRP</td>
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<td>1:5000</td>
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<td>Anti goat</td>
<td>AP1069</td>
<td>rabbit</td>
<td>HRP</td>
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<td>1:3000</td>
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2.8.5 Denaturing urea polyacrylamide RNA-Gel

2.8.5.1 Casting Gel

In order to maintain RNase free conditions, all glass plates and gel comb was washed with 5 M NaOH (Sigma). Furthermore, the washed part were rinsed twice with DEPC treated H2O to remove excess NaOH. DEPC treated H2O was generated by treating deionised H2O with 0.1% (v/v) DEPC (Sigma) over night. DEPC containing H2O was inactivated by autoclaving the solution. After gel-casting parts were cleaned, glass plates were fixed in the gel casting rack. 10 ml RNA-Gel solution was poured into a 15 ml Falcon and 5 µl TEMED and 50 µl 10 % APS were added. Solution was mixed and added between the glass plates, and a gel comb was inserted.

RNA-Gel solution (100ml)
15% (v/v) Acrylamide/bis-Acrylamide 40 % (Sigma)
48 g Urea (AppliChem)
1x TBE-buffer
Filled up to 100 ml with DEPC treated H2O
Filter sterilise (0.22 µm filer) – store protected from light at 4 °C

10x TBE buffer (autoclaved)
89 mM Tris base (pH~8)
89 mM Boric acid
2 mM EDTA

2.8.5.2 RNA gel prerun and sample preparation

Polymerised RNA gel was rinsed with DEPC treated H2O, and inserted into the gel chamber. Gel chamber was filled up with 0.5x TBE buffer, and comb was removed. Each well was rinsed, and RNA gel was prerun at constant current (200 V) for 1h at 4 °C (cold room). 10 µg extracted RNA (2.7.5) was diluted in 10 µl DEPC treated H2O and mixed with 10 µl 2x loading buffer (90% (v/v) formamide [Ambion], 11 mM EDTA). Samples and Small RNA Marker Easy (Abnova) were denaturated at 95 °C for 5 min, span down, and stored on ice.
2.8.5.3 RNA gel running and sample detection

Each well was rinsed again, and samples and marker were loaded. RNA gel was run at constant current (200 V) for 100 min at 4 °C (cold room). Gel was extracted from the glass plates, and incubated in 50 ml DEPC with 1x SYBR gold gel stain (Invitrogen) for 10 min. RNA gel was rinsed with DEPC treated H₂O, and imaged on LAS3000 (FujiFilm).

2.8.6 Agarose gel electrophoresis

2.8.6.1 Casting gel

Agarose was weighed in a clean Erlenmeyer flask and appropriate running buffer volume (TEA buffer) was added to generate a 2% gel solution. Agarose solution was melt by heating for ~1 min in the microwave or until agarose has completely dissolved. SYBR™ Safe DNA Gel Stain was added to achieve a 1x working solution. Gel solution was poured into a gel mold, gel comb was inserted, and gel was allowed to polymerase at RT.

Running buffer (TEA buffer)
40 mM Tris-Base (adjust pH 8.5)
0.075% (v/v) Glacial acetic acid
2 mM EDTA

2.8.6.2 Sample loading and gel running

Polymerised gel was put into the electrophoresis chamber, enough running buffer was added to cover the surface of the gel, and wells were rinsed with the running buffer. 6x DNA dye was added to samples and loaded into the gel. Samples were run at constant current for 60 min at 100 V. Gel was documented by LAS3000 (FujiFilm).

6x DNA dye
30% (v/v) glycerol
0.25% (w/v) bromophenol blue
0.25% (w/v) xylene cyanol (Sigma)
2.8.6.3 DNA fragment extraction from agarose gel

Gel was visualised on a transilluminator (Biometra), bands of interest were cut with EtOH-cleaned scalpel, and stored in weighed 1.5 ml tubes. DNA was extracted using GeneJET Gel Extraction Kit (Thermo Fisher Scientific) following manufacturer’s instructions. Therefore, 0.1% (v/w [gel]) binding buffer (e.g. 100 µl binding buffer on 100 mg gel) was added to the gel, and gel was incubated for 10 min at 55 °C on thermomixer (Eppendorf). After gel was dissolved, 0.1% (v/w[gel]) isopropanol (Sigma) was added, column was loaded with the solution, and centrifuged for 1 min at RT and maximum speed (16.1 X kg). Column was washed with 100 µl binding buffer followed by centrifugation step for 1 min at RT and maximum speed (16.1 X kg), washed with 700 µl washing buffer followed by centrifugation step, and centrifuged again for 1 min at RT and maximum speed (16.1 X kg) to entirely remove EtOH from the washing buffer. DNA was eluted twice by incubating the column with 10 µl 65 °C warm distilled H₂O (Thermo Fisher Scientific) for 1 min followed by centrifugation step.

2.8.6.4 Verification of the missense mutations in stable cell lines

Gel-extracted PCR products were sequenced by GATC Biotech using CMV-fwd primer.

2.8.7 Densitometric analysis

Densitometric analysis was performed with the software ImageJ. To reduce signal-to-background ratio, area which determines the signal intensity was also measured for the background, which was in close proximity to signal of interest. Appropriate background intensity was subtracted from each signal intensity.
2.9 Microscopy

2.9.1 Dissecting microscopes

Zoom Stereo Microscope SZ51 (Olympus) with a magnification from 4 to 20 X was employed for dissecting the neural plate from E12-14 embryos from C57Bl6 mice (2.2.2) and cortices of 1 to 2 days old C57Bl6 mouse pups (2.2.3). An external light source (Olympus) illuminated the objects.

2.9.2 Confocal microscopy

All immunocytochemical stainings were visualised on the confocal microscope LSM 710 (Zeiss). The strength of confocal microscopes is to obtain images/scans from one focal plane. The optical slice thickness is defined by full width at half maximum (FWHM) and dependent on the pinhole size. The smaller the FWHM/pinhole value the thinner is the optical slice and the more likely are two signals from the same focal plane. Since less light is collected from the sample stronger excitation sources are required. Lasers deliver photons with high frequency of a specific wavelength and are able to excite more fluorophores than UV lamps. The drawback of high power lasers is that fluorophores can irreversible fade when exposed to excitation light, becoming bleached. Therefore, it is important to balance exposure time and laser power. Dapi stained DNA was excited with 405 nm Laser and emission was detected in the wave length range of 415-480 nm. Alexa Fluorophore 488 was excited with 488 nm Laser and emission was detected in the wave length range of 480-560 nm. Alexa Fluorophore 568 and ROX conjugated tiRNA were excited with 561 nm Laser and emission was detected in the wave length range of 570-620 nm. Alexa Fluorophore 647 was excited with 633 nm Laser and emission was detected in the wave length range of 650-760 nm.
<table>
<thead>
<tr>
<th>Experiment</th>
<th>Objective</th>
<th>AB</th>
<th>Fluorophore</th>
<th>Slice thickness</th>
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<td>Angiogenin localisation in stable SH-SY5Y cells</td>
<td>63x</td>
<td>α-tubulin</td>
<td>Alexa 488</td>
<td>1 μm/section</td>
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<tr>
<td></td>
<td></td>
<td>Angiogenin</td>
<td>Alexa 568</td>
<td></td>
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<tr>
<td>SG formation in stable SH-SY5Y cells</td>
<td>63x</td>
<td>G3BP</td>
<td>Alexa 488</td>
<td>1 μm/section</td>
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<tr>
<td></td>
<td></td>
<td>Angiogenin</td>
<td>Alexa 568</td>
<td></td>
</tr>
<tr>
<td>tiRNA transfection optimisation in SH-SY5Y cells</td>
<td>63x</td>
<td>tiRNA</td>
<td>ROX</td>
<td>0.7 μm/section</td>
</tr>
<tr>
<td>tiRNA localisation in SH-SY5Y cells</td>
<td>63x</td>
<td>Fibrillarin</td>
<td>Alexa 488</td>
<td>0.7 μm/section</td>
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<tr>
<td></td>
<td></td>
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<td>ROX</td>
<td></td>
</tr>
<tr>
<td>SG formation in tiRNA transfected SH-SY5Y and MZ-294 cells</td>
<td>63x</td>
<td>G3BP</td>
<td>Alexa 488</td>
<td>0.7 μm/section</td>
</tr>
<tr>
<td></td>
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<td>ROX</td>
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<td>Motoneuron protection</td>
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<td>ROX</td>
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<tr>
<td></td>
<td></td>
<td>SMI32</td>
<td>Alexa 647</td>
<td></td>
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<tr>
<td>tiRNA localisation in SH-SY5Y cells</td>
<td>63x</td>
<td>YB-1</td>
<td>Alexa 488</td>
<td>1 μm/section</td>
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<tr>
<td></td>
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<td>ROX</td>
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<td>Alexa 647</td>
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<tr>
<td>SG formation in primary astrocytes</td>
<td>40x</td>
<td>GFAP &amp; S100b</td>
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<td>1.2 μm/section</td>
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<td></td>
<td>tiRNA</td>
<td>ROX</td>
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<td>Alexa 568</td>
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<td>Protein translation assessment in primary astrocytes</td>
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<td>Click-iT</td>
<td>Alexa 488</td>
<td>14.2 μm/section (opened pinhole)</td>
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<td></td>
<td></td>
<td>Angiogenin</td>
<td>Alexa 568</td>
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<td>tiRNA</td>
<td>ROX</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>GFAP &amp; S100b</td>
<td>Alexa 647</td>
<td></td>
</tr>
</tbody>
</table>
2.10 Flow cytometry

SH-SY5Y cells were seeded at a density of $1 \times 10^5$ cells/well in a 24 well plate with a working volume of 0.5 ml/well and incubated for 24 h at 37 °C and 5 % CO₂. Cells were either transfected with different tiRNAs (described in 2.3.2) or directly cultured under different stress conditions.

2.10.1 Cell Stress Induction

Different stress types were employed to investigate whether tiRNAs exhibit neuroprotective effect in SHSY5Y cells during ALS pathology-related stress conditions. A broad concentration range of stresses were firstly examined. Cells were either treated with high dose and short exposure time (pulse-like stress) followed by a longer recovery period or low dose for a longer period of time with no recovery. It was aimed to achieve 30-40% cell death for each stress condition, allowing either negative or positive effects of tiRNA on cell death to be accurately measured.

2.10.1.1 Oxidative stress

We employed sodium arsenite (NaAsO₂, Sigma) since it has been reported to cause ROS generation, DNA oxidative damage, cell cycle arrest as well as apoptosis ((Ruiz-Ramos et al. 2009), (Jiang et al. 2013)). Furthermore, it is commonly used to induce SG formation ((Emara et al. 2010), (Ivanov et al. 2011), (Higashi et al. 2013)). However, the exact mechanism is still unknown. For a pulse-like stress, cells were treated with 125 µM and 500 µM sodium arsenite for 1 h at 37 °C and 5 % CO₂. Then cells were washed with 0.5 ml warm HBSS and recovered for 23 h at 37 °C and 5 % CO₂. Continuously treated cells were cultured with sodium arsenite (10-50 µM) for 24 h at 37 °C and 5 % CO₂.

2.10.1.2 Proteasomal stress

Proteasomal stress was achieved by treating the cells with the proteasome inhibitor epoxomicin (Sigma). Epoxomicin is a natural product produced in
Actinomycetes strain, which selectively inhibits the proteasome catalytic activity (Meng et al. 1999).

For a pulse-like stress, cells were treated with 100 µM epoxomicin for 1 h at 37 °C and 5 % CO₂. Then cells were washed with 0.5 ml warm HBSS and recovered for 23 h at 37 °C and 5 % CO₂. Continuously treated cells were cultured with epoxomicin (25-100 µM) for 24 h at 37 °C and 5 % CO₂.

2.10.1.3 Endoplasmic reticulum stress

Thapsigargin (Sigma) containing medium was employed to induce endoplasmic reticulum stress by inhibiting sarco/endoplasmic reticulum Ca²⁺-ATPase resulting in accumulation of Ca²⁺ in the cytosol (Wictome et al. 1992). Cells were continuously treated with thapsigargin (1-10 µM) for 24 h and 48 h at 37 °C and 5 % CO₂.

2.10.1.4 Glucose/Serum deprivation

To investigate whether glucose-serum deprivation could be used as cell stress, cells were cultured in glucose-free RPMI 1640 medium (Thermo Fisher Scientific) with 1% (v/v) P/S, and 0.1% (v/v) FBS for 24 h, 48 h, and 72 h at 37 °C and 5 % CO₂. Control cells were cultured in RPMI medium containing additionally 2 g/l D-(+)-Glucose for 72 h.

2.10.2 Flow cytometry measurement

After the cell treatment the supernatant of a well was transferred to labelled tube, washed with 200 µl HBSS and transferred to appropriate supernatant tube and stored on ice. Ice cold Trypsin/EDTA was added and after all cells were detached trypsinisation was stopped by adding appropriate supernatant to the well and transferred back to the tube and stored on ice. The tubes were centrifuged for 6 min at RT and 290 g. The supernatant was discarded and the cell pellet resuspended in 200 µl 1x Binding Buffer (BioVision) with 0.01 % (v/v) Annexin V-FITC (BioVision). SYTOX® Blue (Life Technologies) was added following manufacturer’s instructions briefly before measurements. SYTOX® Blue was
added to the cells to a final concentration of 1 µM and cells were incubated at RT for 15 min in the dark.

Cells were measured on the flow cytometer LSR II (BD). 10,000 cells, defined by their forward and side scatter signal intensity (Laser λ = 488 nm; BP 488/10), were counted for each well. Annexin V-FITC (Ex = 488 nm; Em = 530 nm) binding was detected by using FITC signal detector (Laser λ = 488 nm; BP 525/50; LP 505). SYTOX® Blue (Ex = 444 nm; Em = 480 nm) was detected by using CFP signal detector (Laser λ = 488 nm; BP 450/50). The RNA conjugated fluorophore ROX (Ex = 578 nm; Em = 604 nm) was detected by using PI561 signal detector (Laser λ = 561 nm; BP 605/40; LP 570). Data were analysed in the program Cyflogic version 1.2.1.

2.10.3 Flow cytometry data analysis

Data generated from flow cytometer was imported into Cyflogic. Single well forward and side scatter measurements were compared and the area which represented the cells was defined (Figure 2.3A, all cells). Only signal of these cells were employed for further analysis. Annexin V-FITC, and SYTOX® Blue signals were compared between treated and control wells and areas were defined which exhibit apoptotic cells (Figure 2.3B, SYTOX® Blue positive cells). ROX signals were compared between tiRNA and MOCK transfected cells and tiRNA positive cells were determined (Figure 2.3C). Dot plots of Annexin V-FITC/ROX signals, and SYTOX® Blue/ROX signals were generated to define quadrants which indicate the cell number of apoptotic tiRNA-transfected cell (Figure 2.3D, SYTOX® Blue/ROX positive cells) and the total number of transfected cells (Figure 2.3E, ROX positive cells). Cell counts were exported and the ratio was calculated in Excel. SEM was calculated from separated wells of the independent experiments.

To account for differences in transfection toxicity, tiRNA baseline toxicity percentage of each experiment was subtracted from corresponding cell death data of each well.
Figure 2.3: Exemplary flow data analysis in Cylogic. One well of 5’Ala transfected SH-SY5Y cells were pulse-treated with 500 µM sodium arsenite followed by 23 h recovery. (A) Forward (x-axis) and side (y-axis) scatter signals were gated to define the ~10,000 cells. All other graphs include the signals of only these cells. (B) SYTOX® Blue signal intensity (x-axis) and the frequency (y-axis) were employed to define the apoptotic cells. (C) ROX intensity (x-axis) and the frequency (y-axis) were used to identify 5’Ala containing cells. (D & E) ROX (x-axis) and SYTOX® Blue (y-axis) signal were utilised to define the quadrant which represents the cell number of SYTOX® Blue/ROX positive cells (upper-right). Both quadrants in the right were employed to define the total cell number and quantify the cell death percentage. Blue dots represent SYTOX® Blue positive cells. Purple dots represent ROX positive cells.
Material and Methods

2.11 CellProfiler pipelines

2.11.1 SG formation analysis

Stress granules (SG) were detected using a custom designed image processing pipeline implemented in CellProfiler (Kamentsky et al. 2011). The pipeline identified and counted SG speckles in SH-SY5Y and MZ-294 cells. G3BP staining was employed as a SG marker. All fluorescence staining produced negligible crosstalk into other channels with the sensitivity settings used. With those also the secondary antibody control produced no detectable signal. At the first step all channels (Figure 2.4A) were smoothed and added into one image. This sum of all images was used to segment cell regions (Figure 2.4B). Next cell nuclei were segmented from the DAPI channel (Figure 2.4C). To identify SG speckles the feature enhancement tool for speckles was used on the G3BP images (Figure 2.4D). Subsequently those processed G3BP images allowed to segment speckles in cell regions (Figure 2.4E). CellProfiler readout was a text file format, which was imported into Excel. Cells with more than one speckle were defined as SG positive cells. SG positive cells were normalised to the total cell number.
Figure 2.4: SG recognition workflow of image processing pipeline in CellProfiler. (A) Confocal images of sodium arsenite dependent SG formation in 5’Ala tRNA (250 nM or 7.5x10^8 copies/cell) transfected SH-SY5Y cells. The red channel shows the fluorophore carboxy-X-rhodamine, which is conjugated to the tRNA. Cell nuclei were stained with DAPI (in cyan). G3BP (in green) was used to recognise SGs in the cells. (B) Identification of individual cells from tRNA, DAPI and G3BP channels with colour coding representing the separated cell areas. (C) Cell nuclei identified from DAPI channel. Colour coding represents individual cell nuclei. (D) Enhancement of speckles from G3BP images generated by CellProfiler tool. (E) Identification of individual speckles subsequently used to identify cells with SGs and cells without SGs. Colour coding aids the visualisation of the individual speckles.

2.11.2 Protein translation analysis in primary astrocyte cultures

Click-iT® labelled nascent synthesised proteins were analysed by employing a custom designed image processing pipeline implemented in CellProfiler. The pipeline identified primary astrocytes, which were co-labelled with the primary astrocyte markers GFAP and S100b, and measured the intensities of ROX conjugated tRNAs and Alexa 488 modified nascent synthesised proteins in those cells.
Material and Methods

At the first step cell nuclei were segmented from the DAPI channel. Next GFAP/S100b-channel crosstalk threshold was determined from primary astrocytes incubated with only 2nd ABs during ICC steps. GFAP/S100b intensities above the determined threshold were smoothed (Figure 2.5A) and employed to segment cell regions (Figure 2.5B).

To account for tiRNA transfection efficiency, Click-iT intensity of each cell was normalised to the tiRNA intensity of this cell. Since tiRNA transfection exhibited intense tiRNA signals at the cell surface (Figure 2.5C, red arrows), which are presumably unreleased tiRNA molecules in Lipofectamine complexes. We assumed that this in complexes contained tiRNA is not accessible to be functional and therefore cannot be taken into account as effective tiRNA. For this purpose, tiRNA intensities were corrected by subtracting tiRNA intensities above the adjacent tiRNA intensities. Therefore, tiRNA-channel was smoothed and subtracted from the raw tiRNA-channel. In the next step this tiRNA intensity difference was subtracted from the raw tiRNA intensity (Figure 2.5D). Corrected tiRNA signals as well as nascent synthesised proteins were measured in the segmented cell regions.

The Click-iT and ROX intensities were corrected for channel crosstalk by subtracting the intensities of the corresponding controls. ROX intensities of the MOCK transfected cells were subtracted from 5’ & 3’tiRNAs intensities. Click-iT intensities of primary astrocytes incubated with AHA free medium were subtracted from all Click-iT measurements. Corrected single cell Click-iT values were either compared relatively to: (i) the average baseline Click-iT value in the case of angiogenin, BSA and cycloheximide treated primary astrocytes, or (ii) the normalised average Ctrl tiRNA Click-iT value in the case of transfected primary astrocytes. Cell regions were weighted with the nuclei count of each cell region to consider merged cells, when cells where not segmented as separate objects using the astrocyte marker stain, and were displayed as box-and-whisker diagram.
Figure 2.5: Protein translation alteration correction workflow of automated image processing pipeline in CellProfiler. Protein translation alteration assessment of 5`Ala transfected primary astrocytes co-stained with the astrocyte markers GFAP and S100b. (A) Smoothed GFAP and S100b intensities above determined background threshold. (B) Cell regions were segmented from smoothed GFAP and S100b intensities above determined background threshold. Colour coding represents the separated cell areas. (C) Raw image of 5`Ala transfected primary astrocytes. Red arrows illustrate tiRNA-Lipofectamine aggregates at the cell surface. (D) tiRNA intensities were corrected for untransfected tiRNA-Lipofectamine aggregates at the cell surface by subtracting tiRNA intensities above the adjacent tiRNA intensities.
2.11.3 Quantification of primary astrocyte culture purity

Purity of primary astrocyte culture was determined by employing the same CellProfiler pipeline as described in 2.11.2. The pipeline identified cell nuclei and determined whether the nuclei were located within primary astrocytes. GFAP and S100b co-staining was employed as primary astrocyte markers. Nuclei within primary astrocytes were normalised to total nuclei quantity.

2.12 Statistical analysis

For statistical comparison, data were tested for normality by employing Shapiro-Wilk and Kalmogorov-Smirnov test. Normal distributed data (P>0.05) were analysed by one-way ANOVA pairwise comparison with Tukey's post hoc test and non-normal distributed data (P<0.05) by one-way ANOVA pairwise comparison with Kruskal-Wallis post hoc test. p values were determined by employing the software SPSS version 21 and P<0.05 were considered to be statistically significant.
3. Chapter 3: Angiogenin and ALS related angiogenin mutations characteristics in a human neuronal cell line

3.1 Introduction

Angiogenin (ANG) is a secreted ribonuclease, and was initially isolated from culture medium from colon carcinoma cells (Fett et al. 1985). Angiogenin belongs to the pancreatic RNase A superfamily. Genes encoding for all members are located on human chromosome 14 (reviewed (Dyer & Rosenberg 2006)). Each protein has a ~20-28 amino acid signal pro-domain, which is common among secretory proteins. This is cleaved to produce the mature angiogenin consisting of 123 amino acids and a molecular weight of 14.1 kDa. Angiogenin has three distinct functional sites: (i) a catalytic triad consisting of His13, Lys40 and His114 responsible for its ribonucleolytic activity; (ii) a nuclear localisation sequence $^{31}$RRRGL$^{35}$, involved in nuclear translocation; and (iii) a receptor-binding site $^{60}$NKNGNPHREN$^{68}$ that mediates the endocytosis of angiogenin (reviewed in (Sheng & Xu 2016)).

