MEDIZINISCHE UNIVERSITÄT
INNSBRUCK

July 2015-December 2016

# MUI-START Report

Medical University of Innsbruck

July 2015-December 2016

# Inhalt

# 1 Background and aim of the programme

MUI-START is the follow-up programme of the MFI (Medizinische Forschungsfonds Innsbruck) that ended in 2011.

The MUI-START programme is devised as a start-up fund for young scientists. The aim of the programme is to offer young scientists the opportunity of developing new project ideas, within the MUI research focuses, that could serve as basis for a subsequent successful application for external funding (e. g. FWF, ÖNB).

According to the present guidelines, eligible candidates must: 1) have a working contract with the Medical University of Innsbruck, 2) have completed their doctoral studies, and 3) not be older than 35 years by the application deadline. Fully justified career breaks can be taken into account (e. g. parental leave). Professors and PIs of third-party funded (FWF; OeNB, FFG and EU) projects are not eligible. Applicants' track record must be commensurate with their academic age. However, two peer-reviewed international publications as first author are compulsory**.**

The guidelines of the program have been substantially modified along the years to adapt the program to the high standards applied by external funding agencies (e. g. FWF). Since 2016 proposals undergo a three-step evaluation procedure: 1) Selection of proposals by the MUI-START jury, 2) International peer-review of the pre-selected proposals, and 3) Hearing of the shortlisted applicants by the MUI-START Jury. Final decisions are based on the reviewers' scores as well as on the outcome of the interviews.

Moreover, since the 7$^{th}$ call (2016) the submission of an external funding proposal before end of the funding period has become compulsory for all MUI-START grantees. Failure to submit such an application translates into the cancellation of the payment of last quarter of the MUI-START grant budget.

# 2 Overview on MUI-START calls

The first MUI-START call was announced in the summer 2010 and supported 42% of the submitted proposals. Since then, the approval rates have been oscillating from year to year (Table 1) depending on both the available budget and the quality of the submitted proposals.

Table 1. Overview on all MUI-START calls

| Call | Proposals submitted | Proposals granted (Male/Female) | Funding rate | Total funding requested | Total funding granted |
|---|---|---|---|---|---|
| **1. Call** | 31 | 13 (7M/6F) | 42% | € 2.074.365,7 | € 667.054,80 |
| **2.Call** | 11 | 5 (2M/3F) | 45% | € 629.968,95 | € 173.171,00 |

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|---|---|---|---|---|---|
| **3. Call** | 29 | 9 (4M/5F) | 31% | € 742.808,21 | € 240.000,00 |
| **4.Call** | 28 | 14(11M/3F) | 50% | € 713.652,93 | € 323.484,66 |
| **5. Call** | 31 | 12(4M/8F) | 39% | € 771.750,48 | € 260.826.60 |
| **6. Call** | 28 | 8 (4M/4F) | 28% | € 711.035,41 | € 176.726,00 |
| **7. Call** | 9 | 3 (1M/2F) | 33% | € 248.945,01 | € 85.000,00 |
| **8. Call** | 15 | 7 (5M/2F) | 47% | € 365.189,29 | € 162.208,80 |

## 3 MUI-START jury members and reviewers

The MUI START jury members are Professors and associate Professors of the Medical University of Innsbruck working in both basic and clinical research topics. The jury members are chosen according to their proven expertise in a specific research field. The composition of the Jury is not fixed, but changes as a result of the variety of topics covered by the proposals submitted to a particular call.

The following jury members helped in the selection of the projects presented in this report (4th and 5th MUI-START Call). Their help and commitment is warmly acknowledged.

| | |
|---|---|
| Univ.-Prof. Dr. Gottfried BAIER | Sektion für Zellgenetik |
| Univ.-Prof. Dr. Christine BANDTLOW | Sektion für Neurobiochemie |
| Univ.-Prof. Dr. Georg DECHANT | Gemeinsame Einrichtung für Neurowissenschaften |
| Univ.-Prof. Dr. Francesco FERRAGUTI | Institut für Pharmakologie |
| Univ.-Prof. Dr. Ludger HENGST | Sektion für Medizinische Biochemie |
| o. Univ.-Prof. Dr. Werner JASCHKE | Universitätsklinik für Radiologie |
| Univ.-Prof. Dr. Michaela KRESS | Sektion für Physiologie |
| Ao. Univ.-Prof. Dr. Alexandra LUSSER | Sektion für Molekularbiologie |
| Univ.-Prof. Dr. Gert MAYER | Universitätsklinik Innere Medizin IV |
| Univ.-Prof. Dr. Matthias SCHMUTH | Universitätsklinik für Dermatologie und Venerologie |
| Univ.-Prof. Dr. Erich SCHMUTZHARD | Universitätsklinik für Neurologie |
| Ao. Univ.-Prof. Dr. Michael SCHOCKE | Universitätsklinik für Radiologie |

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|---|---|
| Univ.-Prof. Dr. Günther SPERK | Institut für Pharmakologie |
| Priv.-Doz. Dr. Patrizia STOITZNER | Universitätsklinik für Dermatologie |
| Ao. Univ.-Prof. Dr. Günter WEISS | Universitätsklinik Innere Medizin I |
| Ao. Univ.-Prof. Dr. Johann WILLEIT | Universitätsklinik für Neurologie |
| Univ.-Prof. Dr. Johannes ZSCHOCKE | Sektion für Humangenetik |

The tasks of the jury members comprise: 1) internal review of the proposals, 2) nomination of the reviewers at the suggestion of the Research Office (SCF), and 3) presentation of proposals during the decision meeting.

The reviewers of the MUI-START Projects are international experts active in their field of research. At least two reviews per proposal are necessary to help the jury members to take a decision.

## 4 MUI-START symposium and project evaluation

By accepting the MUI-START grant, the grant recipients commit themselves to take part in the annual MUI-START Symposium organized by the MUI Research Office (SCF). This event represents also an ideal occasion for the MUI-START jury members, which were involved in the selection of the projects, to assess the progress achieved by the grant holders in their respective projects. Additionally, the symposium is expected to have a mentoring effect on the grant holders who profit from the expertise and good advice of the jury members.

At present, the symposium is organized following a format in which poster presentations illustrate the progress in projects that are half way through the funding period. The PIs of projects close to their end-phase or recently completed present their results during an oral presentation.

During the 4$^{th}$ and 5$^{th}$ MUI-START Symposium a total of 26 projects of the 4$^{th}$, 5$^{th}$ and 6$^{th}$ funding period were evaluated. The results of this evaluation are summarized in Fig. 1

In more than 80% of the cases the project development and the PI qualifications were considered as good or excellent. The same trend was observed in the assessment of the research environment. However, despite these positive results the jury members judged that only two thirds of the projects could translate into an external funding application. This discrepancy is due to the fact that several PIs quit the MUI earlier than expected or immediately after the end of the funding period. In few other cases the jury members thought that it will be difficult to find a suited funding program to submit a proposal for external funding.

Links: 4$^{th}$ MUI-START Symposium Programme
5$^{th}$ MUI-START Symposium Programme