Previous studies, conducted in our laboratory, showed that angiogenin treatments in transgenic mouse model of ALS (SOD1$^{G93A}$) increased lifespan and motoneuron survival by restoring the disease-associated decrease in Akt-1 survival signalling, and reversing a pathophysiological increase in ICAM-1 expression (Kieran et al. 2008). Additionally, angiogenin treatment in mixed primary motoneuron cultures afforded neuroprotection against endoplasmic reticulum (ER) stress induced by tunicamycin, hypoxic-stress induced by exposure to 1 % O$_2$, and AMPA induced excitotoxicity cell death. Furthermore, our group demonstrated that angiogenin is secreted by motoneurons under stress conditions, such as serum withdrawal, and endocytosed by neighbouring astroglial cells in mixed primary motoneuron cultures (Skorupa et al. 2012), leading to the hypothesis that the neuroprotection of angiogenin is mediated in paracrine (Figure 3.1).
**Figure 3.1: Paracrine neuroprotection of angiogenin.** The hypothesis reveals that motoneurons secrete angiogenin under stress conditions. Angiogenin is than taken up by neighbouring astroglial cells which, in turn, directly or indirectly mediate neuroprotection of motoneurons (adapted from (Skorupa et al. 2013)).

The ribonucleolytic activity of angiogenin has been shown to be required for the *in vitro* protection of mouse motoneuron-like (NSC-34) cells against ER- and oxidative stress ((Kieran et al. 2008), (Cho et al. 2010)). Unpublished next generation sequencing data from glioblastoma cell line (MZ-294) treated with angiogenin under serum deprived conditions identified tRNA as main cleavage targets. Other groups have revealed that tRNAs are cleaved in response to different type of stress (e.g. nutrient deprivation, oxidative and thermal stress, and upon innate immunoactivation) (reviewed in (Kirchner & Ignatova 2015)). RNA cleavage product analysis identified specific pattern with distinct fragments of nucleotide lengths between 30 nt and 47 nt ((Yamasaki et al. 2009), (Skorupa et al. 2012)) and confirmed the finding that angiogenin cleaves tRNA in or near the anticodon loop in mammalian cells (Czech et al. 2013). Studies of stress-induced cleavage of tRNA (tiRNA) have shown that some of the phosphorylated 5’ but none of the 3’ tiRNA fragments can repress protein translation by interfering with
the assembly of the cap binding complex eukaryotic initiation factor 4F (eIF4F). 5’
tiRNAs also induces stress granules (SGs) formation ((Yamasaki et al. 2009),
(Emara et al. 2010), (Ivanov et al. 2011)). SGs are transient cytosolic aggregations
of preinitiation and translation-related factors, mRNA-binding proteins, proteins
associated with mRNA metabolism and RNA molecules (Kedersha & Anderson
2007).
In order to model angiogenin production and secretion in motoneurons, the human
neuroblastoma SH-SY5Y cell line was employed, which possess an established
and stable neuronal background (Biedler et al. 1973). Stable overexpression of
ANG was achieved by transfecting a plasmid with a neomycin resistance
(neomycin phosphotransferase gene) containing an ANG insert. Neomycin
phosphotransferase gene mediates resistance of transformants to geneticin
(G418) selection pressure in mammalian cells. To investigate ALS associated
ANG mutations, stable SH-SY5Y cell lines overexpressing ANG WT, K40I, R31K
and empty plasmid control (pcDNA) were generated. It has to be highlighted that
the selected ANG missense mutations exhibit only low structural changes to native
angiogenin (compare Figure 3.2).

![Structural comparison of hANG and chosen ALS-associated variants.](http://www.delanoscientific.com)

**Figure 3.2:** Structural comparison of hANG and chosen ALS-associated variants. 3D structures of hANG mutants (orange except for mutated amino acid in black) were taken from PDB (K40I – 4AHI; R31K – 4AHH) and aligned to native hANG structure (PDB ID: 4AOH, cyan). Residues in 5 Å surrounding to mutation are displayed to show the low structural changes to native hANG. Figures were created using the programme PyMOL. (http://www.delanoscientific.com)
However, in previous work our group showed that both mutations did not protect NSC-34 cells against hypoxic stress (Sebastià et al. 2009). However, the missense mutations affect different functional sites of angiogenin. Since the K40I mutation affects the catalytic triad (Greenway et al. 2006) and several reports demonstrated the loss of ribonucleolytic activity ((Skorupa et al. 2012), (Thiyagarajan et al. 2012)), it represents the ideal candidate to investigate ribonucleolytic mediated functions of angiogenin. ANG R31K exhibits the mutation in the nuclear localisation sequence. However, R31K in vitro investigations reported that the mutation did not compromise transport into the nucleus (Thiyagarajan et al. 2012). Furthermore, ribonucleolytic activity of R31K was examined in an extracellular system, where tRNA cleavage was only slightly lower (91%) compared to wild-type angiogenin (Thiyagarajan et al. 2012). These observations leave the pathological mechanism of R31K unanswered.

Therefore, we investigate the relationship between ANG expression and cell morphology, and whether the produced ANG mutants varying in the subcellular localisation by employing confocal microscopy. Total RNA cleavage was measured on denaturing urea polyacrylamide gels and the generation of specific tiRNA was quantified by custom designed TaqMan assays, to characterise whether angiogenin ribonucleolytic activity in an intracellular environment. Furthermore, we investigated how the ribonucleolytic activity and tiRNA generation contributed to SG formation. Additionally, we determined the paracrine activity of stable SH-SY5Y cell lines by analysing the secretion of angiogenin and different tiRNAs in conditioned medium of the stable SH-SY5Y cell lines.
3.2 Results

3.2.1 Generation of stable cell lines overexpressing ANG WT and ALS associated ANG mutants

ANG WT and two different ALS-associated mutants (K40I, R31K) were employed to examine their ribonucleolytic activity and secretional characteristics in stable overexpressing human neuroblastoma cell line SH-SY5Y. To generate stable SH-SY5Y cell lines, cells were transfected for each of the following condition: ANG WT; ANG K40I; ANG R31K; empty control vector. Transfected cells and controls were cultured under 0.5 g/l G418 selection pressure for 27 days. After the selection period with high G418 concentration, stable SH-SY5Y cells were cultured under 0.1 g/l G418 (Figure 3.3).

Figure 3.3: Stable overexpressing SH-SY5Y cell line generation. Workflow to generate stable SH-SY5Y cells. pcDNA 3.1 vector backbone with neomycin resistance was used to select and culture stable clones under G418 selection pressure.
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To select the clone, from different replicates, that has the highest angiogenin content produced under antibiotic pressure, western blotting analysis was performed probing for angiogenin protein. The empty plasmid (pcDNA) was used as negative control to ensure that its expression was not enhancing the endogenous angiogenin levels.

As result, ANG WT, pcDNA control clone 2, K40I clone 1, and R31K clone 1 were employed (Figure 3.4) for further experiments. To simplify the nomenclature, selected stable SH-SY5Y cells overexpressing the ANG variant were named for the specific ANG insert: Ang WT; K40I or R31K. In the case of empty plasmid, cell line was named pcDNA. The difference between the separated clones was marginal, suggesting however R31K cells produced the double amount of ANG compared to K40I. This discrepancy indicates that the difference between the mutants is not due to the different plasmid copy number, but suggests either different angiogenin variant turnover or higher levels of R31K were favoured by SH-SY5Y cells.

![Western Blot Analysis](image)

**Figure 3.4: Selection of ANG overexpressing SH-SY5Y stable clones.** SH-SY5Y cells were transfected with ANG WT; ANG K40I; ANG R31K; empty control vector (pcDNA). Angiogenin levels were measured by western blotting (A) and densitometric analysis (B). Beta-actin was used as loading control and for densitometry normalisation. Recombinant human angiogenin (rhANG) was added as positive control of an angiogenin signal.
To verify the missense mutations of ANG K40I and R31K, selected stable SH-SY5Y clones were sequenced. To do this, DNA was extracted from the selected clones using High Pure PCR Template Preparation Kit, and purified ANG DNA was amplified by PCR. Forward and reverse primers (CMV-fwd & pcDNA3_rev) aligned to the vector backbone DNA and PCR products were analysed on an agarose gel. The products had the expected nucleotide sizes, 670 nt for ANG sequence in vector and 273 nt for empty vector (compare Figure 3.5A). Bands were isolated from the gel, and DNA was extracted using GeneJET Gel Extraction Kit. Gel purified PCR products were sequenced by GATC Biotech using CMV-fwd primer. Sequencing results were aligned to human ANG sequence (NM_001097577), and showed the specific missense mutations in ANG K40I (in green) and ANG R31K (in red - Figure 3.5B).
Results

Figure 3.5: Selected Ang K40I and R31K clones showed the specific missense mutations. (A) Agarose gel of PCR products from selected, stable SH-SY5Y clones. PCR products were amplified from extracted DNA using CMV-fwd and pcDNA3_rev primer. Bands matched with predicted fragment size of 670 nt for ANG sequence in vector and 273 nt for empty vector. (B) Sequencing results of the stable clones (Ang-K40I, Ang-WT, Ang-R31K) showed the specific missense mutations in ANG K40I (in green) and ANG R31K (in red), and complete sequence coverage for the gene compared to reference human ANG sequence (NM_001097577).
Results

To model the observed production and secretion of angiogenin in primary motoneurons (Skorupa et al. 2012), stable SH-SY5Y and plain SH-SY5Y cells were seeded at the same concentration (3x10^6 cells/10 cm dish) and serum deprived for 24 h. Cells were lysed, and protein amounts were determined by BCA assay. pcDNA had the same angiogenin level as plain SH-SY5Y cells, confirming that culturing under G418 did not influence the endogenous levels of ANG (Figure 3.6 A&B). Surprisingly, angiogenin producing stable SH-SY5Y cell lines showed different angiogenin pattern under serum-deprived conditions. Especially, R31K levels were dramatically reduced compared to ANG WT. To examine whether the different ANG levels were due to mRNA levels or regulated differently on the protein level, mRNA levels and ANG secretion were further investigated.

![Figure 3.6: Ang R31K clones have reduced angiogenin protein levels under serum deprived conditions.](image)

Stable SH-SY5Y clones were serum deprived for 24 h. Angiogenin levels were assessed by western blotting (A) and densitometric analysis (B). Beta-actin was used as loading control and for densitometry normalisation, and 5 ng recombinant human angiogenin (rhANG) as a positive control. Data ± SEM represent protein lysates from 4 repeats. Under serum deprivation, Ang R31K protein levels were significantly reduced compared to Ang WT (One-way ANOVA p < 0.001; post-hoc Tukey HSD p(R31K to Ang WT) < 0.001; p(K40I to Ang WT) < 0.001; p(pcDNA to Ang WT) < 0.001; p(plain SH-SY5Y to Ang WT) < 0.001).

To investigate whether the mRNA levels of ANG correlated with the angiogenin protein levels, RNA was extracted from serum deprived stable SH-SY5Y cultures. DNase I treated RNA samples were quantified by real-time two-step RT-PCR method using SYBR Green. C_T values were normalised to the commonly used
Results

The housekeeping gene GAPDH and compared relatively to pcDNA control using the $2^{\Delta\Delta C_T}$-method (Livak & Schmittgen 2001). All ANG overexpressing cell lines had significantly higher mRNA level of ANG compared to pcDNA (Figure 3.7). The mRNA level of Ang WT and R31K were comparable, indicating that the low angiogenin protein level in serum deprived Ang R31K were regulated on the protein level. mRNA levels in K40I were lower than Ang WT and R31K, but correlated roughly with the reduced protein levels.

![Relative hANG fold change](image)

**Figure 3.7: Relative human ANG fold change in stable SH-SY5Y.** Stable SH-SY5Y were seeded at the same density ($3 \times 10^6$ cells/10 cm dish), and serum deprived for 24 h. RNA was extracted from three separate experiments using TRIzol RNA extraction method. DNaseI treated RNA samples were relative quantified by real-time two-step RT-PCR method using SYBR Green. hANG mRNA was normalised to GAPDH mRNA levels and compared relatively to pcDNA using the $2^{\Delta\Delta CT}$ method. Data ± SEM represent RNA from 3 repeats. ANG R31K expression levels were significantly reduced compared to ANG WT (One-way ANOVA p < 0.001; post-hoc Tukey HSD p(Ang WT to pcDNA) < 0.001; p(K40I to pcDNA) < 0.001; p(R31K to pcDNA) < 0.001).
Stable SH-SY5Y cells, stained with alpha-tubulin and angiogenin antibodies, were examined for morphology and angiogenin localisation in normal culture conditions. Differential interference contrast (DIC) images showed no morphological changes between stable SH–SY5Y and plain SH-SY5Y cells (Figure 3.8). Angiogenin localised mainly in the cytosol of Ang WT, K40I and R31K cells. We also observed the presence intense punctuate structure of angiogenin in perinuclear and neurite regions (Figure 3.8, white arrows), suggesting formation of resembling secretory granules. pcDNA and plain SH-SY5Y cells displayed weak angiogenin staining, as previously revealed by western blotting analysis in Figure 3.4, indicating once again that angiogenin endogenous levels are relatively low in these cells. The lack of nuclear localisation is not surprising, since reports display contradictory results in SH-SY5Y cells (nucleus - (Thiyagarajan et al. 2012), cytosol - (Steidinger et al. 2013)). In conclusion, it is demonstrated here that ANG overexpression does not influence cell morphology and the observed angiogenin signals in immunofluorescence images correlated with the detected angiogenin protein levels by western blotting.
Results

Figure 3.8: Angiogenin is mainly localized in cytosol, and no morphological changes are observed between stable SH-SY5Y and plain SH-SY5Y cells. Confocal images of stable SH-SY5Y and plain SH-SY5Y cells under normal culturing conditions. Tubulin (in green) and DAPI (in cyan) were used as cytosolic and nuclear markers, respectively. Antibody against angiogenin shows angiogenin localisation in red. White arrows highlight intense punctuate structure of angiogenin in perinuclear and neurite regions.
3.2.2 Characterisation of stable SH-SY5Y cells overexpressing WT & ALS-associated ANG mutants

3.2.2.1 Generation of 5´Val tiRNA in Ang WT and R31K cells

To characterise the ribonucleolytic activity of Ang WT, K40I and R31K during nutrient starvation, stable SH-SY5Y cell lines and plain SH-SY5Y cells were seeded at the same concentration (3x10^6 cells/10 cm dish) and serum deprived for 24 h. RNA was extracted from cells, and RNA content was analysed on denaturing urea polyacrylamide gel. Gels were loaded equally with 20 µg RNA, and loading control was unaffected by ANG overexpression (Figure 3.9A, upper panel). As expected, all SH-SY5Y cell lines revealed RNA cleavage products at 32 nt and 40 nt (Figure 3.9A, lower panel), since SH-SY5Y cells express endogenous ANG and cells stress was induced. However, Ang WT and R31K had increased levels of RNA cleavage compared to pcDNA control, indicating that both angiogenin variants were ribonucleolytic active. This confirms previous findings in angiogenin treated primary astrocytes, where angiogenin treatment showed RNA cleavage products at ~34 nt and ~43 nt (Skorupa et al. 2012). Interestingly, R31K displayed similar RNA cleavage as Ang WT, despite lower angiogenin protein levels (Figure 3.9B). This observation suggests an increased activity of R31.
Results

Figure 3.9: Increased RNA cleavage in Ang WT and R31K cell lines. Stable overexpressing SH-SY5Y cell lines were seeded at the same density (3x10^6 cells/10 cm dish), and serum deprived for 24 h. TRIzol was used to extract RNA from cells. (A) Representative image of denaturing urea polyacrylamide gel showing increased RNA cleavage products at 32 nt and 40 nt in Ang WT and R31K compared to pcDNA. Gel was loaded with 20 µg RNA/well, and stained with SYBR Gold. (B) Densitometric analysis of RNA cleavage products was performed using the software ImageJ. Data represent median intensity ± SEM compared relatively to empty vector control (pcDNA) of three separate experiments. The difference in RNA cleavage was not statistically significant when comparing the cleavage bands between different samples to pcDNA, although there was a trend towards increased RNA cleavage in ANG WT and R31K (One-way ANOVA p = 0.053; post-hoc Tukey HSD p (Ang WT to pcDNA) = 0.379; p(R31K to pcDNA) = 0.168).

To examine whether increased RNA cleavage shows generation of specific tiRNAs, RNA extracts were quantified by custom designed TaqMan assays. C_T values were normalised to U6 snRNA housekeeping RNA (RNU6B - (Benz et al. 2013)) and compared relatively to pcDNA control using the 2^(-ΔΔC_T)-method. tiRNA sequences were chosen on the basis of their functional analysis from the literature data (5´Ala – (Emara et al. 2010), (Ivanov et al. 2011)), and from those identified by our laboratory in angiogenin treated MZ-294 cells, and primary astrocytes (5´Gly; 5´Val; 3´Arg). 5´Ala was not detected in any of the cell lines (N.D. Figure 3.10). However, the 5´Ala assay was able to detect synthetic spiked-in tiRNA sequences, indicating that 5´Ala tiRNA was not generated in SH-SY5Y cells. ANG WT had increased 5´Val, whereas 5´Gly and 3´Arg levels were similar compared to pcDNA control. The fold change difference between 5´Val and 5´Gly
and 3’Arg suggested that angiogenin generates specific tiRNA. Ribonucleolytic inactive K40I displayed similar tiRNA fold change as pcDNA control, confirming that tiRNAs are produced by ribonucleolytic active angiogenin. R31K had increased tiRNA fold change in 5’Val, and slightly in 5’Gly, indicating that ribonucleolytic activity was not impaired by the missense mutation.

In conclusion, production of ribonucleolytic active angiogenin showed an up-regulation of 5’Val and not 5’Gly and 3’Arg, suggesting a sequence/codon specific tRNA cleavage under stress conditions.

**Figure 3.10: 5’Val tiRNA is generated in ANG WT and R31K cells.** Stable overexpressing SH-SY5Y cell lines were seeded at the same density (3x10^6 cells/10 cm dish), and serum deprived for 24 h. RNA was extracted using TRIzol RNA extraction method. DNase I treated RNA samples were quantified by custom designed TaqMan assays. C_T values of different tiRNAs were normalised to U6 snRNA (RNU6B, 45 nt) and compared relatively using the 2^-ΔΔC_T- method. Data ± SEM represent three experiments. The difference in tiRNA fold change was not statistically significant when comparing the different samples to pcDNA, although there was a trend towards increased 5’Val fold change in ANG WT and R31K (One-way ANOVA p(5’Val) = 0.042; post-hoc Tukey HSD p(Ang WT to pcDNA) = 0.971; p(K40I to pcDNA) = 0.109; p(R31K to pcDNA) = 0.310).
3.2.2.2 Wild-type angiogenin and ALS associated ANG mutations do not enhance SG formation during sodium arsenite stress

To examine whether the increased 5`Val generation correlates with SG formation, stable SH-SY5Y and plain SH-SY5Y cells were treated with different concentrations of sodium arsenite (20 µM and 30 µM NaAsO₂) for 1h. SGs are transient cytosolic aggregations of preinitiation and translation-related factors, mRNA-binding proteins, proteins associated with mRNA metabolism and stalled ribosomes (Anderson & Ivanov 2014). It has been previously shown that angiogenin treated U2OS cells display enhanced SG formation under sodium arsenite stress (Emara et al. 2010), (Ivanov et al. 2011)). In detail, SG formation was the result of tiRNA generation and associated with translation repression by interfering with the cap binding complex assembly. SG formation was analysed by immunocytochemistry using a G3BP (GTPase activating protein (SH3 domain) binding protein 1) antibody to recognise SGs, and imaged using confocal microscopy. G3BP is a sequence-specific, phosphorylation-dependent helicase and endoribonuclease. Under normal conditions, G3BP is distributed evenly in the cytosol (Figure 3.11B, cyan arrows), where it is involved in mRNA metabolism (Tourrière et al. 2003). However, under stress conditions, including sodium arsenite and thapsigargin stress, heat and osmotic shock, UV and viral infection, G3BP forms SGs (Figure 3.11B, white arrows) with other proteins and mRNAs, and it is a commonly used as SG marker (Kedersha & Anderson 2007). SG formation was quantified with the custom designed image processing CellProfiler pipeline validated in chapter 4.2.2 (pipeline detail in chapter 2.11.1).

Stable SH-SY5Y cells showed neglectable SG formation in the absence of sodium arsenite stress, and increased sodium arsenite concentration correlated with increased SG formation. ~80% of the cells were SG positive with high sodium arsenite stress (30 µM NaAsO₂). pcDNA and plain SH-SY5Y cells both had a similar response during all sodium arsenite treatments (Figure 3.11A), indicating that G418 selection did not alter SG formation in the SH-SY5Y cells. Surprisingly, ANG overexpressing stable SH-SY5Y cells showed numerically less but not
Results

statistically significant SG positive cells compared to pcDNA control. ANG WT and K40I overexpressing cells showed similar SG formation properties during moderate (20 µM NaAsO₂) sodium arsenite stress, whereas R31K had during all sodium arsenite treatments fewer SG positive cells. Ang WT revealed increased SG formation at high sodium arsenite concentration. However, these results did not reach statistical significance employing one-way ANOVA pairwise comparison with Tukey’s post hoc test. The reduced SG formation during moderate sodium arsenite stress was contradictory to the reported ANG effects in osteosarcoma U2OS cells ((Emara et al. 2010), (Ivanov et al. 2011)). However, previous examination in SH-SY5Y cells demonstrated that angiogenin enhanced SG formation only at high sodium arsenite concentration (1 mM for 2.5 h - (Thiyagarajan et al. 2012)), which correlates with our data, and indicates a cell type specific effect of angiogenin.
Figure 3.11: Overexpressed ANG variants repress SG formation in stable SH-SY5Y cells. Ang WT and mutants overexpressing stable SH-SY5Y cell lines were treated with indicated sodium arsenite (NaAsO₂) concentration for 1 h before immunocytochemistry. Images were taken on LSM 7.10 confocal microscope. (A) SG formation was quantified using a custom designed CellProfiler pipeline. Six fields of view were imaged per cover slip with 200-300 cells/cover slip. All conditions were repeated in triplicate, and an average for each separate experiment was calculated. Data represent mean ± S.E.M from at least three experiments. One-way ANOVA p(20 µM NaAsO₂) = 0.096; post-hoc Tukey HSD p(Ang WT to pcDNA) = 0.949; p(K40I to pcDNA) = 0.947; p(R31K to pcDNA) = 0.222. One-way ANOVA p(30 µM NaAsO₂) = 0.586; post-hoc Tukey HSD p(Ang WT to pcDNA) = 0.992; p(K40I to pcDNA) = 0.990; p(R31K to pcDNA) = 0.586). (B) Representative confocal images of G3BP channel (in green), and SG identified by the CellProfiler approach (in red). Stable SH-SY5Y cells treated with 20 µM sodium arsenite for 1 h. White arrows highlighted SG formations and cyan arrows normal distributed G3BP.
To examine whether angiogenin co-localises with SGs, stable SH-SY5Y cells were treated with 30 μM sodium arsenite for 1h. Cells were stained for G3BP and angiogenin, and analysed on a confocal microscope. Similar to the observation in Figure 3.8, angiogenin was located in intense punctuate structures (Figure 3.12, white arrows). However, angiogenin did not co-localised with SGs. This observation illustrates that angiogenin itself is not aggregated in the SGs.

Figure 3.12: Overexpressed ANG variants do not co-localise with SG in stable SH-SY5Y cell lines. Stable SH-SY5Y cells were incubated with 30 μM sodium arsenite for 1 h before immunocytochemistry. Images were taken on LSM 7.10 confocal microscope. Cells were stained for G3BP (in green), a well-established SG marker, and for angiogenin (in red). Cell nuclei were highlighted by DNA stained with DAPI. White arrows highlighted punctuate structure of angiogenin in cells. Scale bars = 10 μm
3.2.2.3 Stable SH-SY5Y under stress conditions

In previous work our group has shown that angiogenin protected motoneurons against cell-stress induced by tunicamycin, hypoxia, and AMPA. This protection was mediated by restoring the disease-associated decrease in Akt survival signalling (Kieran et al. 2008). Furthermore, our group has shown that overexpressing ANG K40I did not provide this protection. To investigate whether plasmid overexpressed ANG alters the native cell behaviour of SH-SY5Y cells, Akt phosphorylation and the down-stream associated proteins were examined in stable SH-SY5Y under serum deprivation. Stable and plain SH-SY5Y cells were seeded at the same density (3x10^6 cells/10 cm dish), and serum deprived for 24 h. Cell lysates were analysed by western blotting. Akt is a critical regulator of cell survival and proliferation. Phosphorylated Akt inhibits a various numbers of pro-apoptotic proteins, such as Bcl-2-associated death promoter (BAD), Caspase-9 and Yes-associated protein (YAP). P-Akt activates also the transcription factor cyclic AMP-response element binding protein (CREB), which promotes the expression of anti-apoptotic Bcl-2 genes (reviewed in (Song et al. 2005)). Protein levels of pro- and anti-apoptotic Bcl-2 family members were also monitored. Bcl-XL, Bcl-2, and Mcl-1 were investigated, as they bind directly to anti-apoptotic proteins Bim, Bax and Bak, and inhibit indirectly cytochrome c release (reviewed in (Zhou et al. 1997)). Protein levels of apoptotic protease activating factor 1 (Apaf-1) were measured as Apaf-1 forms together with cytochrome c, pro-caspase 9, and dATP the apoptosome that leads to cell death via the activation of a caspase cascade (reviewed in (Baliga & Kumar 2003) and (Portt et al. 2011)).

Ribonuclease/angiogenin inhibitor 1 (RNH1) was also investigated whether the ANG overexpression trigger RNH1 up-regulation. Surprisingly, Ang WT did not show a clear difference in Akt phosphorylation compare to K40I and control cell lines (plain SH-SY5Y and pcDNA - Figure 3.13A). This might be due to either late time point, since Akt phosphorylation has been shown to occur time-dependent with highest detection within the first 3 h ((Dimmeler et al. 1998), (Steidinger et al. 2011)), or because SH-SY5Y cells are showing resistance against serum-deprivation (Figure 4.6D) suggesting this stress stimulus was not sufficient to
trigger the up-stream activation of Akt. Since the expression changes were marginal (Figure 3.13D), we carried out these experiments only once. However, Ang WT and R31K displayed a slight increased p-Akt levels compare to K40I, pcDNA and plain SH-SY5Y cells. Akt down-stream regulated proteins did also not differ between ANG overexpressing cell lines and controls (Figure 3.13A&B). This suggested that elevated angiogenin levels neither induce phosphorylation of Akt nor influence the expression of Akt regulated proteins down-stream under serum deprivation. Surprisingly, also RNH 1 levels were not affected (Figure 3.13C), since ANG overexpression could mediate angiogenin activity compensation by up-regulation of angiogenin inhibitor RNH 1.

In conclusion, ANG overexpression did not influence the Akt pathway and the endogenous angiogenin inhibition through RNH 1 under serum deprivation. These results indicate that plasmid overexpressed ANG did not alter the native cell behaviour of SH-SY5Y cells.
Results

Figure 3.13: Plasmid overexpressed ANG did not alter the native cell behaviour of SH-SY5Y. Stable overexpressing SH-SY5Y cell lines were seeded at the same density (3x10^6 cells/10 cm dish), and serum deprived for 24 h. Cells were lysed, and protein amount was determined by BCA assay. (A) & (B) No difference in pro- and anti-apoptotic protein levels were observed in stable SH-SY5Y compared to empty vector control (pcDNA) and plain SH-SY5Y cells by western blotting analysis. (C) Representative western blot indicating that the ribonuclease/angiogenin inhibitor 1 (RNH1) levels were not regulated by ANG overexpression through expression vector in SH-SY5Y cells. Actin was used as loading control. (D) Densitometric quantification of different SH-SY5Y cell lines normalised to actin. Data represent median intensity compared relatively to empty vector control (pcDNA) of a single experiment.
3.2.3 Paracrine activity of stable SH-SY5Y cells overexpressing WT & ALS-associated ANG mutants

In previous studies, our group demonstrated that motoneurons secrete angiogenin under serum deprivation, and secreted angiogenin is endocytosed by neighbouring astroglial cells (Skorupa et al. 2012). Our group proposes that the neuroprotection of angiogenin is mediated in paracrine. To examine whether the generated stable SH-SY5Y cell showed secretion of angiogenin, and determine how the ALS associated ANG mutations differ from wild-type ANG, stable and plain SH-SY5Y cells were serum deprived and the conditioned medium was further analysed.

Cells were seeded at the same density (3x10^6 cells/10 cm dish) and volume of the conditioned medium was kept constant (10 ml/10 cm dish). Total protein content of 1 ml conditioned medium was precipitated with trichloroacetic acid (TCA) and analysed by western blotting. Several secreted proteins such as cytokines (IL-1β, IL-1ra, TNFα), exosomal marker (Alix, flotillin, TSG10), and others (NGF, MMP3, S100b, albumin, Col4a) were examined in order to compare the secretion levels between the cell lines. Only collagen 4a (Col4a) was sufficiently secreted in SH-SY5Y cells. Col4a assembles with laminin, heparan sulfate proteoglycans, perlecan and agrin basement membrane (reviewed in (Yurchenco & Patton 2009)). The key role of basement membrane is to form a linkage layer that protects from disruptive shear forces. In line to previous observation, angiogenin was secreted in all SH-SY5Y cells under serum deprivation. By normalising densitometric signals of angiogenin to Col4a (Figure 3.14B), pcDNA control and plain SH-SY5Y showed a strong difference, which were due to low Col4a levels in plain SH-SY5Y cells. Ang WT and R31K display the strongest angiogenin secretion, when normalised to recombinant human angiogenin. Interestingly, R31K showed increased Col4a levels compared to the other stable SH-SY5Y cell lines (Figure 3.14A, lower panel). In contrast, Ang WT and K40I did not alter the Col4a secretion compared to pcDNA control. This observation suggested that the Col4a
secretion is not regulated by ribonucleolytic activity of angiogenin, but rather an effect of the missense mutation of R31K.

To compare how much angiogenin was secreted from the stable overexpressing SH-SY5Y cell lines, secreted angiogenin was normalised to the cytosolic angiogenin levels of the same experiment. Since Col4a levels were increased in the CM of Ang R31K cells, secreted angiogenin levels normalised to Col4a as well as to a rhANG standard (2 ng) were compared (Figure 3.14C). The comparison of secreted angiogenin normalised to Col4a with cytosolic angiogenin showed variation between experiments, however, these results indicated that ANG K40I was secreted more than ANG WT whereas ANG R31K was secreted less than ANG WT. In contrast, when secreted angiogenin normalised to rhANG standard was compared with cytosolic angiogenin. These ratios revealed that both angiogenin mutant stable overexpressing cell lines displayed higher secretion of angiogenin than Ang WT cells. In particular, Ang R31K cells showed almost fivefold higher angiogenin secretion compared to the Ang WT cells, which could explain the reduced cellular angiogenin levels observed in serum-deprived conditions compared to normal culturing conditions (Figure 3.4 and Figure 3.6).
Figure 3.14: Angiogenin secretion under serum deprivation conditions. Stable overexpressing SH-SY5Y and plain SH-SY5Y cells were seeded at the same density (3x10^6 cells/10 cm dish), and serum deprived for 24 h in same volume. Conditioned medium (CM) was collected from three separate experiments and proteins were precipitated from 1 ml CM using the trichloroacetic acid (TCA) precipitation method. (A) Representative western blot of TCA precipitated conditioned medium (Protein amount from 1 ml CM) showing angiogenin secretion under serum deprivation in different SH-SY5Y cell lines. Collagen (Col4a) was used as a loading control as it is known to be secreted, and indicated increased secretion in R31K cell lines. (B) Densitometric quantification of angiogenin secretion in different SH-SY5Y cell lines normalised to Col4a or rhANG control. Data ± SEM represent three separate CM generation experiments. (C) Relative angiogenin ratio, between secreted angiogenin normalised to Col4a or rhANG control and the cytosolic angiogenin, are illustrated.
To examine whether the cellular generated tiRNAs are secreted from stable cell lines, tiRNA amount in the conditioned medium was determined. Therefore, RNA was isolated from conditioned medium using a miRCURY™ RNA Isolation Kit. RNA samples were DNasel treated and absolute copy number was quantified by custom designed TaqMan assays. Standard curves were generated from synthetic tiRNAs. A subset of tiRNA sequences were used to investigate whether the cellular detected distribution correlated with the secretion profile. 5`Val was employed, as it was highly generated by ribonucleolytic active stable cell lines (Ang WT, and R31K). 3`Arg was selected, because the fold change was not increased in the stable cell lines. Furthermore, 5`Ala was determined in the conditioned medium in order to examine whether it was entirely secreted, and therefore not detectable in the cytosol of stable cell lines. However, similar to cellular tiRNA investigation, 5`Ala was not detected in the conditioned medium, suggesting that 5`Ala is not generated by angiogenin in SH-SY5Y cells. In the conditioned medium of ANG WT and R31K were more 5`Val copies detected than in pcDNA and K40I (Figure 3.15A). However, these results did not reach statistical significance employing one-way ANOVA pairwise comparison with Tukey’s post hoc test. Nevertheless, these results correlated with cellular detected 5`Val fold change, although R31K showed higher secretion compared to Ang WT. Surprisingly, 3`Arg was also numerically greater but not statistically significant secreted by ribonucleolytic active Ang WT and R31K cells compared to pcDNA (Figure 3.15B). However, the 3`Arg copy number ratio between ANG WT & R31K and pcDNA was lower than the 5`Val ratio, indicating cellular tiRNA concentration determined how much tiRNA will be secreted.
Figure 3.15: Increased tiRNA secretion in Ang WT and R31K overexpressing stable SH-SY5Y cells. Stable overexpressing SH-SY5Y cells were seeded at the same density (3x10^6 cells/10 cm dish), and serum deprived for 24 h in same volume. Conditioned medium (CM) was collected from separate experiments, and RNA from 2 ml CM was isolated using miRCURY™ RNA Isolation Kit. Absolute copy number was quantified from DNase I treated RNA samples by custom designed TaqMan assays. Standard curves were generated from synthetic tiRNAs. (A) 5`Val copy number quantification. Data ± SEM represent isolated RNA from conditioned medium of three experiments. The difference in 5`Val copy number was not statistically significant when comparing the different samples to pcDNA, although there was a trend towards increased 5`Val fold change in ANG WT and R31K (One-way ANOVA p(5`Val) = 0.079; post-hoc Tukey HSD p(Ang WT to pcDNA) = 0.639; p(K40I to pcDNA) = 0.998; p(R31K to pcDNA) = 0.117). (B) 3`Arg copy number quantification. Data ± SEM represent isolated RNA from conditioned medium of three experiments. One-way ANOVA post-hoc Tukey HSD showed no significance. The difference in 3`Arg copy number was not statistically significant when comparing the different samples to pcDNA, although there was a trend towards increased 3`Arg fold change in Ang WT and R31K (One-way ANOVA p(3`Arg) = 0.052; post-hoc Tukey HSD p(Ang WT to pcDNA) = 0.163; p(K40I to pcDNA) = 0.8; p(R31K to pcDNA) = 0.692).
3.3 Discussion

To model ANG expression and secretion observed in motoneurons, and to characterise the effects of wild-type ANG and ALS associated ANG mutants, stable cell lines overexpressing ANG WT, K40I and R31K were generated. SH-SY5Y cell line was employed as a human in vitro model with an established and stable neuronal background. K40I ANG mutation was used, since the mutation affects the catalytic triad, and our group has previously shown to be ribonucleolytic inactive in NSC-34 cells (Skorupa et al. 2012). R31K ANG exhibits the mutation in the nuclear localisation sequence, however SH-SY5Y treatments with R31K illustrated only a delayed nuclear translocation (Thiyagarajan et al. 2012). Furthermore, ribonucleolytic activity towards tRNA cleavage in a buffered environment showed neglectable reduction in the ribonucleolytic activity (Crabtree et al. 2007), (Thiyagarajan et al. 2012)). Interestingly, our group revealed that R31K does not protect NSC-34 cells against hypoxic stress compared to wild-type angiogenin (Sebastià et al. 2009). This observation indicated that R31K ribonucleolytic activity might differ in an intracellular environment and/or the mutation alters other angiogenin functions. By analysing secreted amount of angiogenin in stable SH-SY5Y, R31K cells displayed higher protein secretion from the same cell number and volume as Ang WT, K40I and pcDNA. Both secreted angiogenin and Col4a were highly enriched in the conditioned medium. Increased angiogenin secretion could explain the lower cellular levels of angiogenin in R31K cells during serum deprivation. However, Col4a was constant in Ang WT, K40I, and pcDNA cells, but highly secreted in R31K. These results suggest that increased secretion could be caused by missense mutation of R31K and was not a general ribonucleolytic effect. However, so far the R31K mutation impact on protein secretion has not been reported and was not further analysed in this study. But in previous work our group showed that angiogenin treatment modifies the secretome of primary astrocytes (Skorupa et al. 2013). Proteins residing in the extracellular compartment, proteins involved in multicellular organismal development and proteins contributing to cell response to stress and extracellular stimuli were altered in astrocyte supernatants. The secretion alteration in R31K
Results

cells implicates a pathological mechanism, which could lead to ALS. However, since our results represent single clone investigations in a plasmid mediated angiogenin production model, further validation would be necessary to ensure it is not an artefact.

To address the ribonucleolytic activity in the stable SH-SY5Y cells, RNA content of serum-deprived stable SH-SY5Y was analysed. Denaturing urea polyacrylamide gels showed bands at 32 nt and 40 nt. These bands were more intense in Ang WT and R31K cells compared to ribonucleolytic inactive K40I and control cell lines, but lacked to reach statistical significance. Nevertheless, it suggests that the wild-type angiogenin and R31K are ribonucleolytic active, and generated specific fragments. These results correlated with previous findings, where angiogenin treated primary astrocytes also generated specific RNA cleavage pattern (Skorupa et al. 2012).

The lower protein levels of angiogenin in R31K expressing cells under serum-deprived conditions in our results suggest an increased RNase activity. This observation contradicts the observed slightly reduced ribonucleolytic activity (91%) of R31K in a buffered system (Thiyagarajan et al. 2012), and suggests a more comprehensive function of angiogenin in an intracellular environment. To examine whether specific tiRNAs were generated in stable SH-SY5Y cells, cellular tiRNA levels were determined using custom designed TaqMan assays. Quantitative analysis showed numerically greater but not statistically significant 5`Val copy numbers in Ang WT and R31K, and not in K40I compared to pcDNA control. Whereas, 5`Gly and 3`Arg levels had negligible difference in Ang WT, K40I and R31K compared to pcDNA control. These findings suggest that the ribonucleolytic active angiogenin generates specific tiRNAs. By quantifying tiRNA levels in the conditioned medium, we observed numerically greater but not statistically significant secretion of 5`Val and surprisingly also 3`Arg in Ang WT and R31K, and not in K40I and pcDNA. However, the 3`Arg copy number ratio between Ang WT & R31K and pcDNA was lower than the 5`Val ratio, suggesting cellular tiRNA concentration determines how much tiRNA get secreted. Furthermore, the repeatedly reported functional 5`Ala was not detected in our system, indicating
that 5`Ala is not generated in SH-SY5Y cells. Our results indicate that the ribonucleolytic activity of angiogenin generates specific tiRNAs, which are also secreted by the cells. These findings add new aspects to the documented loss-of-function mutation of \textit{ANG}, and suggest a mechanism in which angiogenin mediate tiRNA secretion in response to stress. This could be a paracrine mechanism similar to secreted miRNAs, which can be delivered to neighbouring cells where they regulate multiple targets or signalling events.

Angiogenin has been shown in osteosarcoma cell lines (U2OS) to enhance SG formation under sodium arsenite stress ((Emara et al. 2010), (Ivanov et al. 2011)). The Anderson laboratory revealed that the angiogenin mediated enhancement of SG formation in U2OS cells was mediated by tiRNAs interfering with the assembly of the cap binding complex, and was associated with translation repression. SGs are transient cytosolic aggregations of preinitiation and translation-related factors, mRNA-binding proteins, proteins associated to mRNA metabolism and stalled mRNA molecules (Anderson & Ivanov 2014). In our experimental set-up Ang WT cells showed increased SG formation compared to control cell lines only under high sodium arsenite stress (30 µM NaAsO$_2$ for 1 h), however it reached not statistical significance. Under moderate sodium arsenite conditions (20 µM NaAsO$_2$ for 1 h) ANG overexpressing cell lines exhibited numerically less but not statistically significant SG positive cells. These findings contradict the Anderson laboratory results from U2OS cells, but are in line with previous observation in SH-SY5Y cells from (Thiyagarajan et al. 2012), suggesting a cell line specific function of angiogenin.

Taken together, R31K cells showed increased protein secretion under serum-deprived conditions, suggesting a mutation specific effect. ANG WT and R31K cells generated specific tiRNAs and showed increased secretion. K40I cells lacked to generate and secrete tiRNAs, indicating the K40I loss-of-function mutation affect not only the intracellular RNA cleavage, but also the secretional stress response.
This lead us to further investigate the function of tiRNAs, especially focusing on understanding whether transfected tiRNAs could induce SG formation in SH-SY5Y cells and display neuroprotection properties in SH-SY5Y cells and primary mixed motoneurons.
4. **Chapter 4: Characterisation of tiRNAs in neurons and primary mixed motoneuron cultures**

4.1 Introduction

4.1.1 Stress induced tRNA cleavage

Several common genetic causes of ALS suggest a correlation between RNA processing defects and ALS (reviewed in (Polymenidou et al. 2012)). In the case of ANG, it was observed that loss-of-function mutation in angiogenin could cause ALS. Previous studies in our group revealed that the ribonucleolytic inactive ANG K40I mutant failed to protect NSC-34 cells against tunicamycin-induced ER stress (500 nM, 24 h) and serum-deprivation (for 24 h) when compared to ANG WT treatment (Kieran et al. 2008). In Chapter 3, we demonstrated that ribonucleolytic active ANG WT and R31K, and not K40I, generate a specific RNA cleavage pattern (Figure 3.9). This was in line with previous findings ((Yamasaki et al. 2009), (Skorupa et al. 2012)). Custom designed TaqMan assays in Figure 3.10 revealed that Ang WT and R31K generate specific tiRNAs under stress conditions, and have further influence on tiRNA homeostasis in the cell. These results confirmed the findings that angiogenin cleaves preferably tRNA in or near the anticodon loop in mammalian cells (Czech et al. 2013). Our group and an increasing number of reports identified several tiRNAs generated by ANG (Figure 4.1). The revealed tiRNA sequences differ between cell types, suggesting that this might be due to the altered tRNA expression levels in the different cell types. Furthermore, unpublished next generation sequencing data detected only one of the halves, indicating a mechanism by which some tiRNA halves are protected from degradation.
Figure 4.1: Project related tRNAs and their cleavage sites. Homo sapiens tRNA sequences and structures were extracted from tRNA database (http://gtrnadb.ucsc.edu/). Coloured arrowheads present different proposed cleavage sites.
4.1.2 tiRNA function

Because tRNA cleavage events do not decrease the level of mature, full-length tRNA ((Thompson & Parker 2009a), (Emara et al. 2010), (Saikia et al. 2012)), meaning that the cellular tRNA pool depletion is not the mechanism of action, an increasing number of reports have been allotting biological functions to tiRNAs. tiRNA studies from the Anderson laboratory have shown that phosphorylated 5`tiRNAs, especially those with a terminal oligo-guanine (TOG) motif (4–5 guanine residues) at their 5` ends, such as 5`Ala (compare Figure 4.1), inhibit cap-dependent protein synthesis by interfering with the assembly of the cap binding complex eukaryotic initiation factor 4F (eIF4F), and induce SG formation ((Yamasaki et al. 2009), (Emara et al. 2010), (Ivanov et al. 2011)). Several reports illustrated that translation repression was associated with SG formation, however, it is unclear which event occurs first ((McEwen et al. 2005), (Reineke et al. 2012), (Yamamoto & Izawa 2013), (Liem & Liu 2016)). It has been hypothesised that stress-induced tRNA cleavage inhibits cap-mediated global protein synthesis and activates a cytoprotective stress response pathway (Anderson & Ivanov 2014). A recent study in sex hormone-dependent breast and prostate cancers examined angiogenin-mediated tRNA cleavage, and illustrated cell proliferation enhancement mediated by 5` and not 3` tiRNA fragments ((Honda et al. 2015) – compare Figure 4.1).