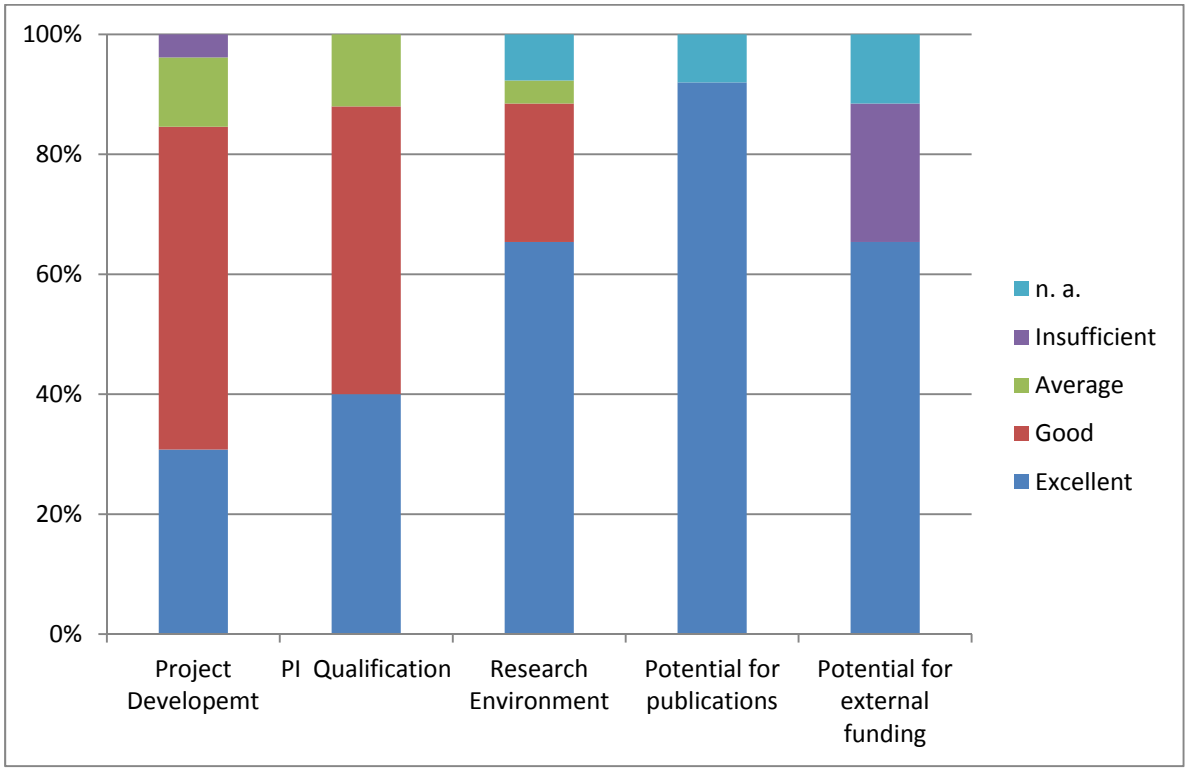


Fig. 1. Results of the evaluation of projects during the MUI-START Symposium. n.a. no answer.

## 5 External funding granted to MUI-START grant recipients

As stated in the first section of this report the aim of the programme is to help young scientists to develop new project ideas that could serve as basis for a subsequent application for third-party funding.

So far (Stand 31.12.2016) 48 MUI-START funded projects are closed. Nine PIs quit the MUI either before the planed end or immediately after the end of the project. Approximately 71 % (28 PIs) of the remaining PIs applied for external funding. Given the stringency of the current funding landscape not all applications could translate into funded projects. Table 2 and Table 3 provide an overview on the funds acquired by the MUI-START grantees to date.

Table 2. FWF and ÖNB funded projects acquired by former MUI-START grant holders.

| Applicant | Project Title | Funding Agency-Project number | Duration (Months) | Funds granted |
|---|---|---|---|---|
| Natasha Kleiter | Orphan receptor NR2F6 as a negative regulator of T cell effector functions | FWF – P23537 | 36 | € 255.858,75 |
| Natasha Kleiter | NR2F6 governs immune defense against microbial pathogens | FWF-P 28694 | 36 | € 317.627,10 |
| Galina Apostolova | Role of genome organizer | FWF - P25014 | 36 | € 299.817,00 |