As generated stable SH-SY5Y cell lines showed up-regulation of 5`Val tiRNA in Ang WT and R31K, but not in ribonucleolytic inactive K40I mutant and pcDNA, we further characterised tiRNA sequences on the basis of their functional analysis from literature data (5`Ala – (Emara et al. 2010), (Ivanov et al. 2011)) and from those identified in house, in angiogenin treated MZ-294 cells and primary astrocytes (3`Gly, 3`Asp, 5`Val, 3`Arg). 5`Ala tiRNA is a fragment showing the strongest response in SG formation and translation repression ((Ivanov et al. 2014) - Figure 4.2), however, the information regarding its biogenesis is still uncertain. Ctrl tiRNA sequence has been commonly used in the literature ((Emara 2009a), (Emara et al. 2010), (Saikia et al. 2012)).

Ctrl tiRNA sequence has been commonly used in the literature ((Emara 2009a), (Emara et al. 2010), (Saikia et al. 2012)).
et al. 2010), (Ivanov et al. 2011), (Ivanov et al. 2014) - Figure 4.2) and its sequence homology to other tRNAs was lower than 65%. 5` & 3`Gly and 5` & 3`Asp were employed at the beginning of this project and the sequence length of 5’Gly and 5’Asp were adjusted to the published 5’Ala and Ctrl tiRNA length of 29 nt (Figure 4.2). As the project progressed and our group identified new ANG regulated tiRNAs (Figure 4.1, red arrows), we next utilised Val & Arg tiRNA sequences corresponding to the new findings (Figure 4.2). Although, mainly one of the cleaved tiRNA halves was detected, we also employed for our experiments the other matching part in order to be able to compare their functions. All synthetic tiRNAs were labelled at the 3’ end with the fluorophore ROX. ROX has similar excitation/emission properties as Alexa 568 fluorophore. It was used to localise tiRNAs during ICC experiments and for the identification of tiRNA transfected cells in flow cytometry experiments. Since native tRNAs are phosphorylated at the 5` end, and the transphosphorylation reaction catalysed by angiogenin produces an unphosphorylated 3` product (Leland et al. 2002), only Ctrl and 5` tiRNAs were phosphorylated at the 5` end.

In this chapter, tiRNAs properties were characterised in two different in vitro models. First, SH-SY5Y cells were employed to examine neuroprotection of tiRNA against a broad range of ALS pathology related stress conditions. In order to investigate whether the protection properties of tiRNAs correlated with the ability to induce SG formation, SH-SY5Y cells were transfected with different tiRNA concentrations. Furthermore, to examine whether the new tiRNAs might enhance SG formation during stress, transfected SH-SY5Y cells were additionally exposed to 20 µM sodium arsenite for 1 h. SG formation was analysed by immunocytochemistry using an antibody that recognises G3BP, which is well established SG marker. To quantify SG formation, an automated approach was established using CellProfiler software.

Next, primary ventral horn motoneuron cultures from E12-E14 wild type (C57Bl/6) mouse embryos have been purified and used as a more accurate model of the cellular interactions within the spinal cord. As primary motoneurons are very
sensitive, different transfection methods were examined. The transfection method with the lowest cytotoxicity and highest transfection efficiency was used to investigate whether the neuroprotection of angiogenin is due to angiogenin generated tiRNAs. Motoneurons were then cultured under hypoxic condition (1% O₂ for 24 h), and survival was quantified by immunocytochemistry using an antibody against SMI-32 that recognises motoneurons (Tsang et al. 2000).
Figure 4.2: Characterised tiRNA sequences and their predicted secondary structure. Secondary structure was predicted using RNAfold web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi). This tool calculated the partition function and base pairing probability matrix in addition to the minimum free energy structure.
4.2 Results

4.2.1 Assessing neuroprotective effects of tiRNAs in SH-SY5Y cells

4.2.1.1 Transfection optimisation in a human in vitro model

To investigate whether tiRNAs display neuroprotection properties in a human in vitro model, tiRNA transfection was optimised in SH-SY5Y cells. Therefore, SH-SY5Y cells were transfected with different concentrations of tiRNAs, and subcellular localisation was determined by immunocytochemistry employing the confocal microscope LSM 710 (Zeiss).

Two different kinds of tiRNA signals were observed in all scans: (i) intense tiRNA signals at the cell surface (Figure 4.3, cyan arrows), which are presumably unreleased tiRNA molecules in Lipofectamine complexes; and (ii) intracellular tiRNA signals within the cells (Figure 4.3, white arrows), which indicated successful tiRNA transfection. Increasing 5’- and 3’ Asp tiRNA concentrations increased the intracellular tiRNA signals within the cells. However, intracellular tiRNA signals within the cells were detected only at 250 nM (7.5x10^8 copies/cell) of Ctrl tiRNA. 5’ & 3’ Asp tiRNAs showed intracellular tiRNA signals within the cells also at lower concentrations (50 nM – 1.5x10^8 copies/cell). These findings suggested that the secondary structure of tiRNAs may be important for successful transfections, as folded sequences may aggregate less to each other.
Figure 4.3: 250 nM tiRNA or 7.5x10^8 copies/cell showed a detectable intracellular tiRNA signal within SH-SY5Y cells. Representative confocal images of transfected SH-SY5Y cells with increasing tiRNA concentration using the same volume of 2.5 µl of 2. Lipofectamine. ROX conjugated tiRNAs are displayed in red. Cell nucleus was highlighted by DNA stained with DAPI (in cyan). Cyan arrows illustrate tiRNA-Lipofectamine aggregates at the cell surface. White arrows designate intracellular tiRNA signals as indication of successful transfection.
Although tiRNAs showed consistent distribution in the cytosol, transfection optimisation experiments revealed that in the nucleus, tiRNAs were concentrated only in distinct areas. To examine whether these areas were the nucleoli, transfected SH-SY5Y cells were stained for fibrillarin. Fibrillarin is a 2’-O-methyltransferase located in dense fibrillar component of the nucleolus ((Tollervey et al. 1993), (Boulon et al. 2010)). It is involved in pre-rRNA processing, pre-rRNA methylation and ribosome assembly (Lafontaine & Tollervey 2000). Immunocytochemistry analysis confirmed that tiRNA were enriched in nucleoli (white arrows in Figure 4.4). However, also Ctrl tiRNA showed in nucleoli staining, suggesting the presence of a sequence unspecific mechanism.
Figure 4.4: tiRNA are enriched in cell nucleoli. SH-SY5Y cells were transfected with 250 nM tiRNA or 7.5x10^8 copies/cell for 5 h with Lipofectamine. Cells were stained for fibrillarin (in green), a commonly used nucleolar marker. ROX conjugated tiRNAs are displayed in red. Cell nuclei were stained with DAPI (in cyan). White arrows highlighted nucleolar tiRNA enrichment.
4.2.1.2 tiRNAs are detectable in transfected cells for over 24 h

To assess tiRNA stability in transfected cells, SH-SY5Y cells were transfected with 250 nM or 7.5x10^8 copies/cell 5`Gly tiRNA for 5 h with 2.5 µl Lipofectamine, and RNA was extracted at different time points from transfected and control cells using TRIzol. tiRNA fragments were relatively quantified using custom designed TaqMan assays. C_T values were normalised to U6 spliceosomal RNA (RNU6B, 45 nt) housekeeping gene (Benz et al. 2013). SH-SY5Y cells did not show detectable levels of 5`Gly. When we compared the 5`Gly tiRNA fold change immediately after transfection and 24 h following transfection, a decrease of 80% was observed. However, this difference might be explained with a detachment of unreleased tiRNA molecules in Lipofectamine complexes (indicated by cyan arrow in Figure 4.3) during the following 24 h of culturing. Therefore, a tiRNA function-based stability assay could be a more accurate method to investigate the tiRNA stability. However, studies on tiRNA showed translation repression in HeLa cells 16 h after transfection of 200 nM 5`tiRNA in luciferase reporter assays (Sobala & Hutvagner 2013). In general tRNAs have shown to be very stable, and the turnover was estimated to be between 48 and 72 h (reviewed in (Phizicky & Hopper 2010)). In conclusion, transfected tiRNAs are detectable over the 24 h, as their secondary structure prevents fast degradation.
Results

Figure 4.5: 5`Gly is detectable in SH-SY5Y cells 24 h after transfection. SH-SY5Y cells were transfected with 250 nM 5`Gly tiRNA or 7.5x10^8 copies/cell for 5 h with 2.5 µl Lipofectamine. RNA was extracted from transfected cells immediately and 24 h after transfection using TRIzol RNA extraction method. DNase I treated RNA samples were relative quantified by real-time two-step RT-PCR method using custom designed TaqMan assay. C_T values of different extraction time points were normalised to their U6 snRNA (RNU6B, 45 nt) levels. Data ± SEM represent a single experiment.

4.2.1.3 Cell stress optimisation in SH-SY5Y cells

To determine the optimal drug dose, cell death curves were generated over a range of concentrations (compare Figure 4.6). Four different cell-induced stess treatments were employed, including epoxomicin (proteasomal stress), thapsigargin (ER stress and Ca^{2+} depletion), sodium arsenite (oxidative stress), and glucose/serum-deprivation. SH-SY5Y cells were either treated with high dose and short exposure time (pulse-like stress) followed by a longer recovery period or low dose for a longer period of time with no recovery. Cell death was measured by flow cytometry, using SYTOX® Blue and Annexin V-FITC staining. Annexin V binds to phosphatidylserine (PS), which is normally restricted to the inner layer of the plasma membrane. During apoptosis, when the fluidity of the cell-membrane is increased, PS is translocated to the cell surface, where it can be detected as an indicator of early stages of apoptosis. SYTOX® Blue has similar properties to propidium iodide (PI), it penetrates dying cells with compromised plasma and nucleus membranes and binds to DNA by intercalating between the nucleobases.
To exclude debris during flow cytometry measurements, events were gated for defined forward and side scatter signal intensity (in detail compare chapter 2.10) and 10,000 events were counted for each well.

SH-SY5Y cells responded to all stresses. Sodium arsenite (NaAsO$_2$) and epoxomicin (Epoxo) treatments for 24 h induced dose-dependent cell death. However, only sodium arsenite displayed cell death response after pulse-like stress in SH-SY5Y cells (Figure 4.6A). SH-SY5Y cells treated for 1 h with 500 µM sodium arsenite followed by 23 h recovery showed cell death percentage of ~30%.

In order to observe whether tiRNA resulted in an alteration in cell death, our aim was ideally to induce a mild stress to the cells and obtain 30-40% cell death as the optimal drug-induced response. Proteasomal stress by epoxomicin yielded an optimal level of cell death at concentrations between 25 nM and 50 nM (Figure 4.6B) and therefore 35 nM epoxomicin treatment for 24 h condition was selected for further experiments. Different concentrations of thapsigargin were tested over 24 h and 48 h time periods. Thapsigargin treatment for 24 h showed no significant difference in the percentage of cell death, however, 48 h treatment showed over 60 % cell death at low concentration (Figure 4.6C). Since the ER-stress protein marker Grp78 has been previously shown to be induced following 3 µM thapsigargin treatment for 12 h in SH-SY5Y cells (Kitamura et al. 2003), this concentration was selected for our experiments. Glucose/Serum deprivation in SH-SY5Y showed a low stress response. The percentage of cell death after 72 h of Glucose/Serum deprivation did not exceed 7% (Figure 4.6D), hence this cell death stimulus was not further employed for neuroprotection experiments with tiRNAs.

In conclusion, the optimisation experiments showed that appropriate stress conditions for studying the neuroprotective effects of tiRNAs in SH-SY5Ys are: 1 h incubation with 500 µM sodium arsenite followed by a 23 h recovery period; 35 nM epoxomicin for 24 h; and 3 µM thapsigargin treatment over 24 h.
**Results**

Figure 4.6: Survival of SH-SY5Y cells in full culture medium DMEM/F12. Cell death was assessed by flow cytometry, using SYTOX® Blue and Annexin V-FITC staining. They both showed similar cell death trend, however here, we only illustrated SYTOX® Blue cell death data. Results are analysed with the program Cyflogic. (A) Flow cytometry quantification of SH-SY5Y cells undergoing cell death following oxidative-stress. Cells were treated over 24 h with sodium arsenite (NaAsO$_2$) or for 1 h with sodium arsenite, before recovering in culture medium for 23 h. (B) Flow cytometry quantification of SH-SY5Y cells undergoing cell death following proteasomal-stress. Cells were treated over 24 h with epoxomicin (Epoxo), or for 1 h with Epoxomicin and allowed to recover for 23 h. (C) Flow cytometry quantification of SH-SY5Y cells undergoing cell death following ER-stress. Cells were treated over 24 h and 48 h with thapsigargin (Thapsi). (D) Flow cytometry quantification of SH-SY5Y cells undergoing cell death following glucose/serum-deprivation. Cells were cultured over 24 h, 48 h and 72 h in glucose-free and reduced serum DMEM/F12 medium. Data are represented as mean ± SEM (3 wells) from a single experiment repeated in triplicate. *p<0.05 - One-way ANOVA post-hoc Tukey HSD.
4.2.1.4 Neuroprotection of tiRNA in SH-SY5Y cells

SH-SY5Y cells were transfected with 250 nM tiRNA or 7.5x10^8 copies/cell, optimal concentration able to show a detectable and consistent ROX/tiRNA signal within the cells. Figure 4.7 illustrates the experimental procedure of neuroprotection assessment with tiRNAs in SH-SY5Y cells. 24 h after seeding, cells were transfected with different tiRNAs using 2.5 µl Lipofectamine2000® for 5 h in OptiMEM. Selected stress stimuli were then added to the transfected cells and used for cell death assay assessed by flow cytometry. Since the transfected tiRNA fragments were conjugated to a fluorophore (ROX), cells were gated for ROX signal and only ROX positive cells were included in the analysis (compare Figure 2.3). 10,000 events, defined by their forward and side scatter signal intensity, were counted for each well. SYTOX® Blue/ROX and Annexin V/ROX positive quadrants were quantified showing similar cell death trend. Again, we are illustrating only SYTOX® Blue/ROX positive quantification.
Figure 4.7: Flow chart of the established neuroprotection assessment in tiRNA transfected SH-SY5Y cells.

Under control conditions, transfection of different tiRNAs had variation in the basal cell death levels (Figure 4.8A), suggesting that individual tiRNAs may induce toxicity differently. Under proteasomal stress, 5` Val showed a significant protection, whereas the other tiRNAs showed no difference compared to Ctrl tiRNA. In thapsigargin-induced ER stress, none of the tiRNAs showed protection. In contrast, we observed a significant increased toxicity with 3` Val and 5`&3` Arg. As tiRNAs had different basal cell death levels, it was necessary to correct the resulted treatments for differences in transfection toxicity. To this aim, tiRNA
Results

Baseline toxicity percentage of each experiment was subtracted from corresponding epoxomicin and thapsigargin cell death data (Figure 4.8B). After subtraction, 5` Val still showed significant protection, but also, 5` & 3` Gly, 3` Val and 5` & 3` Arg resulted in a significant protection against epoxomicin stress. In the case of thapsigargin, none of the tiRNA afforded protection after transfection toxicity correction. However, 5` Ala, 5` & 3` Val and 5` & 3` Arg still displayed increased cell death levels.

In the case of sodium arsenite cell death assessment, 5` & 3` Val and Arg tiRNA were not investigated, since these tiRNAs were not available in our group at the time these experiments were performed. tiRNAs transfection at sodium arsenite treatment control revealed also different toxicity (Figure 4.8C). Furthermore, none of the tiRNA demonstrated significant protection against pulsed sodium arsenite stress. 5` Gly displayed in contrast marginally increased toxicity. After transfection toxicity subtraction (Figure 4.8D), all tiRNAs had the same cell death compared to Ctrl tiRNA. These results indicate that tiRNAs do not protect SH-SY5Y cells against sodium arsenite induced cell stress.

In conclusion, transfection toxicity showed slight variation in the controls. In the case of sodium arsenite, where controls were washed as the corresponding treatments, even higher cell death was observed, indicating that SH-SY5Y cells, under normal conditions, are extremely sensitive to the washing step during media exchange. 5` Val alone showed significant protection in epoxomicin stressed SH-SY5Y cells. As it is one of the latest identified tiRNA fragment, it protection properties were characterized additionally only against thapsigargin stress, and in this case it showed no protection. None of the tiRNA displayed protection in thapsigargin stressed cells, most of the tiRNAs showed higher cell death percentage compared to Ctrl tiRNA transfected SH-SY5Y cells. 3` Val and 5` & 3` Arg showed significant toxicity. tiRNAs revealed no significant protection against sodium arsenite stress. 5` Gly and 5` & 3` Asp indicated slight protection compared to Ctrl tiRNA. 5` Gly showed some protection against epoxomicin in raw data results, and this reached significance in transfection toxicity corrected results. 5` & 3` Asp showed also slight protection against thapsigargin stress.
**Results**

**Figure 4.8**: 5' Val transfected SH-SY5Y cells are protected against proteasomal stress. SH-SY5Y cells were transfected with 250 nM tiRNA or 7.5x10⁸ copies/cell for 5 h using Lipofectamine. Cell death was measured by flow cytometry, using SYTOX® Blue and Annexin V-FITC staining. 10,000 events, defined by their forward and side scatter signal intensity, were counted for each well. SYTOX® Blue/ROX positive and Annexin V/ROX positive quadrants were quantified, and both quantification methods showed the same trends.

(Figure legend continues on the next page.)
Results

However, cell death percentages shown are from SYTOX® Blue/ROX quantification. Data were analysed with the program CytoLogic. *p<0.05 - One-way ANOVA post-hoc Tukey HSD. (A) Flow cytometry quantification of tiRNAs transfected SH-SY5Y cells undergoing cell death following proteasomal- (35 nM epoxomicin for 24 h) and ER-stress (3 µM thapsigargin for 24 h). Data are represented as mean ± SEM (6-9 wells) from three separate experiments, except 5’ & 3’Val and 5’ & 3’Arg of two separate experiments. Each experiment consisted of 3 wells. (B) Raw data from epoxomicin and thapsigargin treatments were corrected for tiRNA toxicity measured in Control condition. (C) Flow cytometry quantification of tiRNA-transfected SH-SY5Y cells undergoing cell death following oxidative-stress (500 µM sodium arsenite for 1 h and 23 h recovery in culture medium). Data are represented as mean ± SEM (6-9 wells) from three separate experiments, except 5’Gly and 3’Gly of two separate experiments. Each experiment consisted of three wells. (D) Raw data from sodium arsenite treatment were corrected for tiRNA toxicity measured in Control condition.

4.2.2 Stress granules are not induced by tiRNAs in SH-SY5Y cells

It has been previously observed that tiRNAs repress translation by interfering with the assembly of the cap binding complex and contributing the formation of SGs (Emara et al. 2010), (Ivanov et al. 2011)). It was hypothesised that translation repression and SG formation are a cell-protection mechanism adopted by the cells to reduce energy expenditure and react correctly to stress. To examine whether the protection properties of tiRNA correlated with SG formation, SH-SY5Y cells were transfected with different tiRNA concentrations. To investigate whether the new identified tiRNAs could enhance SG formation during stress, transfected SH-SY5Y cells were treated with 20 µM sodium arsenite (NaAsO₂) for 1 h. SG formation was analysed by immunocytochemistry using a G3BP antibody identifying SGs and the images were taken using the LSM 7.10 confocal microscope. Similar to the observation in Figure 3.11, G3PB was present both evenly distributed in the cytosol (Figure 4.9, cyan arrows) and aggregated in form of SG speckles (Figure 4.9, white arrows). Cells with SGs exhibited always several SG speckles, therefore, only cells with more than one G3BP speckles were defined as SG positive for the SG quantification. To investigate whether tiRNAs aggregate in SGs, tiRNAs co-localisation with G3BP were qualitatively examined.
Lipofectamine transfections showed comparable tiRNA signals as described in chapter 4.2.1.1. Intense tiRNA signals at the cell surface are presumably unreleased tiRNA molecules in Lipofectamine complexes, whereas intracellular tiRNA signals within the cells represented successful tiRNA transfection. Confocal images exhibited that tiRNA distribution in the cytosol was even and we did not observe any increase tiRNA signals in SGs (Figure 4.9). These findings indicated that tiRNAs are not enriched in SGs.
Results

Figure 4.9: tiRNA did not aggregate in SG. SH-SY5Y cells were transfected with 250 nM tiRNA or 7.5x10^8 copies/cell for 5 h with Lipofectamine and stained for G3BP, a well-established SG marker. Cell nuclei were highlighted by DAPI staining. White arrows indicate SG formations and cyan arrows normal distributed G3BP.
SG formation was quantified with two different approaches: (i) a manual approach, where 200-300 cells per treatment were counted in a blind experiment; (ii) an automated approach using a CellProfiler pipeline able for high throughput analysis quantification. To verify the CellProfiler pipeline, SH-SY5Y cells were treated with different sodium arsenite concentration (20-30 µM NaAsO₂) for 1 h and analysed by immunocytochemistry using a G3BP antibody. Cells were imaged on the confocal microscope LSM 7.10 and analysed automatically. Therefore, a custom designed automated image processing pipeline was created (in detail at chapter 2.11.1). The results showed an increase in the number of cells with SGs with increasing concentrations of sodium arsenite (Figure 4.10A). Representative images illustrate the appropriate overlap of SGs identified by the CellProfiler pipeline with those observed in the G3BP channel (Figure 4.10B, white arrows). In conclusion the designed pipeline is a valuable tool for high throughput analysis.
Results

Figure 4.10: Custom designed CellProfiler pipeline identifies reliable different levels of SG formation. SH-SY5Y cells were exposed to different sodium arsenite (NaAsO$_2$) concentration for 1 h. SGs were measured by confocal microscopy using an anti-G3BP as antibody. Six different fields of view were imaged per cover slip, in which 200-300 cells were quantified. (A) SG formation was identified by a custom designed image processing pipeline in CellProfiler. All concentrations were repeated in triplicate, and an average for each separate experiment was calculated. Data are represented as mean ± SEM from at least 3 independent experiments. (B) Representative confocal images of either G3BP channel, SGs identified by the pipeline and merged images with DAPI nuclear staining are illustrated. White arrows highlight the overlap of SGs identified by the CellProfiler pipeline with those observed in the G3BP channel.
Results

SH-SY5Y cells were transfected with different concentration tiRNA: (i) 7.5x10^8 copies/cell (as used in previous experiments); (ii) 22.5x10^8 copies/cell (as previously reported by (Emara et al. 2010), (Ivanov et al. 2011)); and (iii) 15x10^8 copies/cell (a concentration in between the preceding two). When we compared manual versus automated analysis, we observed that the automated analysis identified 2-3 times more SG positive cells than the manual approach (Figure 4.11A&B). This might due to higher sensitivity detection of SGs in the automated approach, as SG identified by the pipeline co-localised with SGs in G3BP channel (Figure 4.10B, white arrows). All tiRNAs, including also Ctrl tiRNA, induced SG formation in SH-SY5Y cells showing SG positive cells. However, 5`Ala showed the strongest effect on SG formation. At 7.5x10^8 copies/cell ~24% of 5`Ala transfected cells were SG positive. This effect was not concentration dependent and decreased in 15 & 22.5x10^8 copies/cell. 5`Gly showed a strong effect at 22.5x10^8 copies/cell, and low percentages of SG positive cells during 7.5 & 15x10^8 copies/cell. 3`Gly displayed high variability between experiments at 7.5 & 22.5x10^8 copies/cell, and low effect at 15x10^8 copies/cell. 5` & 3`Asp had at all concentrations constant 5 – 10% SG positive cells with slightly more in 3`Asp transfected SH-SY5Y cells.

In conclusion, both methods showed same tendencies and indicated that only 5`Ala induced SG formation at 7.5x10^8 copies/cell compared to Ctrl tiRNA transfected SH-SY5Y cells. SG formation was not concentration dependent, and there were also no difference between 5` and 3` tiRNA, suggest that SGs are not induced by tiRNAs in SH-SY5Y cells.
Figure 4.11: Stress granules are not induced by tiRNAs in SH-SY5Y cells. Different concentrations and tiRNAs were transfected with Lipofectamine for 5 h in SH-SY5Y cells to examine SG formation. SGs were measured by confocal microscopy using an anti-G3BP antibody. Six different fields of view were imaged per cover slip with 350-600 cells/cover slip. tiRNA concentrations were carried out once per experiment. (A) SG formation was quantified blinded by counting SG positive cells. SGs positive cells were normalised to total cell number. Data are represented as mean ± SEM of three separate experiments. (B) SG formation was quantified using the automated image processing pipeline in CellProfiler. Data are represented as mean ± SEM of three separate experiments. (C) Representative confocal images of either G3BP channel, SGs identified by the pipeline and merged images with DAPI nuclear staining are shown. White arrows illustrate the overlap of SGs identified by the CellProfiler pipeline with those observed in the G3BP channel.
As our group recently identified new strongly ANG generated tiRNAs (Figure 4.1), and our findings, in generated stable SH-SY5Y, indicated that 5`Val tiRNA was highly generated and secreted (Chapter 3.2.2.1 and 3.2.3), additional SG formation experiments were carried out. 20 µM sodium arsenite (NaAsO\textsubscript{2}) for 1 h treatment was employed to the experimental procedure in order to examine whether tiRNAs might enhance SG formation under stress conditions. SH-SY5Y cells were transfected only at the concentration of 7.5x10\textsuperscript{8} copies/cell, as higher concentrations had no effect on SG formation in the experiments described above. Ctrl tiRNA afforded the highest impact on SG formation directly after transfection (Figure 4.12A), as shown in previous experiments. Neither 5` nor 3` of the new tiRNAs induced SG formation compared to Ctrl tiRNA transfected SH-SY5Y cells. 20 µM sodium arsenite treatments for 1 h on transfected SH-SY5Y cells showed high percentage of SG positive cells. 5` & 3`Val had both a slight impact on SG formation compared to Ctrl tiRNA. 5` & 3`Arg showed numerically less SG positive cells compared to Ctrl tiRNA transfected SH-SY5Y cells. Comparing 5` & 3` fragment effects, 3` fragments induce SG formation in slightly more cells than the corresponding 5` fragment, however it did not reach statistical significance.

In conclusion, the new tiRNAs confirmed that SGs are not induced by tiRNAs in SH-SY5Y cells and tiRNAs do not enhance the SG formation also under oxidative stress conditions.
Figure 4.12: Stress granules are neither induced nor enhanced by tiRNAs in SH-SY5Y cells. SH-SY5Y cell were transfected with 250 nM tiRNA or 7.5x10^8 copies/cell for 5 h using Lipofectamine. Transfected SH-SY5Y cells were either directly fixed after transfection, or treated with sodium arsenite (20 µM NaAsO_2) for 1 h after transfection and fixed after the treatment. SGs were measured by confocal microscopy using G3BP as antibody. Several fields of view were imaged per cover slip with 200-300 cells/cover slip. (A) SG formation was quantified by a custom designed automated image processing pipeline in CellProfiler. All concentrations were performed in triplicate, and an average for each separate experiment was calculated. Data are represented as mean ± SEM of two separate experiments. (B) Representative confocal images of either G3BP channel (in green), SGs identified by the pipeline (in red) and merged images with DAPI nuclear staining are illustrated. White arrows illustrate the overlap of SGs identified by the CellProfiler pipeline with those observed in the G3BP channel.
4.2.3 Angiogenin protection is not mediated by single tiRNAs in primary mixed motoneuron cultures

4.2.3.1 Transfection optimisation in primary mixed motoneuron cultures

In previous studies, our group showed that systemic angiogenin treatments in the transgenic SOD1<sup>G93A</sup> mouse model of ALS increased lifespan and motoneuron survival by restoring the disease-associated decrease in Akt-1 survival signalling (Kieran et al. 2008). Additionally, angiogenin treatment of primary ventral horn motoneuron cultures from E12 mouse embryos showed neuroprotection against a broad spectrum of stresses: hypoxia (12 h – 48 h, 1 % O<sub>2</sub>) (Sebastià et al. 2009), AMPA treatment-induced excitotoxicity (50 µM, 24 h) and tunicamycin-induced ER-stress (500 nM, 24 h) (Kieran et al. 2008). To examine whether a single angiogenin generated tiRNA provides neuroprotection under hypoxic conditions (1% O<sub>2</sub> for 24 h), primary ventral horn motoneuron cultures from E12-E14 wild type (C57Bl/6) mouse embryos have been employed.

Primary motoneurons are well known to be very sensitive and challenging to transfect <i>in vitro</i>, hence, different transfection reagents and methods were tested to select the optimal transfection technique with the lowest toxic effects. Lipofectamine®, Viromer® BLACK, NeuroMag®, and calcium phosphate (CaPO<sub>4</sub>) precipitation were chosen. All four transfection reagents are using electrostatic interaction to form complexes with negatively charged nucleic acid. However, the cationic formulation differs from each reagent. Lipofectamine consists of cationic lipid molecules formulated with a neutral co-lipid (Dalby et al. 2004). Viromer BLACK molecules are alkyl moieties in combination with long chain fatty acids. NeuroMag are iron nanoparticles coated with styrene copolymers. Ca<sup>2+</sup>-ions form complexes with the PO<sub>4</sub>-backbone of tiRNAs, where the low solubility of CaPO<sub>4</sub> in water solution forces Ca-tiRNA complexes to precipitate. In contrast to CaPO<sub>4</sub> precipitation, Lipofectamine, and Viromer BLACK which all precipitate by gravity, NeuroMag are magnetic particles and are concentrated onto the cells by an external magnetic field using a specific magnetic plate for 15 min. The incubation of reagent-tiRNA solution on cells varied between the methods according to the
manufacturer’s protocols and it is illustrated in Figure 4.13. Viromer BLACK and NeuroMag® transfection solutions were left for 24 h on the primary motoneurons. In case of Lipofectamine, Lipofectamine-tiRNA complex solution was added to primary motoneuron cultures either directly to their culture medium or this was changed to OptiMEM for the entire time of transfection. After 1 h transfection with Lipofectamine, transfection media was entirely removed and replaced with conditioned media and primary motoneurons were allowed to recover for 23 h. CaPO$_4$-precipitation was performed following (Goetze et al. 2004). Briefly, Ca-tiRNA complexes, or in the case of CaPO$_4$-precipitation control only CaCl$_2$-solution, are added after short period to primary motoneuron in transfection medium. To keep the pH of the transfection medium constant, primary motoneuron cultures were incubated in a CO$_2$ free incubator for 2 h. Transfection medium was changed to the conditioned media that had been removed earlier and cells were allowed to recover for 24 h. To examine whether tiRNA fragments are endocytosed by primary motoneuron without transfection reagents, tiRNA was added without any transfection reagent to culture medium at day in vitro (DIV) 7 for 72 h.
Figure 4.13: tiRNA transfection workflow of different transfection reagents and methods in primary motoneurons.
To compare all different transfection methods (fully listed in section 2.3.3), 5` Asp tiRNA (250 nM or $2.15 \times 10^8$ copies/cell) was transfected in 24 well plates cultured primary mixed cultured motoneurons on DIV 8. Each transfection method was performed in triplicate. CaCl$_2$ solution without tiRNA was used as additional control for the CaPO$_4$ transfection method. To evaluate transfection toxicity, transfected cells were analysed by immunocytochemistry. SMI-32 antibody was used to recognise non-phosphorylated neurofilaments, enriched in motoneuron cell bodies and neurites (Tsang et al. 2000). Trypan blue, penetrates dying cells with compromised plasma membranes and stains the cytoplasm of nonviable cells blue (Strober 2001), was used to determine healthy motoneurons. SMI-32 positive cells with distinct neurites and not stained by trypan blue were defined as healthy motoneurons. Motoneurons were counted blinded in five different fields of view of each well. Motoneuron counts of all different transfection methods were normalised to control primary motoneuron counts. Control primary motoneurons were cultured for 9 DIV without any transfection treatment.

Lipofectamine transfections and transfection with Viromer BLACK had the lowest survival rate with 40%. CaPO$_4$ precipitation control treatment showed already 20% decreased motoneuron survival, indicating that the transfection procedure itself was harmful to motoneurons. Only NeuroMag transfections, and tiRNA added to culture medium without transfection reagents exhibited survival of over 90% (Figure 4.14).
Figure 4.14: Transfection toxicity in primary mixed cultured motoneurons. Primary mixed cultured motoneurons were transfected with different transfection methods and reagents using 5`Asp tiRNA/well (250 nM or 2.15x10^8 copies/cell) in 24 well plates. Motoneurons were counted blinded in five different fields of view of each well. SMI-32 positive cells with distinct neurites and trypan blue negative were defined as healthy motoneurons. CaPO_4 precipitated cells have an additional control without adding CaCl_2-tiRNA solution. Data ± SEM represent normalised mean values of 3 wells (N=3). Motoneuron counts of all different transfection methods were normalised to control primary motoneuron counts. Control primary motoneurons were cultured for 9 DIV without any transfection treatments. *p(to control)<0.05 - One-way ANOVA post-hoc Tukey HSD.
Results

Transfection efficiency in primary mixed cultured motoneurons was analysed by confocal microscopy. Transfection efficiency was calculated as median ROX signal of SMI-32 defined area after subtracting background and channel crosstalk. Only transfection with 3 µl NeuroMag and Lipofectamine demonstrated significant efficiency. Incubation for 72 h with tiRNA showed no tiRNA signal in primary motoneurons, suggesting that primary motoneurons do not endocytose tiRNA from the medium. Additionally, different transfection medium showed strong variation in the efficiency of Lipofectamine transfections. Motoneurons left in OptiMEM media only, during transfection period, died more and had lower transfection efficiency than those left in preconditioned culture media. Transfection of 250 nM 5`Asp tiRNA (2.15x10^8 copies/cell) with 3 µl NeuroMag resulted to be overall the best transfection reagent for primary mixed motoneuron cultures, as the highest transfection efficiency with better survival outcome was obtained with this approach.

Figure 4.15: Transfection efficiency in primary mixed motoneuron cultures. Primary mixed cultured motoneurons were transfected with different transfection methods and reagents using 250 nM 5`Asp tiRNA/well (2.15x10^8 copies/cell) in 24 well plates. 17-20 SMI-32 positive cells with distinct neurites and Trypan Blue negative staining were imaged using the LSM 7.10 confocal microscope. Data represent tiRNA conjugated ROX median signal in SMI-32 defined area ± SEM, after channel crosstalk correction. *p(to untransfected control)<0.05 - One-way ANOVA post-hoc Tukey HSD.
4.2.3.2 All tiRNAs are successful transfected into primary motoneurons

TiRNA transfection levels were analysed in hypoxia treated primary motoneurons to examine whether all tiRNAs were successfully transfected and tiRNA levels were compared. Therefore, primary motoneurons were transfected with 3 µl NeuroMag and different tiRNAs (250 nM or 2.15x10^8 copies/cell) at DIV 8 for 24 h, and were exposed to hypoxic conditions (1% O₂ for 24 h). 10-15 fields of view were imaged on the confocal microscope LSM 7.10 and ROX intensities were analysed in SMI-32 positive cells with distinct neurites and Trypan Blue negative staining.

Transfection with NeuroMag indicated similar transfection pattern as Lipofectamine transfections in SH-SY5Y. Intense tiRNA signals at the surface (Figure 4.16A, cyan arrows), which are presumably unreleased tiRNA molecules in NeuroMag complexes, and consistent tiRNA signals within the cells, which indicated successful tiRNA transfection (Figure 4.16A, white arrows), were observed.

TiRNA signals were corrected for the channel crosstalk and calculated as median ROX signal in the SMI-32 defined area (in detail chapter 2.3.3.6). TiRNA transfected primary motoneurons exhibited different tiRNA levels in hypoxia treated primary motoneurons, but did not reach statistical significance employing One-way ANOVA post-hoc Tukey HSD. 5` & 3`Val and 3`Arg had slightly higher tiRNA signals compared to Ctrl tiRNA (Figure 4.16B). Whereas, 5`Ala, 5` & 3`Gly and 5`Arg displayed slightly lower tiRNA signals compared to Ctrl tiRNA. These tiRNA levels did not correlated with either the nucleotide length or whether they were 5` or 3` fragment (Figure 4.16C), and suggested that the secondary structures of 5` & 3`Val and 3`Arg were favoured in either the NeuroMag-tiRNA complex forming or release from the complexes. However, since all tiRNAs displayed successful transfection, primary motoneuron protection assessment was carried out with all tiRNAs.
Results

Figure 4.16: tiRNA transfected primary motoneurons displayed different tiRNA levels after hypoxia treatment. Primary mixed motoneuron cultures were transfected with different tiRNAs (250 nM or 2.15x10^8 copies/cell) employing 3 µL NeuroMag/well in 24 well plates for 24 h. Transfected motoneurons were subsequently exposed to hypoxic stress (1% O₂ for 24 h). SMI-32 positive cells with distinct neurites and trypan blue negative staining were defined as healthy motoneurons, and were imaged on the confocal microscope LSM 7.10. (A) Representative confocal images of healthy primary motoneurons stained against SMI-32 (in green), tiRNA (in red) and DAPI (in cyan). Cyan arrows illustrate tiRNA-NeuroMag aggregates at the cell surface. White arrows designate intracellular tiRNA signals as indication of successful transfection. Scale bars 10 µm. (B) tiRNA signals were quantified in the SMI-32 defined cell bodies. Data represent tiRNA median signals ± SEM in 20-30 motoneurons, after channel crosstalk subtraction. (C) Nucleotide length of the different tiRNAs.
4.2.3.3 Angiogenin protects primary motoneurons against hypoxic stress

Next, primary ventral horn motoneuron cultures from E12-E14 wild type (C57Bl/6) mouse embryos were firstly employed to explore, in our hands, the already known protection properties exerted by angiogenin during hypoxia stress. Motoneurons were exposed to hypoxic stress (1% O\(_2\) for 24 h) and angiogenin protection was assessed at DIV 9. Serum-free supplemented culture medium, in the presence and the absence of ANG/BSA, was equilibrated for 4 h in hypoxic chamber and primary motoneurons were then exposed to hypoxic conditions (1% O\(_2\)) for 24 h. Control cultures were kept in parallel in normal conditions (normoxia) for 24 h. Motoneuron protection was quantified by counting blinded healthy motoneurons in five different fields of view of each well. SMI-32 positive cells with distinct neurites and trypan blue negative were defined as healthy motoneurons. Motoneuron counts were normalised to normoxia baseline counts for each experiment separately. Baseline counts represented motoneurons exposed to normoxic or hypoxic conditions in serum-free supplemented culture medium for 24 h. Motoneuron count comparison of angiogenin treatments (100 ng/ml and 500 ng/ml) relative to controls (baseline and BSA) indicated a protection against hypoxic stress (Figure 4.17). The relative motoneuron count of 500 ng/ml angiogenin showed 18% protection compared to hypoxia baseline/BSA treatment, however, this did not reach statistical significance employing one-way ANOVA pairwise comparison with Tukey’s post hoc test.
Results

Figure 4.17: Angiogenin is protective in mixed motoneuron cultures against hypoxic stress. Primary mixed motoneuron cultures were treated with BSA or recombinant human angiogenin (100 ng/ml and 500 ng/ml ANG) and exposed to hypoxic stress (1% O₂ for 24h). (A) Motoneuron survival was quantified by counting healthy motoneurons blinded in five different fields of view of each well. SMI-32 positive cells with distinct neurites and trypan blue negative were defined as healthy motoneurons. Data represent normalised mean values ± SEM of 4 separate experiments with 2-3 wells per experiment. Baseline counts were motoneurons, which were cultured 24h under normal (normoxia) or hypoxic conditions in serum-free supplemented culture medium. Motoneuron counts were normalised to normoxia mean baseline count for each experiment separately. (One-way ANOVA post-hoc Tukey HSD $p(\text{BSA to Baseline-Hypoxia}) = 1$; $p(100 \text{ ng/ml ANG to Baseline-Hypoxia}) = 0.991$; $p(500 \text{ ng/ml ANG to Baseline-Hypoxia}) = 0.696$) (B) Representative images of SMI-32 stained primary motoneurons under normoxic and hypoxic conditions.
4.2.3.4 Single tiRNAs are not responsible for ANG protection in primary motoneurons

To investigate whether the neuroprotection of angiogenin is due to single tiRNA, primary motoneuron, from the same preparation in above experiments, were transfected with 3 µl NeuroMag and different tiRNAs (250 nM or 2.15x10^8 copies/cell) at DIV 8 for 24 h. tiRNA transfected motoneurons were exposed to hypoxic conditions (1% O_2 for 24 h) at the same time as angiogenin treated motoneuron cultures. Motoneuron protection was quantified the same way as angiogenin treated motoneurons. Transfection control treatments were compared under normoxic conditions in Figure 4.18 and we found that MOCK transfected motoneurons showed slightly lower survival than baseline. This observation indicating that NeuroMag transfection method stressed the cells additionally, as it was already observed during toxicity and transfection efficiency methods validation (Figure 4.14). Ctrl tiRNA transfected motoneuron had again lower counts than MOCK transfected. Comparing other tiRNA transfected motoneuron counts to Ctrl tiRNA under normoxic conditions, we noticed fluctuation in the survival rate: 3´Gly transfected motoneurons showed slightly higher survival compared to Ctrl tiRNA, and 5´ & 3´Val and especially 3´Arg had a lower survival compared to Ctrl tiRNA. Hypoxia related relative motoneuron counts did not correlate with tiRNA transfected motoneuron survival under normoxic conditions. 5´Val and 3´Arg showed slightly higher survival compared to Ctrl tiRNA and, 5´Gly had a lower survival compared to Ctrl tiRNA. Despite the observed trend, none of the motoneuron counts reached statistical significance employing one-way ANOVA pairwise comparison with Tukey’s post hoc test.