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|---|---|---|---|---|
| | Satb2 in adult brain function | | | |
| Birgit Frauscher | Motor activity during sleep in health and disease | FWF – KLI236 | 36 | € 203.609,70 |
| Birgit Frauscher | REM sleep without atonia: early sign of neurodegeneration | ÖNB - 15127 | 30 | € 90.000 |
| Martin Puhr | Functional significance of PIAS1 and BIRC5 in docetaxel resistant prostate cancer | FWF – P25639 | 30 | € 337.233,75 |
| Markus Theurl | Catestatin for the treatment of myocardial ischemia | FWF – P26251 | 24 | € 262.731,00 |
| Rupert Oberhuber | Evaluierung der Leberorganqualität vor Transplantation | ÖNB-17287 | 36 | € 110.000,00 |
| Manfred Nairz | Die Rolle von Innate Response Activator B Zellen bei Sepsis | FWF - J3486 | 24 | € 69.700,00 |
| James Wood | Dopamine and NPY signaling in a fear extinction circuit | FWF - M1783 | 24 | € 157.380,00 |
| Sebastian Herzog | Molecular regulation of the oncogenic miR-17-92 cluster | FWF P 30194 | 48 | € 297.304,89 |
| Sebastian Herzog | Unraveling miR-15 function in health and disease | FWF P 30196 | 42 | € 364.019,25 |
| Ramon Tasan | Role of the neurokinin B-expressing neurons in the bed nucleus of the stria terminalis | FWF-P 29952 | 36 | € 399.441,00 |

Table 3. Additional funding acquired by former MUI-START grant holders.

| Applicant | Project Title | 3 | Duration (Months) | Funds granted |
|---|---|---|---|---|
| Wegene Borena | Genital HPV infection among HIV - positive men in West Austria in the Austrian HIV Cohort Study | MFF – Nr.262 | 18 | € 13.728,00 |
| Selma Tuzlak | Bcl-2 family | ÖAW - 23949 | 36 | € 105.000,00 |
| Christian Ploner | MADI | Land Tirol | 51 | € 73.466,98 |
| Peter Lackner | The role of voltage gated Ca channels for neuroprotection in experimental subarachnoid hemorrhage | Land Tirol | 24 | € 75.400,00 |
| Rupert Oberhuber | Tetrahydrobiopterin as novel therapeutic strategy to improve outcome after the transplantation of organs from brain death donors | TWF-2013-1-17 | 24 | € 20.000,00 |
| Sebastian Herzog | Systematic analysis of the miR-26 family in lymphocyte | TWF-2014-1-17 | 24 | € 26.500,00 |

| | | | | |
|---|---|---|---|---|
| | development and cancer | | | |
| Michael Blatzer | Alternative regulatory circuits of secondary metabolite production in *Aspergillus terreus* | TWF-2016-1-1 | 24 | € 39.500,00 |
| Beno Cardini | The impact of simvastatin on the ischemia reperfusion injury in the murine heart transplantation model | TWF-2016-1-6 | 18 | € 32.069, 00 |
| Martin Bodner | Helena's many daughters - Massively parallel sequencing provides highest-resolution insights into the most common West Eurasian mtDNA control region haplotype | TWF-2016-1-2 | 24 | € 30.122,00 |
| Martin Bodner | Helena's many daughters: massively parallel sequencing provides further insights into the most common West Eurasian mtDNA control region haplotypre at the highest resolution | DSF-2015-1-1 | 12 | € 5.000,00 |
| Lourdes Rocamora-Reverte | Role of Glucocorticoides on B cell development and function | TWF-2016-1-23 | 24 | € 37.390,00 |
| Luca Fava | How do cells count their centrosomes? A mechanistic study | Armenise-Harvard Foundation | 60 | $1.000.000,0 |

## 6 Publications acknowledging the MUI-START programme

**This section lists publications issued from the projects with end date July 2015 and onwards. A list of publications of projects that finished before that time frame can be found in the former MUI-START report (January 2013- June 2015).**

Bodner, Martin; Iuvaro, Alessandra; Strobl, Christina; Nagl, Simone; Huber, Gabriela; Pelotti, Susi et al. (2015): Helena, the hidden beauty: Resolving the most common West Eurasian mtDNA control region haplotype by massively parallel sequencing an Italian population sample. In: *Special Issue: DNA in Forensics 2014* 15, S. 21–26. DOI: 10.1016/j.fsigen.2014.09.012.