In conclusion, angiogenin protection in hypoxia-treated primary motoneurons was not mediated by a single tiRNA. Furthermore, no tendencies towards a difference between 5´ and 3´ tiRNA was observed.
Figure 4.18: tiRNAs have no effect on protection against hypoxic stress in mixed motoneuron cultures. Primary mixed motoneuron cultures were transfected with different tiRNAs (250 nM or 2.15x10⁸ copies/cell) employing 3 μL NeuroMag/well in 24 well plates for 24 h. Motoneuron survival was quantified by counting healthy motoneurons blinded in five different fields of view of each well. SMI-32 positive cells with distinct neurites and Trypan Blue negative staining were defined as healthy motoneurons. Data represents normalised mean values ± SEM of three separate experiments with two to three wells per experiment. Baseline counts were motoneurons, which were cultured 24 h under normal (normoxia) or hypoxic conditions in serum-free supplemented culture medium. Motoneuron counts were normalised to normoxia mean baseline count for each experiment separately. None of the tiRNAs displayed significant difference compared to Ctrl-tiRNA employing one-way ANOVA pairwise comparison with Tukey’s post hoc test.
4.3 Discussion

Stable SH-SY5Ys cells overexpressing ANG WT and ALS associated ANG mutant (R31K), but not the ribonucleolytic inactive K40I mutant and the empty vector control, displayed up-regulation of tiRNAs. For this reason, tiRNAs function was further characterised in this chapter. Epoxomicin, thapsigargin and sodium arsenite were stress-induced cell death drugs selected to further examine tiRNAs neuroprotective effect in SHSY5Y cells during ALS pathology-related stress conditions. Epoxomicin is a proteasome inhibitor, and was used to model proteasomal failure, and accumulation of ubiquitinated proteins (Meng et al. 1999). Thapsigargin induces ER stress through inhibition of ER Ca\(^{2+}\) ATPase (Lytton et al. 1991), and was used to model accumulation of unfolded or misfolded proteins. We also investigated sodium arsenite stress as this has been shown to induce SG formation. SGs are transient cytosolic aggregations of pre-initiation and translation-related factors, mRNA-binding proteins, proteins associated to mRNA metabolism and stalled mRNA molecules (Anderson & Ivanov 2014). SG formation and ALS pathology could be linked through DNA-RNA-binding proteins FUS and/or TDP-43 (reviewed in (Li et al. 2013)). Both proteins are generally localised in the nucleus and have prion-like domains. However, stress conditions and ALS-causing mutations lead to cytoplasmic displacement and protein aggregation. Sodium arsenite treated HeLa cells showed that short- (500 µM for 0.5 h) and long-pulsed (500 µM for 2 h) cell stress leads to insoluble TDP-43 aggregates. However, after a recovery period of 4.5 h without sodium arsenite, only cells stressed for 2 h exhibited insoluble TDP-43 aggregates (Higashi et al. 2013). Therefore, tiRNA protection was investigated in sodium arsenite treated cells.

After optimising the transfection and the treatment conditions, flow cytometry quantification data of SH-SY5Y transfected cells with tiRNAs showed that 5`Val was protective against epoxomicin stress (Figure 4.8A). Protection properties of 5`Val were additionally studied against thapsigargin stress, showing no protection. Previously, our group has demonstrated that SH-SY5Y cells exposed to 50 nM epoxomicin had impaired protein degradation, and subsequently triggering
caspase-3 activation and programmed cell death (Concannon et al. 2007). The proteasome inhibition was associated with the induction of the proapoptotic BH3-only family members, including PUMA and Bim, through the activation of the transcription factor p53. p53 stimulates proteins that inhibit cell division (reviewed in (Ryan et al. 2001)). In contrast, thapsigargin induced apoptosis has been shown to be p53 independent (Reimertz et al. 2003). A recent tiRNA study revealed that tiRNAs play a significant role in cell proliferation, as 5`tiRNA fragments silencing experiments resulted in lower proliferation in breast and prostate cancer cell lines (Honda et al. 2015). Taken together, these data led us to hypothesise that 5`Val may protect cells against epoxomicin stress via directly and/or indirectly repression of p53.

To examine whether the neuroprotection properties of 5`Val correlated with SG formation, tiRNAs ability to induce SG formation was investigated in SH-SY5Y cells. It has been previously observed that phosphorylated 5’ and none of the 3` tiRNA fragments repress translation in an eukaryotic initiation factor 2α (eIF2α) independent manner by interfering with the assembly of the cap binding complex eIF4F. 5` tiRNAs also induced SGs formation ((Emara et al. 2010), (Ivanov et al. 2011)). Several studies have reported SG formation associated with translation repression, however, it is unclear which event occurs first ((McEwen et al. 2005), (Reineke et al. 2012), (Yamamoto & Izawa 2013), (Liem & Liu 2016)). tiRNAs show suppression of cap-mediated protein translation, but not internal ribosome entry sequence (IRES)-mediated translation, which is often used by pro-survival and anti-apoptotic genes ((King et al. 2010), (Komar & Hatzoglou 2011)). Therefore, it was hypothesised that tiRNAs play an important role in the stress response program (reviewed in (Anderson & Ivanov 2014), (Li & Hu 2012)). A CellProfiler pipeline was designed and evaluated to be a robust tool for high throughput analysis. SG quantification showed that protection by 5`Val did not correlate with the ability to induce SG formation. In general, SG formation analysis showed that only 5`Ala had slight impact on SG formation (Figure 4.11 A&B). However, the effect of 5`Ala was not concentration dependent, as 5`Ala induced at
the lowest concentration (7.5x10^8 copies/cell) SG formation and not at higher concentration. These results are in contradiction with published data indicating that 5’Ala has a significant impact on SG formation in a concentration dependent manner (2.5x10^8 – 33.3x10^8 copies/cell). However, these experiments were performed in human bone osteosarcoma epithelial U2OS cells (Emara et al. 2010) and the information regarding the biogenesis of the assayed 5’Ala remained elusive. As Ctrl tiRNA showed similar impact on SG formation in our experiments, the observed SG formation effects might be induced by the transfection procedure itself, and not by tiRNAs in SH-SY5Y cells.

In previous studies, our group demonstrated that angiogenin treatment increased lifespan and motor function in SOD1^{G93A} mice, an established and well characterised ALS mouse model, which shows accumulation of ubiquitinated SOD1 aggregates in motoneurons (Stieber et al. 2000). It has been shown that this protection was mediated by Akt-1 phosphorylation (Kieran et al. 2008). To model more accurate the cellular interactions that occur within the spinal cord, primary ventral horn motoneuron cultures from E12-E14 wild type (C57Bl/6) mouse embryos were employed. In our model, hypoxic stress (1% O_2 for 24 h) was selected to investigate whether angiogenin mediated protection is due to a single tiRNA. The relative motoneuron count of angiogenin treated primary mixed motoneuron cultures showed 18% protection compared to hypoxia baseline/BSA treatment, but did not reach statistical significance (Figure 4.17A). This finding were in line with previous observation that angiogenin treatment of primary ventral horn motoneuron cultures afforded neuroprotection against a broad spectrum of stresses, such as hypoxia (Sebastià et al. 2009), AMPA-induced excitotoxicity and tunicamycin-induced ER-stress (Kieran et al. 2008). After testing different transfection reagents and approaches, NeuroMag was identified as the optimal transfection reagent for transfecting primary motoneuron cultures. tiRNA transfections under normal conditions exhibited variation in survival rate similar to SH-SY5Y tiRNA transfections. Survival experiments in primary mixed motoneurons indicated that transfection toxicity in normal conditions might
correlate with the amount tiRNA present following transfection. A trend in lower motoneurons counts is observed when a higher amount of tiRNA is transfected, however, this observation is not noticed under hypoxic conditions. Moreover, none of the tiRNAs showed significant protection compared to Ctrl tiRNA and no difference between 5` and 3` tiRNA was observed (Figure 4.18). Therefore, angiogenin protection, in hypoxia treated primary motoneurons, was not mediated by a single tiRNA.

In conclusion, 5`Val tiRNA showed protection against epoxomicin stress, which we hypothesise may associated with p53 signalling. Ctrl tiRNA displayed a similar impact to 5` & 3` tiRNAs on SG formation, indicating that the observed SG formation effects might be induced by the transfection procedure itself and not by the specific tiRNAs in SH-SY5Y cells. Angiogenin protection in hypoxia stressed primary motoneurons was not mediated by a single tiRNA, and may require a combination of different tiRNA to provide protection.

As angiogenin is secreted by motoneurons and endocytosed by astroglial cells, our group proposed that the neuroprotection is mediated in paracrine (Skorupa et al. 2012). Therefore, tiRNA functions were further characterised in astrocyte cultures.
5. **Chapter 5: tiRNA effect in astrocyte cultures**

5.1 **Introduction**

5.1.1 **Protein translation**

In endothelial cells angiogenin undergoes nuclear translocation where it stimulates rRNA transcription by binding the ribosomal DNA promoter (Xu et al. 2002). rRNA transcription is essential for ribosome synthesis, and inhibition of angiogenin-stimulated transcription of rRNA results in reduced protein translation and cell proliferation (Kishimoto et al. 2005). This has been suggested to occur during neovascularisation. Our group revealed that angiogenin treatments increased lifespan in transgenic SOD1^{G93A} mouse model of ALS restoring the disease-associated decrease in Akt-1 survival signalling (Kieran et al. 2008). Beside activation of Akt survival pathway, our group and others revealed that angiogenin cleaves tRNA under stress conditions in the cytosol of several cell types ((Yamasaki et al. 2009), (Skorupa et al. 2012), (Saikia et al. 2014)). In Chapter 3, we demonstrated that under serum deprivation angiogenin generates specific tiRNAs, and that these are subsequently secreted. In Chapter 4, we revealed 5´Val has neuroprotective effects against epoxomicin stress in SH-SY5Y cells. Furthermore, we observed that angiogenin protects motoneurons against hypoxic stress, which confirms previous findings, from our laboratory (Kieran et al. 2008). The Anderson Lab reported that specific tiRNAs inhibit cap-dependent protein synthesis by interfering with the assembly of the cap binding complex eIF4F. Translation repression was accompanied by SG formation (Kedersha & Anderson 2007). Several studies reported SG formation associated with translation repression, however it is unclear which occurs first ((McEwen et al. 2005), (Reineke et al. 2012), (Yamamoto & Izawa 2013), (Liem & Liu 2016)). To investigate whether angiogenin enhance or repress protein translation in astrocytes, nascent protein synthesis was examined in primary astrocyte cultures. A recent study addressed dual roles of angiogenin in the hematopoietic system. (Goncalves et al. 2016) showed that protein synthesis was up-regulated in
angiogenin treated myeloid-restricted progenitor (MyoPro) cells, but down-regulated in ANG treated hematopoietic stem cells (HSCs). Angiogenin treatment showed in HSCs an increase of tiRNA (5`Gly) generation, but levels of rRNA (47S and 28S) were not altered. In contrast, MyoPro cells treated with angiogenin showed increased 47S and 28S rRNA levels, but no generation of 5`Gly tiRNA was observed. Cell cycle analysis showed angiogenin restricted cell proliferation in HSCs, but promoted proliferation of MyePro cells. The authors proposed that angiogenin has a cell-type-specific role, promoting quiescence by repressing translation in hematopoietic stem cells, and stimulating proliferation in differentiated cells.

The aim of this chapter was therefore to characterise angiogenin and tiRNA functions in astrocyte cultures. Angiogenin uptake and RNA cleavage was examined in MZ-294 cells by gel electrophoresis, to investigate cell type specific differences. Furthermore, tiRNA impact on SG formation in MZ-294 cells was characterised in the same way as in SH-SY5Y cells in chapter 4.2.2. tiRNA co-localisation with SG associated proteins G3BP and Y-box binding protein 1 (YB-1) was examined by immunolabelling. To investigate whether the paracrine neuroprotection of angiogenin in mixed primary motoneuron cultures is mediated by translation alteration, primary astrocytes cultures from P1-P2 mice pups were employed as a more related in vitro model. To ensure high astrocyte purity, primary astrocyte preparation and culturing protocol was validated. To examine SG formation in cultured primary astrocytes, a broad range of established SG inducing stress conditions were tested, however, we could not detect SGs in primary astrocytes by immunolabelling using G3BP and TIA-1 antibodies. Angiogenin and tiRNA influence to alter protein translation was investigated by using Click-iT® labelling technology. Click-iT® labelling technology uses a non-radioactive amino acid analog (L-Azidohomoalanine – AHA) that contains an azide modification that was fed to the cells (Figure 5.1, Step 1) and incorporated into proteins during active protein synthesis (Figure 5.1, Step 2). After fixation and permeabilisation, a chemoselective reaction (“click” reaction - copper(I)-catalysed azide alkyne cycloaddition (Meldal & Tornøe 2008)) was performed, which
modified the proteins containing the azide modification and inserted an alkyne modified fluorophore (Figure 5.1, Step 3). Fluorescence intensities of nascent synthesised proteins were imaged on LSM 7.10 confocal microscope and quantified by customised CellProfiler pipeline.

**Step 1: Incubation of cells with AHA containing medium**

![L-Azidohomoalanine (AHA) - azide](image)

**Step 2: AHA incorporation into proteins during active protein synthesis**

![mRNA and protein synthesis](image)

**Step 3: Click azide/alkyne reaction**

![Click reaction](image)

**Figure 5.1: Non-radioactive “Click-iT®” labelling of nascent synthesised proteins.** Essential “Click-iT®” steps to label nascent synthesised proteins.
5.2 Results

5.2.1 Angiogenin cleaves RNA under serum-deprived conditions in MZ-294 cells

Our group demonstrated previously that angiogenin is secreted by primary motoneurons under stress conditions (serum withdrawal), and endocytosed by neighbouring astroglial cells ((Skorupa et al. 2012), (Skorupa et al. 2013)). Angiogenin uptake was therefore examined in a human *in vitro* model by employing glioblastoma-derived MZ-294 cell line. Angiogenin treatment was adapted to the previous findings where ideal angiogenin uptake occurred within 6 h (Skorupa et al. 2012). MZ-294 cells were treated for 6 h with different concentrations of rhANG, BSA or serum-free neurobasal medium (compare paragraph 2.7.4), and angiogenin uptake was examined by western blotting. Angiogenin uptake in MZ-294 cells under serum-free conditions was proportional to the angiogenin concentration in the medium.

![Western blot demonstrating angiogenin uptake](image)

**Figure 5.2: ANG uptake in MZ-294 cells under serum-free conditions.** MZ-294 cells (2x10^5 cells/well of 6 well plate) were treated for 6 h with indicated recombinant human angiogenin (rhANG) concentration, BSA or serum-free neurobasal medium. Cells were lysated, and protein amount was determined by BCA assay. Western blots demonstrating that MZ-294 cells endocytose angiogenin proportional to angiogenin concentration in serum-free neurobasal medium.

To investigate whether recombinant human angiogenin generated similar RNA fragment pattern as observed in stable SH-SY5Y cell (compare Figure 3.9), MZ-294 cells were treated for 6 h with different concentration of recombinant human
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angiogenin (10-500 ng/ml rhANG), BSA or serum-free neurobasal medium (compare paragraph 2.7.4). RNA was extracted from cells, and RNA content was analysed on denaturing urea polyacrylamide gel. Angiogenin showed concentration-dependent RNA cleavage with 2 distinct bands at 34 nt and around 40 nt (indicated with arrows in Figure 5.3). These RNA cleavage pattern correlated with the previous findings in primary astrocyte cultures, where ANG treatment showed RNA cleavage products at ~34 nt and ~43 nt (Skorupa et al. 2012). Sequencing analysis of these bands, performed by Dr. Marion C. Hogg, identified new strongly angiogenin-regulated RNA fragments, including up-regulation of 5`Val and 3`Arg (data unpublished).

Figure 5.3: Recombinant human ANG cleaves RNA in a concentration-dependent manner. MZ-294 cells (2x10^6 cells/T75 flask) were treated for 6 h with indicated recombinant human angiogenin (rhANG) concentration, BSA or plain serum-free neurobasal (sf NB) medium. TRIzol was used to extract RNA from cells. Denaturing urea polyacrylamide gel was loaded with 10 µg RNA/well, and stained with SYBR Gold. ANG generated concentration dependent RNA cleavage products at 34 nt and around 40 nt (indicated with arrows).
5.2.2 tiRNA induced SG formation in MZ-294 cells

Considering MZ-294 cells showed similar properties during angiogenin uptake and RNA cleavage to primary astrocyte cultures, tiRNAs effect on SG formation was characterised in MZ-294

The experimental procedure was similar to chapter 4.2.2 (summarised in Figure 5.4). MZ-294 cells were transfected with different concentrations of tiRNAs. SG formation was then analysed by immunocytochemistry using a G3BP antibody to recognise SGs, and imaged by confocal microscopy. SG formation was quantified by the validated CellProfiler pipeline (in detail chapter 2.11.1).

Figure 5.4: tiRNAs characterisation of SG formation in MZ-294 cells.
Similar to the observation in SH-SY5Y cells, G3BP was distributed evenly in the cytosol in cells with normal proteostasis (Figure 5.5B, cyan arrows). Furthermore, tiRNA transfection induced aggregation of G3BP (white arrows in Figure 5.5B). Representative images illustrate the good detection of SGs by the CellProfiler pipeline in the G3BP channel (Figure 5.5B, red arrows). All tiRNAs induced SG formation in MZ-294 cells, and also Ctrl tiRNA showed SG positive cells. Ctrl tiRNA transfected MZ-294 cells had at 30x10^8 and 60x10^8 copies/cell the second highest score on SG positive cells. 5`Ala showed the strongest effect on SG formation at 30x10^8 and 60x10^8 copies/cell. At the highest concentration (90x10^8 copies/cell) 5` & 3` Asp showed the most SG positive cells. However, these results did not reach statistical significance. In summary, none of the tiRNAs showed a concentration-dependent SG formation, and there were also no differences between 5` and 3` tiRNA. Similar to the SG formation results in SH-SY5Y cells, the data suggest that SGs are not induced by tiRNAs in these models. The results contradict with previous publications from the Anderson laboratory, which showed that 5`tiRNAs with 5` terminal oligo guanine (TOG) motives have a concentration dependent, significant impact on SG formation in U2OS cells ((Emara et al. 2010), (Ivanov et al. 2011)).
Figure 5.5: Automated quantification of tiRNA transfected MZ-294 cells.
Different concentrations and tiRNAs were transfected with Lipofectamine for 5 h in MZ-294 cells to examine SG formation. SGs were measured by confocal microscopy using an anti-G3BP antibody. Different fields of view were imaged per cover slip with 200-400 cells/cover slip. tiRNA concentrations were carried out once per experiment. (A) SG formation was quantified using the automated image processing pipeline in CellProfiler. SGs positive cells were normalised to total cell number. Data are represented as mean ± SEM of three separate experiments. (B) Representative confocal images of either G3BP channel (in green), SGs identified by the pipeline (in red) and merged images with DAPI nuclear staining (in cyan) are shown. White arrows illustrate the overlap of SGs identified by the CellProfiler pipeline with observable SGs in the G3BP channel. Cyan arrows highlighted normal distributed G3BP, white arrows SG formations, and red arrows the overlap of SGs identified by the CellProfiler pipeline with observable SGs in the G3BP channel.
The inability to induce SG formation by tiRNAs in our models, lead us to examine the interaction of tiRNA with Y-box binding protein 1 (YB-1). It was shown previously that 5`-TOG-containing tiRNAs bind directly to YB-1 (Ivanov et al. 2011). YB-1 is involved in regulation of mRNA stability and translation (Lyabin et al. 2013). Furthermore, recent study (Lyons et al. 2016) showed that YB-1 is required for SG assembly. It was proposed that the process of SG formation occurs after stalled translation complexes were displaced by 5`-TOG-containing tiRNAs. 5`-TOG-containing tiRNAs bind to cold shock domain of YB-1 (Ivanov et al. 2014), which would lead to conformational changes that increase non-specific RNA binding and subsequently SG formation. To investigate whether tiRNA co-localised with YB-1, tiRNA were transfected into MZ-294 cells and imaged on a confocal microscope. YB-1 is not exclusively detectable in SGs, and it is also weakly measurable in processing bodies (PBs). Similar to SG formation, PBs are formed in response to stress, and mediate translational arrest (reviewed in (Eulalio, Behm-Ansmant & Izaurralde 2007), (Lloyd 2013)). It is difficult to distinguish PBs from SGs, as it has been shown that they interact with each other, and exchange protein components with surrounding cytoplasm (reviewed in (Anderson et al. 2015)). However, PBs are cytoplasmic RNA granules that are enriched in the factors involved in mRNA degradation, mRNA surveillance, translational repression and RNA-mediated gene silencing.

As we observed in tiRNA transfected SH-SY5Y cells, tiRNAs were not enriched in G3BP positive granules in MZ-294 cells (Figure 5.6, G3BP granules panel). Furthermore, G3BP positive granules showed co-localisation with YB-1. However, some YB-1 formed granules were G3BP negative, and showed enrichment of tiRNA (Figure 5.6, G3BP negative granules panel). This enrichment appeared to be sequence unspecific, as Ctrl tiRNA was also incorporated.

In conclusion, our data confirm that tiRNAs have an affinity for YB-1. But the lack of tiRNA enrichment in G3BP positive SG granule suggests that G3BP negative YB-1 granules are either early stage SGs, which release tiRNAs when G3BP bound the tiRNA-YB-1 complex, or they are PBs.
Figure 5.6: tiRNA is enriched in G3BP negative YB-1 granules. MZ-294 cells were transfected with 250 nM tiRNA or 30x10^8 copies/cell (in red) for 5 h with Lipofectamine and stained for G3BP (cyan) and YB-1 (in green). G3BP is a well-established SG marker, and YB-1 is detectable in PBs and SGs, with higher tendency towards SGs under stress conditions. Cell nuclei were highlighted by DNA stained with DAPI. G3BP granules panel showed representative confocal images of MZ-294 cells with G3BP positive granules. G3BP negative granules panel showed representative confocal images of MZ-294 cells with G3BP negative YB-1 granules.
5.2.3 Angiogenin and tiRNAs influence nascent protein synthesis in primary astrocytes cultures

5.2.3.1 Protocol validation for primary astrocyte preparation and culturing

As our results revealed that tiRNA did not induce SG formation, but were enriched in G3BP negative YB-1 granules, we decided to characterise the effect of tiRNAs on nascent protein synthesis. Furthermore, the translational effect of tiRNAs should be comparable to angiogenin treatment, as it has been shown to repress translation in U2OS cells (Emara et al. 2010). To examine whether the translation alteration of angiogenin mediated the paracrine protection in primary mixed motoneurons, primary astrocytes cultures from P1-P2 mice pups were selected as in vitro model.