Fava, Luca L.; Schuler, Fabian; Sladky, Valentina; Haschka, Manuel D.; Soratroi, Claudia; Eiterer, Lisa et al. (2016): The PIDDosome activates p53 in response to supernumerary centrosomes. In: *Genes & Development* 31 (1), S. 34–45. DOI: 10.1101/gad.289728.116.

Gomes, Sibylle M.; Bodner, Martin; Souto, Luis; Zimmermann, Bettina; Huber, Gabriela; Strobl, Christina et al. (2015): Human settlement history between Sunda and Sahul: a focus on East Timor (Timor-Leste) and the Pleistocenic mtDNA diversity. In: *BMC Genomics* 16 (1), S. 70. DOI: 10.1186/s12864-014-1201-x.

Gomes, Sibylle M.; van Oven, Mannis; Souto, Luis; Morreira, Helena; Brauer, Silke; Bodner, Martin et al. (2017): Lack of gene-language correlation due to reciprocal female but directional male admixture in Austronesians and non-Austronesians of East Timor. In: *Eur J Hum Genet* 25 (2), S. 246–252. Online verfügbar unter http://dx.doi.org/10.1038/ejhg.2016.101.

Gostner, Johanna M.; Zeisler, Johannes; Alam, Mohammad Tauqeer; Gruber, Peter; Fuchs, Dietmar; Becker, Kathrin et al. (2016): Cellular reactions to long-term volatile organic compound (VOC) exposures. In: *Scientific Reports* 6, S. 37842. DOI: 10.1038/srep37842.

Just, Rebecca S.; Scheible, Melissa K.; Fast, Spence A.; Sturk-Andreaggi, Kimberly; Röck, Alexander W.; Bush, Jocelyn M. et al. (2015): Full mtGenome reference data: Development and characterization of 588 forensic-quality haplotypes representing three U.S. populations. In: *Forensic Science International: Genetics* 14, S. 141–155. DOI: 10.1016/j.fsigen.2014.09.021.

Knackmuss, Ulla.; Lindner, Silke E.; Aneichyk, Tanya.; Kotkamp, Bianka.; Knust, Zeynep.; Villunger, Andreas.; Herzog, Sebastian. (2015): MAP3K11 is a tumor suppressor targeted by the oncomiR miR-125b in early B cells. In: *Cell Death and Differentiation* 23 (2), S. 242–252. DOI: 10.1038/cdd.2015.87.

Müller, Martin; Schmidt, Oliver; Angelova, Mihaela; Faserl, Klaus; Weys, Sabine; Kremser, Leopold et al. (2015): The coordinated action of the MVB pathway and autophagy ensures cell survival during starvation. In: *eLife* 4, S. e07736. DOI: 10.7554/eLife.07736.

Pfeifhofer-Obermair, Christa; Albrecht-Schgoer, Karin; Peer, Sebastian; Nairz, Manfred; Siegmund, Kerstin; Klepsch, Victoria et al. (2016): Role of PKCtheta in macrophage-mediated immune response to Salmonella typhimurium infection in mice. In: *Cell Communication and Signaling : CCS* 14, S. 14. DOI: 10.1186/s12964-016-0137-y.

Siegmund, Kerstin; Thuille, Nikolaus; Posch, Nina; Fresser, Friedrich; Baier, Gottfried (2015): Novel Protein kinase C θ: Coronin 1A complex in T lymphocytes. In: *Cell Communication and Signaling : CCS* 13, S. 22. DOI: 10.1186/s12964-015-0100-3.

**Publications acknowledging the MUI-START programme that were not listed in the former MUI-START Report (January 2013- June 2015)**

Dietl, Anna-Maria; Amich, Jorge; Leal, Sixto; Beckmann, Nicola; Binder, Ulrike; Beilhack, Andreas et al. (2016): Histidine biosynthesis plays a crucial role in metal homeostasis and virulence of Aspergillus fumigatus. In: *Virulence* 7 (4), S. 465–476. DOI: 10.1080/21505594.2016.1146848.

Fischer, Natalie; Mathonia, Nina Maria; Hoellerich, Georges; Veser, Julian; Pinggera, Leyla; Dejaco, Daniel et al. (2017): Surviving murine experimental sepsis affects the function and morphology of the inner ear. In: *Biology Open* 6 (6), S. 732–740. DOI: 10.1242/bio.024588.

Hagenbuchner, Judith; Lungkofler, Lorena; Kiechl-Kohlendorfer, Ursula; Viola, Giampietro; Ferlin, Maria Grazia; Ausserlechner, Michael J.; Obexer, Petra (2017): The tubulin inhibitor MG-2477 induces autophagy-regulated cell death, ROS accumulation and activation of FOXO3 in neuroblastoma. In: *Oncotarget* 8 (19), S. 32009–32026. DOI: 10.18632/oncotarget.16434.

Hagenbuchner, Judith; Rupp, Martina; Salvador, Christina; Meister, Bernhard; Kiechl-Kohlendorfer, Ursula; Müller, Thomas et al. (2016): Nuclear FOXO3 predicts adverse clinical outcome and promotes tumor angiogenesis in neuroblastoma. In: *Oncotarget* 7 (47), S. 77591–77606. DOI: 10.18632/oncotarget.12728.

Jaitner, Clemens; Reddy, Chethan; Abentung, Andreas; Whittle, Nigel; Rieder, Dietmar; Delekate, Andrea et al. (2016): Satb2 determines miRNA expression and long-term memory in the adult central nervous system. In: *eLife* 5, S. e17361. DOI: 10.7554/eLife.17361.

Nairz, Manfred; Ferring-Appel, Dunja; Casarrubea, Daniela; Sonnweber, Thomas; Viatte, Lydie; Schroll, Andrea et al. (2015): IRON REGULATORY PROTEINS MEDIATE HOST RESISTANCE TO SALMONELLA INFECTION. In: *Cell host & microbe* 18 (2), S. 254–261. DOI: 10.1016/j.chom.2015.06.017.

Nairz, Manfred; Schroll, Andrea; Haschka, David; Dichtl, Stefanie; Sonnweber, Thomas; Theurl, Igor et al. (2015): Lipocalin-2 ensures host defense against Salmonella Typhimurium by controlling macrophage iron homeostasis and immune response. In: *European journal of immunology* 45 (11), S. 3073–3086. DOI: 10.1002/eji.201545569.

Nairz, Manfred; Theurl, Igor; Wolf, Dominik; Weiss, Günter (2016): Iron deficiency or anemia of inflammation?: Differential diagnosis and mechanisms of anemia of inflammation. In: *Wiener Medizinische Wochenschrift (1946)* 166 (13), S. 411–423. DOI: 10.1007/s10354-016-0505-7.

Oberhuber, Rupert; Riede, Gregor; Cardini, Benno; Bernhard, David; Messner, Barbara; Watschinger, Katrin et al. (2016): Impaired Endothelial Nitric Oxide Synthase Homodimer Formation Triggers Development of Transplant Vasculopathy - Insights from a Murine Aortic Transplantation Model. In: *Scientific Reports* 6, S. 37917. DOI: 10.1038/srep37917.

## 7 The MUI-START programme in numbers (Stand december 2016)

★ **64** Proposals granted / **48** Projects completed

★ **33** Male PI's / **31** Female PI's

★ **52** Publications acknowledging the MUI-START programme

**€ 1,7 Mio.** Granted by the MUI START programme

**€ 3,6 Mio.** Funds acquired by MUI-START grant holders

Additionally, 46% of PIs from closed projects have now a permanent position at the MUI or at the Clinic. Another 10% of PIs got positions in other research institutions or at pharmaceutic or high tech companies. The remaining scientists still work at the MUI as project collaborators or hold non-permanent positions.