To ensure high purity of primary astrocytes, preparation and culturing protocol was adopted from (Schildge et al. 2013). In brief, after pups were sacrificed by decapitation, cranium was opened and the brain was transferred into dissection dish. Brain was cut in two, the cortex hemispheres were dissected and the meninges from the cortex hemispheres were peeled away. Cortices were trypsinised, and the cell suspension was passed through a cell strainer. After a centrifugation step, cells were resuspended in culture medium and two cortices/T75 flask were plated. Culture medium was changed on the 2nd day in vitro (DIV), and then every 3rd day until the flask was confluent. To minimise contamination with microglia and oligodendrocyte precursor cells, culture flask was shaken vigorously by hand, and supernatant was discarded. Astrocytes were then trypsinised, and passaged in new flasks. Cells were passaged twice with a total of ~21 DIV to enrich astrocytes in the culture (for details chapter 2.2.3).

To investigate the astrocyte purity, 2x10^4 cells/well were seeded in 24-well plate. Medium was changed next day, and on the 5th day astrocyte purity was examined by immunocytochemistry. Cells were co-stained with antibodies against glial fibrillary acid protein (GFAP) and S100 calcium-binding protein B (S100b) to ensure full astrocyte spectrum. GFAP is an intermediate filament protein, which is a component of the cytoskeleton network that defines and maintains the shape of
astrocytes (reviewed in (Sofroniew & Vinters 2010)). S100b is a member of a multigenic family of Ca^{2+}-binding proteins with helix-loop-helix topology. S100b has been implicated in the regulation of both intracellular and extracellular activities (reviewed in (Marenholz et al. 2004)). Both proteins are prototypically used for immunocyto- & histochemical identification of astrocytes (reviewed in (Sofroniew & Vinters 2010)). Astrocyte purity was quantified by employing a custom designed CellProfiler pipeline (in detail chapter 2.11.3). Immunolabelling of primary mouse astrocyte cultures showed over 90% purity (Figure 5.7A). The adjusted purification and culturing protocol showed a robust procedure to obtain a high purity of astrocytes. Furthermore, the designed CellProfiler pipeline enables high throughput analysis to be processed efficiently.

![Figure 5.7: Purity of primary astrocyte culture.](image)

Primary astrocyte cells were seeded 2x10^4 cells/well in 24-well plate. Culture medium was changed on the 2nd day and on the 5th day cells were immunolabelled with the markers GFAP, and S100b. Cell nuclei were highlighted by DNA staining with DAPI. Six different fields of view were imaged per cover slip with 150-200 cells/cover slip. (A) Purity of primary astrocytes in the culture was quantified by custom designed CellProfiler pipeline. Data are represented as mean ± SEM of three cover slips. (B) Representative image of merged channels, GFAP/S100b (in orange) and DAPI (in cyan).
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5.2.3.2 SG are not assembled under stress conditions in primary astrocytes

To investigate whether paracrine neuroprotection of angiogenin in primary mixed motoneurons was mediated by translational changes in neighbouring cells, primary astrocytes were examined for SG formation ability. SG formation was firstly studied under established SG-inducing conditions (reviewed in (Kedersha & Anderson 2007)). Therefore, different concentration of sodium arsenite, different duration of osmotic shock and heat shock were applied to primary astrocytes. As the used G3BP antibody recognised only human G3BP, T-cell intracellular antigen-1 (TIA-1) was chosen as a SG marker. TIA-1 has been shown to form SG in vitro (Gilks et al. 2004), and tauopathies studies, using GFAP identified astrocyte in mouse tissue, showed SG formation with antibodies against TIA-1 (Vanderweyde et al. 2012). TIA-1 regulates alternative pre-RNA splicing in the nucleus and mediates translational silencing by binding to AU-rich elements located in mRNA 3’ untranslated regions in the cytosol (reviewed in (Matoulkova et al. 2012)). Primary astrocytes were identified by GFAP/S100b immunolabelling. As a positive control for SG formation MZ-294 cells were also treated with sodium arsenite, and were co-stained with antibodies against G3BP and TIA-1. TIA-1 was co-localised in G3BP positive SGs, and formed distinct cytoplasmic granules (Figure 5.8A). However, primary astrocytes did not form distinct granules under stress condition (Figure 5.8B-D). Moreover, under control conditions TIA-1 showed granulated distribution in the cytoplasm (white arrow Figure 5.8B). To obtain a more robust method, translation was measured by using Click-it® labelling technology from Life Technologies.
Results

Figure 5.8: TIA-1 does not aggregate under stress conditions in primary astrocytes. (A) MZ-294 cells were seeded 2x10^4 cells/well in 24-well plate. After cells were stressed with different sodium arsenite concentration (100 µM and 200 µM NaAsO_2) for 1 h. Cells were stained with antibodies against G3BP (in red) and TIA-1 (in green), both common used SG marker. Cell nuclei were highlighted by DNA staining with DAPI (in cyan). (B-D) Primary astrocyte cells were seeded 2x10^4 cells/well in 24-well plate. Culture medium was changed on the 2^{nd} day and on the 5^{th} day cells were treated differently to induce SG formation. (B) Cells were stressed with different concentration of sodium arsenite (50 µM; 300 µM; and 500 µM NaAsO_2) for 1h. (C) Osmotic shock was applied for different duration (1/2 h; 1 h; and 2 h) by changing medium to culture medium with 1 M sorbitol. (D) Cells were cultured for 1 h at 43°C to apply a heat shock. Primary astrocytes were co-immunolabelled with GFAP and S100b (in green) to identify astrocyte cells, TIA-1 (in orange) to investigate SG formation. Cell nuclei were highlighted by DNA staining with DAPI.
5.2.3.3 Translation alteration protocol validation

To measure nascent protein synthesis Click-iT® labelling technology was employed. MZ-294 cell were used to adjust AHA concentrations, because of their easy accessibility. $2 \times 10^4$ cells/well were seeded in 24-well plate, cells were washed 24 h later, and cultured for 1 h in amino acids (AA) free medium to deplete AA reserves. Different AHA concentrations were added to AA-free DMEM and cells were incubated for 1 h. To examine translation repression different cycloheximide (CHX) concentrations were added to AHA containing medium. CHX interrupts protein translation elongation through binding to the E site of the 60S ribosome (Schneider-Poetsch et al. 2010). MZ-294 cells were stained with antibody against G3BP to identify cell bodies, and to examine whether the AHA incubation in serum-free medium caused SG formation. Fluorescence intensities of nascent synthesised proteins were analysed with the program ImageJ (in detail chapter 2.6.1). In brief, cell outlines were defined in the G3BP channel with particles bigger 200 $\mu$m$^2$ and intensity higher 200 AU. Fluorescence intensities of nascent synthesised proteins were measured in the defined cell area. Various AHA concentrations showed a small difference in the translation signal (Figure 5.9A), therefore 50 $\mu$M AHA was chosen to examine the translation alteration in primary astrocytes. The most nascent synthesised proteins were located in the nucleus (white arrows in Figure 5.9B), rather than evenly distributed in the cell. This observation correlated with published fast protein turnover in the nucleus (<6 h) corresponding to ribosomal proteins (Boisvert et al. 2012). This suggests regulation of ribosomal proteins in the nucleus is a main control element in the cell. However, manually examined images showed no SG formation at any treatment. CHX at 50 $\mu$M showed already a very strong inhibition of translation in MZ-294 cells, and did not differed to 500 $\mu$M CHX with 500 $\mu$M AHA.
Results

Figure 5.9: 50 μM AHA incorporation showed sufficient nascent protein synthesis signal. MZ-294 cells were seeded 2x10^4 cells/well in 24-well plate. Cells were washed, and cultured for 1 h in amino acids (AA) free medium to deplete AA reserves. Different non-radioactive amino acid analog (L-Azidohomoalanine – AHA) concentrations were added to AA-free DMEM in the presence or absence of 50 and 500 μM CHX, and were incubated for 1 h. Chemoselective reaction modified AHA incorporated nascent proteins with an Alexa 488 fluorophore. Cells were immunolabelled with G3BP to identify cell bodies, and to examine whether the AHA incubation in serum-free medium caused SG formation. Cell nuclei were highlighted by DNA staining with DAPI. Five different fields of view were imaged per cover slip with 100-150 cells/cover slip. (A) Fluorescence intensities of nascent synthesised proteins were analysed with the program ImageJ. Data are represented as median ± SEM of all cell areas. (B) Representative images of nascent synthesised proteins (green) and merged channels of G3BP (in red) and DAPI (in cyan). White arrows highlight elevated levels of nascent synthesised proteins in the nucleus.

5.2.3.4 ANG enhances protein translation in primary astrocytes

To examine translation alteration in primary astrocytes, cells were purified and cultured using previously validated protocol. Primary astrocytes were seeded in 24-well plate (2x10^4 cells/well), and culture medium was changed next day. On the 5th day cells were washed, and cultured for 1 h in amino acids (AA) free medium to deplete AA reserves. Primary astrocytes were incubated with 50 μM AHA in the presence or absence of recombinant human angiogenin (100 ng/ml and 500 ng/ml rhANG), and controls BSA, and CHX (0.5 μM and 5 μM). The incubation period
was chosen to 6 h as our group showed previously the highest uptake of angiogenin within this period (Skorupa et al. 2012). Chemoselective reaction modified AHA incorporated nascent proteins with an Alexa 488 fluorophore. Cells were co-stained with antibodies against GFAP and S100b to cover as many astrocytes as possible, and against angiogenin to examine the angiogenin uptake (summarised in Figure 5.10). Fluorescence intensities of nascent synthesised proteins were quantified by customised CellProfiler pipeline (in detail chapter 2.11.2).

**Figure 5.10:** Translation alteration assessment in primary astrocytes.
Comparing the relative magnitude of nascent protein synthesis, both CHX treatments showed translation interruption in primary astrocytes. Angiogenin treatments enhanced significant translation in primary astrocytes compared to plain AHA incubation and BSA treatment (Figure 5.11A). Recent study revealed that angiogenin properties are cell type specific (Goncalves et al. 2016). Hence the increased translation in ANG treated primary astrocytes might be due to enhanced rRNA transcription. By examining translation signal distribution in primary astrocytes, nascent synthesised proteins were mainly located in the nucleus (Figure 5.11B, white arrows). Similar to MZ-294 observation, synthesis of nuclear proteins is probably the main regulation of environmental changes. Furthermore, immunolabelling of angiogenin showed it to be mainly restricted to cytoplasm of primary astrocytes (Figure 5.11B, cyan arrows). This observation confirms the previous findings, where angiogenin was taken up into the cytosol, and only a fraction of angiogenin was present in the nucleus (Skorupa et al. 2012).
Figure 5.11: Angiogenin enhances protein translation in primary astrocytes.

Primary astrocyte cells were seeded 2x10^4 cells/well in 24-well plate, and culture medium was changed next day. On the 5th day cells were washed, and cultured for 1 h in amino acids (AA) free medium to deplete AA reserves. Primary astrocytes were incubated with 50 µM non-radioactive amino acid analog (L-Azidohomoalanine – AHA) (baseline) in the presence or absence of recombinant human angiogenin (100 ng/ml and 500 ng/ml rhANG), and controls BSA, and CHX (0.5 µM and 5 µM) for 6 h. Chemoselective reaction modified AHA incorporated nascent proteins with an Alexa 488 fluorophore. Cells were co-stained with antibodies against GFAP and S100b to cover as many astrocytes as possible, and against angiogenin to examine the angiogenin uptake. Cell nuclei were highlighted by DNA staining with DAPI. Fluorescence intensities of nascent proteins were quantified in GFAP and S100b co-stained cells employing a customised CellProfiler pipeline. (A) Quantification of angiogenin influence on protein translation in primary astrocytes. Translation intensities were normalised to median fluorescence intensity of baseline condition of each experiment. Data represent single cell analysis (N=900-1200 cells) of three separate experiments with three cover slips/experiment. P values were calculated by one-way ANOVA pairwise comparison with Kruskal-Wallis post hoc test. (B) Representative images of nascent synthesised proteins (in green), angiogenin (in red), and merged channels of GFAP/S100b (in orange) and DAPI (in cyan). White arrows highlight elevated levels of nascent synthesised proteins in the nucleus. Cyan arrows illustrate angiogenin was mainly located in the cytosol.
5.2.3.5 Transfection optimisation in primary astrocytes

To optimise tiRNA transfection in primary astrocytes, Lipofectamine volume and tiRNA concentration were altered. The transfection duration was kept for 1 h to minimise Lipofectamine toxicity. Transfection protocol described by (Ivanov et al. 2011), was replicated and whereby it was shown that 750 nM (22.5x10^8 copies/cell) 5´Ala tiRNA showed 50% translation reduction in U2OS cells by (35S)-methionine incorporation. Cells were co-stained with antibodies against GFAP and S100b to cover as many astrocytes as possible, and TIA-1 to examine whether SG formation differed to stress induced SG formation in chapter 5.2.3.2.

All Lipofectamine volumes were tolerated by primary astrocytes, and showed no toxicity within 1 h transfection. tiRNA signal was proportional to Lipofectamine volume and tiRNA concentration. The brightest tiRNA signal showed transfection with 2.5 µl Lipofectamine and 750 nM (112.5x10^8 copies/cell) 5´Ala, and these conditions were chosen for the tiRNA translation alteration assessment. However, none of the 5´Ala transfection conditions induced clear TIA-1 granules. This suggests that either TIA-1 is not part of the SG clusters in primary astrocytes or purified primary astrocyte cultures do not regulate translation by SG formation.
Figure 5.12: Transfection optimisation in primary astrocytes. Primary astrocyte cells were seeded 2x10^4 cells/well in 24-well plate, and culture medium was changed next day. On the 5th day cells were transfected with different 5`Ala tiRNA concentrations and Lipofectamine volumes for 1 h. Cells were co-stained with antibodies against GFAP and S100b (in green) to cover as many astrocytes as possible, and TIA-1 (in orange) to examine SG formation. Representative images display tiRNA distribution in grey, and cell nuclei were highlighted by DNA staining with DAPI (in cyan).

5.2.3.6 tiRNA translation alteration assessment

To examine what impact tiRNAs had on translation in primary astrocytes, astrocytes from the same preparation as in section 5.2.3.4 were seeded 2x10^4 cells/well in 24-well plate, and culture medium was changed next day. On the 5th day cells were transfected with the optimised transfection conditions, 750 nM synthetic tiRNAs (112.5x10^8 copies/cell) employing 2.5 μl Lipofectamine for 1 h. The cells were washed, and cultured for 1 h in amino acids (AA) free medium to deplete AA reserves. Primary astrocytes were incubated with 50 μM AHA for 6 h to keep the protocol similar to angiogenin translation assessment. The chemoselective reaction modified AHA incorporated nascent proteins with an Alexa Fluor® 488 fluorophore and cells were co-stained with antibodies against GFAP and S100b to ensure full astrocyte spectrum. Fluorescence intensities of nascent synthesised proteins, and transfection efficiency were quantified by a
customised CellProfiler pipeline (in detail chapter 2.11.2). Relative magnitude of all tiRNAs except 5`Ala were similar compared to Ctrl tiRNA. 5`Ala showed nearly twice higher signal in primary astrocytes (Figure 5.13A), suggesting the secondary structure of 5`Ala was favoured either in Lipofectamine-tiRNA complex forming or in release from the complexes. However, tiRNA transfection did not alter the nascent synthesis protein distribution, as most of the newly synthesised proteins were located in the nucleus (Figure 5.13C, white arrows), in agreement with previous Click-iT experiment observations. Normalised magnitude of nascent protein synthesis of 5`Ala and 5` & 3`Gly displayed significantly repressed the protein translation compared to Ctrl tiRNA. 5` & 3`Val showed slightly reduced protein translation compared to Ctrl tiRNA. Whereas 5` & 3`Arg exhibited lightly increased protein translation compared to Ctrl tiRNA transfected primary astrocytes (Figure 5.13B). The fact that 5` and 3` tiRNAs repressed translation in primary astrocytes, contradict with the findings of the Anderson laboratory, where only 5` tiRNA showed an effect. Taking in account that 3`Arg is highly up-regulated in ANG exposed cells (chapter 3.2.3, and unpublished MZ-294 data), it is most likely that 3`tiRNA also fulfil a function in the host cell.
**Results**

Figure 5.13: 5’ Ala and 5’ & 3’ Gly repress protein translation in primary astrocytes. Primary astrocyte cells were seeded 2x10⁴ cells/well in 24-well plate, and culture medium was changed next day. On the 5th day cells were transfected with different tiRNAs (750 nM or 112.5x10⁶ copies/cell) or MOCK for 1 h. Transfected cells were subsequently cultured for 1 h in amino acids free medium, and were incubated with 50 µM non-radioactive amino acid analog (AHA) for 6 h. Chemoselective reaction modified AHA incorporated nascent synthesised proteins with an Alexa 488 fluorophore. MOCK, Ctrl tiRNA, 5’ Ala, and 5’ & 3’ Gly transfections were performed in three separate experiments with three cover slips/experiment (N=900-1200 cells), and 5’ &3’ Val and 5’ &3’ Arg in two separate experiments (N=500-700 cells). Fluorescence intensities of nascent proteins and ROX were quantified in GFAP and S100b co-stained cells employing a customised CellProfiler pipeline. (A) Quantification of tiRNA transfection efficiency in primary astrocytes. ROX intensities were normalised to median Ctrl tiRNA intensity of each experiment. (B) Quantification of tiRNA influence on protein translation in primary astrocytes. Data represent ROX intensity normalised protein translation of single cells compared relatively to median Ctrl tiRNA normalised protein translation of each experiment. P values were calculated by one-way ANOVA with Kruskal-Wallis post hoc test. (C) Representative images of nascent synthesised proteins (in green), tiRNA (in red), and merged channels of GFAP/S100b (in orange) and DAPI (in cyan). White arrows highlight elevated levels of nascent synthesised proteins in the nucleus.
5.3 Discussion

In previous chapters, angiogenin and tiRNA were characterised in human neuronal cell line and mouse primary mixed motoneuron models. As angiogenin is secreted by motoneurons and endocytosed by astroglial cells, our group proposed that the neuroprotection is mediated in paracrine (Skorupa et al. 2012). To get a broader perspective, angiogenin and tiRNA functions were further characterised in astrocyte cultures. Angiogenin treatment in MZ-294 cells showed a concentration-dependent RNA fragment generation. Sequencing of the cleavage products revealed novel, strongly angiogenin regulated RNA fragments, including 5`Val and 3`Arg tiRNA (data unpublished). Surprisingly, the repeatedly reported functional 5`-TOG-containing tiRNAs were not identified. However, in tiRNA-transfected MZ-294 cells none of the tiRNAs induced-concentration dependent SG formation, and there was also no difference between 5` and 3` tiRNA. Similar to the SG formation results in SH-SY5Y cells, these data suggest that SGs are not induced by tiRNAs in our models. These results are in disagreement with previous findings of the Anderson laboratory (Emara et al. 2010), (Ivanov et al. 2011). They reported that 750 nM (25x10^8 copies/cell) 5`Ala induced in 12.3% U2OS cells SG formation, whereas Ctrl tiRNA, the same sequence as we employed, showed <1% SG positive cells. This contradiction suggests that tiRNA effect on SG formation might be cell type specific.

The lack of induction of SG formation by tiRNAs in these models, led us to examine tiRNA co-localisation with YB-1. It was shown previously that 5`-TOG-containing tiRNAs bind directly to YB-1 (Ivanov et al. 2011). YB-1 is involved in regulation of mRNA stability and translation (Lyabin et al. 2013). Furthermore, recent study (Lyons et al. 2016) showed that YB-1 is required for SG assembling. It was proposed that the process of SG formation occurs after stalled translation complexes were displaced by 5`-TOG-containing tiRNAs. 5`-TOG-containing tiRNAs should bind to cold shock domain of YB-1, which would lead to conformational changes that increase non-specific RNA binding and subsequently SG formation. To investigate whether tiRNA co-localised with YB-1, tiRNA were
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transfected into MZ-294 cells and imaged on a confocal microscope. YB-1 is not exclusively detectable in SGs, and it is also weakly measurable in processing bodies (PBs). Similar to SG formation, PBs are formed in response to stress, and they mediate translational arrest (reviewed in (Eulalio, Behm-Ansmant & Izaurralde 2007), (Lloyd 2013)). It is difficult to distinguish PBs from SGs, as it has been shown that they interact with each other, and exchange protein components with surrounding cytoplasm (reviewed in (Anderson et al. 2015)). However, PBs are cytoplasmic RNA granules that are enriched in the factors involved in mRNA degradation, mRNA surveillance, translational repression and RNA-mediated gene silencing. As we observed in tiRNA transfected SH-SY5Y cells, tiRNAs were not enriched in G3BP positive granules in MZ-294 cells. Furthermore, G3BP positive granules showed co-localisation with YB-1. However, some YB-1 formed granules were G3BP negative, and showed enrichment of tiRNA. Our data confirm that tiRNAs have an affinity for YB-1. However, the lack of tiRNA enrichment in G3BP positive SG granule suggests that G3BP negative YB-1 granules are either PBs or early stage SGs, which release tiRNAs when G3BP bound the tiRNA-YB-1 complex. Additionally, sequence specificity had to be further investigated as the Ctrl tiRNA utilised was also incorporated in G3BP-negative YB-1 granules.