# 8 MUI-START final reports

The principal investigators of the MUI-START projects are responsible for the content of their respective final reports.

## Kerstin Bellaire-Siegmund - Coronin 1A – an intrinsic modulator of T lymphocyte function

*Division of Cell Genetics*

**4. Funding period**

**Project duration:** 01.08.2013 – 31.07.2015

### Project summary

**Results and scientific progress**

An efficient adaptive immune response depends on the activation of T lymphocytes that have encountered their appropriate antigen presented by antigen-presenting cells. On molecular level, several proteins such as kinases contribute to control T cell activation and effector function. Research providing further insight into these signal transduction pathways is important to understand the physiology of T lymphocytes and thus to develop strategies to regulate immune responses.

In the current study, supported by the MUI-START fellowship, the role of coronin 1A for T cell activation was addressed. A physical interaction of coronin 1A and the serine/threonine kinase PKCtheta, which acts down stream of the T cell receptor, was observed. Further analysis of transcriptions factor activation (NF-κB transactivation) suggested that this interaction is of functional relevance for T lymphocyte activation. Our results have been published in May 2015 in the journal "*Cell Communication and Signaling".*

Furthermore, we are currently preparing a manuscript addressing the question whether or not coronin 1A, which is expressed in all leukocyte subsets, is an essential modulator of T cell-intrinsic function. Those results have been obtained by analyzing a T cell-specific *coronin 1a* knockout mouse (coro1afl/fl x CD4[Cre]) that was successfully generated in our laboratory during the course of the fellowship. Our findings strongly suggest an essential T cell-intrinsic function of coronin 1A. In brief, conditional (T cell-specific) *coronin 1a* knockout mice show a strong reduction of T lymphocytes numbers and a shift towards effector/memory T cells in peripheral lymphoid organs, even though less severe than observed with the conventional (full body) knockout mice. In line with these changes in the immune status, both knockout mice strains were resistant to induction autoimmunity using the EAE mouse model of Multiple Sclerosis.

With regard to the results obtained during the fellowship the project title was revised accordingly.

### Publications issued from this project

Siegmund, Kerstin; Thuille, Nikolaus; Posch, Nina; Fresser, Friedrich; Baier, Gottfried (2015): Novel Protein kinase C θ: Coronin 1A complex in T lymphocytes. In: *Cell Communication and Signaling : CCS* 13, S. 22. DOI: 10.1186/s12964-015-0100-3.

### External funding

Lise Meitner Stelle des FWF (M 1636-B23) since 01.02.2014.

## Martin Bodner - Helena, the hidden beauty: Molecular dissection of West Eurasias's most common mitochondrial DNA haplogroup H at the highest resolution