Primary astrocytes were chosen to investigate how angiogenin mediates paracrine neuroprotection in primary mixed motoneurons, as they are the main part of the population in primary mixed motoneurons cultures (Skorupa et al. 2012). Primary astrocyte purification and culturing protocol was validated, and displayed astrocyte purity of over 90 % in our primary astrocyte cultures. As angiogenin showed to induce SG formation (Emara et al. 2010), the ability to detect SG was examined in primary astrocytes. T-cell intracellular antigen-1 (TIA-1) was chosen as a SG marker. TIA-1 has been shown to form SG in vitro (Gilks et al. 2004), and tauopathy studies, using GFAP identified astrocyte in mouse tissue, showed SG formation with antibodies against TIA-1 (Vanderweyde et al. 2012). TIA-1 regulates alternative pre-RNA splicing in the nucleus and mediates translational silencing by binding to AU-rich elements located in mRNA 3’ untranslated regions.
Results

in the cytosol (reviewed in (Matoulkova et al. 2012)). However, various commonly used stresses to induce SG showed no SG formation in the primary astrocyte cultures. Therefore, angiogenin ability to influence translation was investigated in primary astrocytes. To measure nascent protein synthesis Click-iT® labelling technology was employed. Angiogenin treatments enhanced significant translation in primary astrocytes compared to plain AHA incubation and BSA treatment. This enhancement of translation by ANG was contrary to previous finding in Anderson Lab, where angiogenin showed translation repression though generation of tiRNAs in U2OS cells (Emara et al. 2010). Controversially, recent study demonstrated a dichotomously hematopoietic regeneration of stem and progenitor cells by ANG (Goncalves et al. 2016). Protein synthesis was up-regulated in angiogenin treated MyoPro cells, but down-regulated in angiogenin treated HSCs. Furthermore, this study showed differences in the RNA subtype regulation and proliferation. Angiogenin treatment showed in HSCs an up-regulation of tiRNA (5`Gly) generation, but rRNAs (47S and 28S) were not altered. In contrast, MyoPro cells treated with angiogenin showed increased 47S and 28S rRNAs transcription, but no generation of 5`Gly tiRNA. Cell cycle analysis showed angiogenin restricted cell proliferation in HSCs, but promoted proliferation of MyePro cells. When primary astrocytes were transfected with tiRNAs, 5`Ala and 5` & 3`Gly showed translation repression compared to Ctrl tiRNA. Furthermore, none of the tiRNAs showed significantly increased translation compared to Ctrl tiRNA, what suggests the increased protein translation in angiogenin treated astrocytes was not mediated by the examined single tiRNAs. However, angiogenin treated primary astrocytes (Skorupa et al. 2012) showed similar RNA cleavage pattern as stable SH-SY5Y cells (chapter 3.2.2.1), and angiogenin treated MZ-294 cells. This fact suggests that ANG might generate different tiRNAs or other RNA fragments, which mediate the protein translation enhancement. It was previously shown that ANG treatment down-regulated miR-542-3p expression in endothelial cells (He et al. 2015). Furthermore, it was observed that miR-542-3p regulate positively the transcription factor p53 (Wang et al. 2014). It is known that p53 suppresses Pol I & III activity (reviewed in (Ruggero & Pandolfi 2003)), which results in decreased rRNA
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generation and reduced protein translation. Therefore, we hypothesise that angiogenin treatment in primary astrocytes is associated with p53 suppression, which leads to increased rRNA generation, and results in enhanced protein translation.

In conclusion, ANG treated MZ-294 cells revealed a distinct RNA cleavage pattern, which provides evidence that angiogenin generates sequence specific RNA fragments. tiRNAs were enriched in G3BP-negative YB-1 granules, but lacked to induce SG formation in MZ-294 cells. Primary astrocytes treated with ANG showed enhanced protein translation, which was not mediated by single tiRNAs. These results suggest cell type-specific ANG functions, which are most likely associated with the generation of different RNA fragments.
6. Chapter 6: General discussion

6.1 Angiogenin generates specific tiRNAs

In previous work our group identified ANG as an ALS associated gene (Greenway et al. 2006). Since then, several studies have shown that mutations affecting the ribonucleolytic activity or the nuclear translocation of ANG cause ALS ((Wu et al. 2007), (Gellera et al. 2008), (Paubel et al. 2008)). ANG is expressed as a precursor protein with a signal peptide for secretion (Kurachi et al. 1985). Our group revealed that murine ANG1 mRNA and protein are up-regulated in motoneurons in response to hypoxia, and that hypoxia-inducible factor-1α (HIF-1α) is sufficient and required to up-regulate ANG expression during hypoxia (Sebastià et al. 2009). Furthermore, our group demonstrated that ANG over-expressing motoneuronal NSC34 cells secreted angiogenin during serum withdrawal (Skorupa et al. 2012). To investigate whether ALS related ANG missense mutation alter production and secretion of angiogenin in a human neuronal model, ANG overexpressing stable SH-SY5Y cell lines were generated. K40I ANG mutant was employed as a ribonucleolytic inactive control and R31K mutation was selected because the mutation affects the nuclear translocation sequence of ANG. However, in our experiments none of the angiogenin variants were localised in the nucleus. This observation was not surprising, since nuclear translocation of angiogenin is controversial ((Hu et al. 2000), (Tsuji et al. 2005), (Steidinger et al. 2011), (Skeie et al. 2011), (Skorupa et al. 2012)). Studies employing SH-SY5Y cells reported different angiogenin localisations ((Thiyagarajan et al. 2012), (Steidinger et al. 2013)). Since angiogenin was localised mainly in the cytosol in all the models we employed, we did not address this controversial topic experimentally. Under culturing conditions, we observed that Ang WT, K40I and R31K were distributed evenly through the cytoplasm as well as in punctuate structures at perinuclear and neurite regions. Granulated structures such as these have been shown to be associated with secretory granule packaging and regulated release of other secretory angiogenic factors such as VEGF and basic
fibroblast growth factor (bFGF) (Italiano et al. 2008). Under serum withdrawal conditions, stable SH-SY5Y released angiogenin into the media. Surprisingly, R31K cells displayed higher levels of angiogenin secretion than Ang WT and K40I. However, the loading control (here Col4a) was also increased in R31K, whereas, Ang WT and K40I had similar Col4a levels as the pcDNA control in their conditioned media. By comparing the intracellular angiogenin levels under normal and serum withdrawal condition, R31K cells showed a strong decrease of angiogenin levels under serum withdrawal, whereas, Ang WT and K40I displayed similar ANG ratio between both conditions. Taken together, these results suggest that increased secretion of angiogenin is associated with the R31K missense mutation. Until now, the impact of R31K mutation on protein secretion has not been reported. Using a SILAC approach our group showed previously that recombinant human angiogenin treatment modifies the secretome of primary astrocytes (Skorupa et al. 2013). Proteins residing in the extracellular compartment, proteins involved in multicellular-organismal development and proteins contributing to cell response to stress and extracellular stimuli were altered in astrocyte supernatants following 6 h recombinant human angiogenin treatment in serum deprived medium. However, in this study the ANG missense mutation impact on secretome alteration was not addressed. It would be interesting to investigate whether ANG missense mutations modulate the secretome of angiogenin producing and endocytosing cells.

It is known that angiogenin is a member of the pancreatic RNase A superfamily with 35% sequence homology to RNase A ((Strydom et al. 1985), (Kurachi et al. 1985)). However, angiogenin exhibits structural differences at the nucleotide binding site, which results in $10^{-5}$ to $10^{-6}$-fold reduced activity of RNase A ((Harper & Vallee 1989), (Lee & Vallee 1989a)) and divergence in the substrates cleaved ((Shapiro et al. 1986), (Shapiro et al. 1989), (Leland et al. 2002)). Unlike RNase A, which has no sequence specificity, our group and others reported that angiogenin generates a notable RNA cleavage pattern with distinct fragments of nucleotide lengths between 30 nt and 47 nt under stress conditions ((Skorupa et al. 2012),
We and others have demonstrated that these fragments are tRNAs cleaved in or near the anticodon loop ((Saikia et al. 2014), (Goncalves et al. 2016)). In agreement with these findings we observed that angiogenin cleaves RNA in a concentration-dependent manner in MZ-294 cells. Furthermore, we demonstrated that SH-SY5Y cells overexpressing ANG WT and R31K had increased RNA cleavage under serum deprivation. This result suggests that RNA cleavage also occurs in angiogenin producing cells. Although, all models showed distinct cleavage pattern, the size of the fragments were slightly different. This observation suggests variation in cleavage products, which are probably defined by cell-specific expression of RNA species.

By examining the RNA content of stable SH-SY5Y cell lines for specific tiRNAs, custom TaqMan assays revealed that ANG WT and R31K cells generated 5`Val, 5`Gly and 3`Arg tiRNAs. Surprisingly, the repeatedly reported functional 5`Ala ((Emara et al. 2010), (Ivanov et al. 2011)) was not detected in our system, indicating that 5`Ala is not generated by angiogenin in SH-SY5Y cells. Furthermore, unpublished sequencing data of angiogenin treated MZ-294 cells also did not identify the 5`- TOG-containing tRNA fragments Cys and Ala. These findings question the molecular basis behind biogenesis of those 5`- TOG-containing tRNA fragments and the biological relevance of the reported translation repression.

By correlating quantified RNA cleavage products to angiogenin protein levels in stable SH-SY5Y cells, we revealed R31K has enhanced ribonucleolytic activity. Additionally, R31K showed enhanced generation of 5`Gly tiRNAs compared to Ang WT. This finding is contradictory to the reported ribonucleolytic activity of R31K which was shown to be 91% activity of wild-type angiogenin (Thiyagarajan et al. 2012). However, previous publications investigated the ribonucleolytic activity of recombinant angiogenin variants towards purified yeast tRNA in a buffered system. This method examines the ribonucleolytic activity in an isolated environment with defined initial quantity of substrate and angiogenin, however, it does not include the cellular environment. Since our approach confirmed previous
cellular evaluated ribonucleolytic inactivity of K40I ((Thiyagarajan et al. 2012), (Skorupa et al. 2012)), we propose that R31K missense mutation enhances the ribonucleolytic activity of angiogenin. Increased ribonucleolytic activity was also reported on ANG R121H (Thiyagarajan et al. 2012), another missense mutation which was identified in ALS patients (Paubel et al. 2008). It would be interesting to investigate whether R31K and R121H share the revealed properties of increased angiogenin secretion and increased tiRNA generation. In a previous study, (Blanco et al. 2014) linked increased tiRNA generation with neuro-developmental disorders. The authors showed in that loss of cytosine-5 RNA methylation increases the angiogenin-mediated tiRNA generation of 5’ tiRNA fragments. 5’ tiRNA accumulation resulted in reduced cell size and increased apoptosis of cortical, hippocampal and striatal neurons in NSun2-deficient E18.5 embryos mice brains. This finding strengthens the assumption that RNA cleavage is tightly regulated and deviations are crucial for the organism.

Since angiogenin is secreted under stress conditions, we investigated whether tiRNAs are secreted as well and may function similar to miRNAs, which can be delivered to neighbouring cells where they regulate multiple targets. In fact, we identified specific tiRNAs in the supernatant of ANG WT and R31K cell lines under serum deprivation. These findings add new aspects to the documented loss-of-function mutation of ANG, and suggest a mechanism in which angiogenin mediates tiRNA generation and secretion in response to stress.

6.2 Protection of angiogenin is not mediated by a single tiRNA

Our group has reported previously that ribonucleolytic active angiogenin increases lifespan and delays deficits in motor function in SOD1^{G93A} ALS mouse model (Kieran et al. 2008). In this study, we demonstrated that angiogenin protected primary motoneurons against hypoxic stress. This finding was in line with previous observations that angiogenin treatment of primary ventral horn motoneuron cultures afforded neuroprotection against a broad spectrum of stresses, such as hypoxia (Sebastià et al. 2009), AMPA-induced excitotoxicity and tunicamycin-
induced ER-stress (Kieran et al. 2008). Since the ribonucleolytic inactive ANG K40I mutant failed to protect NSC-34 cells against tunicamycin-induced ER stress and serum-deprivation (Kieran et al. 2008), we investigated whether protection of angiogenin is mediated by specific tiRNAs.

In SH-SY5Y cells, we observed that 5`Val was protective against epoxomicin-induced proteasomal stress. This protection was specific to proteasomal stress, as 5`Val did not protect SH-SY5Y cells against thapsigargin-induced ER-stress. These results suggested that 5`Val is not a general protection factor but rather interacts specifically with an epoxomicin-stress-associated cell death pathway. In previous investigations, our group demonstrated that SH-SY5Y cells exposed to epoxomicin had impaired protein degradation, and subsequently triggered caspase-3 activation and programmed cell death (Concannon et al. 2007). Proteasome inhibition by epoxomicin was associated with the induction of the proapoptotic BH3-only family members, including PUMA and Bim, through the activation of the transcription factor p53. In contrast, thapsigargin induced apoptosis has been shown to be p53 independent (Reimertz et al. 2003). Furthermore, angiogenin has been shown to inhibit p53 expression and subsequently mediate anti-apoptosis and cell survival in HEK 293 cells (Sadagopan et al. 2012). 5`Val may protect cells against epoxomicin stress via direct or indirect repression of p53.

In primary motoneurons none of the examined tiRNAs showed protection against hypoxic stress. However, protection against hypoxia might require a combination of different tiRNA in primary motoneurons. The fact that both angiogenin and tiRNA fragments are secreted makes the identification of the specific RNA fragment composition, which mediates protection difficult.

### 6.3 tiRNAs induce specific granule formation

A number of reports have revealed that tRNA-derived small RNAs are involved in various biological processes. The Anderson laboratory illustrated that phosphorylated TOG 5`tiRNAs, in particular 5`Ala and 5`Cys, induce SG formation.
and inhibit cap-dependent protein synthesis by interfering with the assembly of the cap binding complex eIF4F ((Yamasaki et al. 2009), (Emara et al. 2010), (Ivanov et al. 2011)). Several studies reported that translation repression was associated with SG formation, however, it is unclear which event occurs first ((McEwen et al. 2005), (Reineke et al. 2012), (Yamamoto & Izawa 2013), (Liem & Liu 2016)). The Anderson laboratory demonstrated that tiRNAs suppress cap-dependent protein translation which is used by 95–97% of the total cellular mRNAs (reviewed in (Merrick 2004)), but not IRES-mediated translation, which is often employed by pro-survival and anti-apoptotic genes during stress ((King et al. 2010), (Komar & Hatzoglou 2011)). Therefore, it was hypothesised that translation repression and SG formation are part of a protection mechanism adopted by cells to reduce energy expenditure and activate a cytoprotective stress response pathway (reviewed in (Anderson & Ivanov 2014), (Li & Hu 2012)). These findings caused us to investigate whether SG formation and translation repression were the mechanism by which angiogenin mediates protection. Experimental procedures were adapted from (Emara et al. 2010), (Ivanov et al. 2011) to ensure similar conditions. To investigate whether the ribonucleolytic activity of angiogenin or specific tiRNAs induce or enhance SG formation, a custom designed CellProfiler pipeline was established as a valuable tool for high throughput analysis. However, none of the single tiRNAs induced SG formation in either SH-SY5Y or MZ-294 cells. Furthermore, we investigated SG formation in stable SH-SY5Y cells and revealed that only under high sodium arsenite stress Ang WT cells showed enhanced SG formation. Under moderate sodium arsenite conditions the ribonucleolytic activity did not influence SG formation and angiogenin overexpressing cell lines had in general less SG positive cells. These findings contradict to Anderson laboratory investigations in U2OS cells, but are in line with previous observation in SH-SY5Y cells from (Thiyagarajan et al. 2012). These contradictory results suggest a cell type specific effect of angiogenin and lead to the impression that tiRNA generation in neuronal cells may not act on the cells them self. tiRNA are rather secreted as a “help” signal and may stimulate immune response.
Since none of the tiRNAs induce SG formation in our models, we investigated whether the reported interaction of tiRNA with YB-1 is present in our system. The Anderson laboratory has shown that 5’- TOG-containing tiRNAs bind directly to YB-1 and that YB-1 is required for SG assembly ((Ivanov et al. 2011), (Lyons et al. 2016)). They hypothesised that SGs are formed as a consequence of stalled protein translation. In the first step, 5’-TOG-containing tiRNAs would displace the translation initiation factor eIF4F. Next, tiRNAs would bind to cold shock domain of YB-1, leading to conformational changes that increase non-specific RNA binding and aggregation of prone-domain-containing proteins such as G3BP and TIA1/R. Another theory is driven by the fact that YB-1 regulates mRNA stability and translation through binding 5’ and 3’ UTRs (reviewed in (Lyabin et al. 2013)). (Goodarzi et al. 2015) demonstrated in breast cancer cells that CU box motif containing tiRNAs, in particular fragments of Glu$^YTC$, Asp$^GTC$ and Gly$^TTC$, interact with YB-1 and compete with the binding to 5’ and 3’ UTRs. As a result mRNA transcripts are destabilised and subsequently degraded. In the current study we confirmed that tiRNAs co-localise with YB-1, however, tiRNAs were only enriched in G3BP negative YB-1 granules. It is known that YB-1 is not exclusively detectable in SGs, and it is also weakly measurable in PBs (reviewed in (Anderson et al. 2015)). Since all G3BP granules were YB-1 positive but not the opposite, we hypothesise that G3BP negative YB-1 granules are either early stage SGs, which release tiRNAs when G3BP bound the tiRNA-YB-1 complex, or they are PBs. This finding suggests that tiRNAs induce only specific granule formation in our models. Identification of a specific marker which determines G3BP negative YB-1 granules or redesign of the CellProfiler pipeline to exclude G3BP positive granules would be necessary to investigate whether specific tiRNAs have an impact on those granules. Furthermore, it would be interesting to examine whether tiRNAs regulate YB-1-dependent protein translation without forming SG as reported by the Anderson laboratory.
6.4 Angiogenin-regulated protein translation

In previous work our group demonstrated that angiogenin is secreted by motoneurons and endocytosed by astroglial cells (Skorupa et al. 2012). Furthermore, we reveal in this work that angiogenin expressing cells also generate specific tiRNAs and that these tiRNAs are secreted. These results strengthened the hypothesis that the neuroprotection of motoneurons is mediated by neighbouring cells. To investigate whether paracrine neuroprotection of angiogenin in primary mixed motoneurons was mediated by translational changes in neighbouring cells, primary astrocytes cultures from P1/2 mice pups were employed as an in vitro model. Since various commonly used stresses to induce SG showed no SG formation in the primary astrocyte cultures, we characterised the effect of angiogenin and tiRNAs on nascent protein synthesis by employing the Click-iT® labelling technology. Interestingly, angiogenin treatments significantly enhanced translation in primary astrocytes. Although translation enhancement by angiogenin is contradictory to the Anderson laboratory findings, a series of studies have highlighted that angiogenin promote the transcription of 47S pre-rRNA & 45S rRNA ((Shapiro et al. 1986), (Xu et al. 2002), (Sheng et al. 2014)). Furthermore, (Tsuji et al. 2005) demonstrated direct correlation between ANG mediated increased rRNA transcription and cell proliferation. A recent study addressed dual roles of ANG in the hematopoietic system. (Goncalves et al. 2016) showed that protein synthesis was up-regulated in angiogenin treated MyoPro cells, but down-regulated in angiogenin treated HSCs. Angiogenin treated HSCs displayed RNA cleavage such as 5`Gly generation, whereas rRNA levels were not changed. In contrast, angiogenin treated MyePro cells exhibited increased 47S and 28S rRNA levels, but no RNA cleavage products had been observed. Similar contrasting responses were reported for angiogenin effects on proliferation in these cell types. Angiogenin treated HSCs showed cell quiescence, whereas it promoted proliferation of MyePro cells. The authors proposed that angiogenin has a cell-type-specific role, promoting quiescence by repressing translation in hematopoietic stem cells, and stimulating proliferation in differentiated cells. Although, this study
suggest that tiRNA generation represses protein translation, an investigation in sex hormone-dependent breast and prostate cancers revealed proliferation enhancement mediated by angiogenin-generated tRNA fragments (Honda et al. 2015).

However, in our experimental set-up none of the tiRNAs showed increased translation compared to Ctrl tiRNA. On the contrary, 5′Ala and 5′ & 3′Gly repressed protein translation in primary astrocytes. This suggests the increased protein translation in angiogenin treated astrocytes was not mediated by the examined single tiRNAs. Angiogenin treated primary astrocytes (Skorupa et al. 2012) showed slight variation in the RNA cleavage pattern compared to stable SH-SY5Y cells and angiogenin treated MZ-294 cells. This difference suggests that angiogenin might generate different RNA fragments, which mediate the protein translation enhancement. A possible mechanism might be mediated by p53 inhibition. It has been reported that Pol I & III activity can be inhibited by p53 (reviewed in (Ruggero & Pandolfi 2003)), leading to reduction of rRNA levels and ribosome generation, and consequently decreased protein translation. Several studies reported a regulation of p53 through angiogenin. (Sadagopan et al. 2012) demonstrated that silencing endogenous ANG resulted in increased p53 expression in HEK 293 cells. While (Wang et al. 2014) identified miR-542-3p to increase p53 expression in a high-throughput screen of U2OS cells, which itself had reduced expression in angiogenin treated endothelial cells (He et al. 2015). Therefore it would be interesting to determine angiogenin -mediated RNA cleavage products and investigate whether enhanced protein translation is mediated by increased rRNAs production.
6.5 Future Outlook

While this body of work revealed a number of new insights of angiogenin related ALS pathology, it also raises a few interesting questions such as the role of the R31K mutation in modulating the secretome. To investigate this further the secretion pathway should be explored with the focus on ANG expressing cells. By employing the ANG R121H mutant we could examine whether the increased secretion is caused by enhanced ribonucleolytic activity of angiogenin or whether it is specific to R31K missense mutation. Access to ALS patient tissue would permit the ability to investigate this question in a patient derived induced pluripotent stem cell approach and avoid plasmid-based protein overexpression.

Dual roles of angiogenin have been reported in growth and stress. It would be very interesting to determine the RNA cleavage composition of the separated model by a sequencing approach, and investigate whether a cell type specific combination of different RNA fragments would have the same effect in these models.

It would be intriguing to examine whether tiRNAs regulate YB-1-dependent gene expression without classical SG formation. By focusing on YB-1 targeted transcripts, such as EIF4G1, ITGB4, AKT1, and ADAM8, we could verify whether tiRNAs are another form of post-transcriptional regulation.

It would be also interesting to investigate whether enhanced protein translation is mediated by increased rRNA production. Furthermore, by employing p53 deficient cells we could determine whether regulation of p53 through angiogenin is the mechanism of action behind its protein translation enhancement.
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