*Institut für Gerichtliche Medizin*

**4. Antragsperiode**

**Berichts-/Förderzeitraum:** 01.08.2013 – 31.07.2015

### Zusammenfassung

Die Sequenz (der Haplotyp) der humanen mitochondrialen DNA (mtDNA) wird in forensischen Untersuchungen ermittelt, wenn aufgrund hohen Alters, starker Degradierung oder einer zu geringen enthaltenen Zellzahl aus einer Probe kein individuelles Kern-DNA-Mikrosatelliten-Profil (der sog. DNA-„Fingerabdruck") erstellt werden kann, wenn mütterliche Verwandtschaft untersucht werden soll oder kein direktes Referenzmaterial vorhanden ist. Das mitochondriale Genom (mtGenom) liegt in weit höherer Kopienzahl als das Kerngenom vor, ist zirkulär und vermutlich besser vor Abbau geschützt, was eine Typisierung selbst „schwieriger" Proben ermöglicht. Der Marker hat aber auch Eigenschaften, die seine forensische Beweiskraft stark einschränken: mtDNA wird rein mütterlich ohne Rekombination vererbt, auch sehr entfernt maternal verwandte Individuen tragen dieselbe mtDNA-Linie (Haplogruppe). MtDNA kann also zum Ausschluss einer mütterlichen Verwandtschaft oder der Bestätigung identer maternaler Linien führen, aber (auf sich alleine gestellt) nur zur Identifizierung von Individuen verwendet werden, wenn alle infrage kommenden Personen bekannt sind und ggf. unterschiedliche mitochondriale Linien tragen. Die forensische Beweiskraft eines mtDNA-Ergebnisses ist somit direkt von der Anzahl und der Frequenz der auftretenden Linien in der jeweiligen Population oder Stichprobe abhängig. Eine Einschränkung trifft also besonders auf den häufigsten mtDNA-Haplotypen Westeurasiens (inkl. Europas) zu, der in allen bekannten Populationen mit einer Frequenz von 3-4% auftritt und in Haplogruppe H („Helena") fällt. Das bedeutet, dass in jeder westeurasischen Population für 3-4% der Individuen ein solches, nicht unterscheidbares mtDNA-Profil erwartet wird. Diese Angaben beziehen sich allerdings auf die aus technischen, gesetzlichen und/oder finanziellen Gründen weltweit routinemäßig (maximal) analysierte Kontrollregion, einen ~1100 bp langen, nicht codierenden Abschnitt der mtDNA (das ist 1/15 des mtGenoms). Identische Kontrollregionen zweier oder mehrerer Proben bedeuten jedoch nicht, dass auch die übrigen mtGenom-Abschnitte identisch sind oder die mtDNAs derselben phylogenetischen Linie angehören.

Hauptziele des MUI-START-Projekts waren,

*(i)* die Variation des häufigsten Kontrollregion-Haplotyps im gesamten mtGenom zu untersuchen. Aus wenigen vorliegenden, nicht systematisch erhobenen Daten war nicht klar, ob eine „versteckte" genetische Vielfalt oder nur wenige Linien erwartet werden konnten. 100 Proben mit dem häufigsten Haplotyp aus Italien wurden von KooperationspartnerInnen zur Verfügung gestellt. Die Besammlung wurde bewusst auf ein (geographisch und migrationsgeschichtlich besonderes) Land beschränkt, um Einsicht in Ausbreitungswege des Menschen gewinnen zu können;

*(ii)* die zu Projektbeginn bereits am Markt befindlichen *massively parallel/„next*

*generation"*- Sequenziertechniken in die forensische mtDNA-Analytik zu implementieren, wozu Protokolle zur Qualitätssicherung, die den besonders hohen Anforderungen gerichtlicher Verwendbarkeit genügen, erst etabliert werden mussten.

Die 100 mtGenome konnten erfolgreich komplett sequenziert werden. Eine unerwartet große Sequenzvielfalt „hinter" dem häufigsten Kontrollregion-Profil wurde nachgewiesen: nahezu jede der vormals (in der mtDNA-Kontrollregion) identischen Proben war einzigartig, lediglich vier Paare und zwei Trios stimmten komplett überein. **Dadurch stieg die Unterscheidungskraft der Untersuchung *(power of discrimination)*, die bei 0% gelegen war, nun auf 99,6%, was dem Traum einer forensischen Genetikerin/eines forensischen Genetikers, Gleiches zu unterscheiden, sehr nahe kommt**. Die Studie zeigt deutlich den Nutzen der vollständigen Sequenzierung von mtGenomen für forensische Anwendungen, um maximale Diskriminierung zu erreichen. Erste Teilergebnisse (29 Proben) konnten erfolgreich publiziert werden [1]; auch mit etwa dreifacher Probenzahl wurde noch keine Sättigung an neuen Haplotypen erreicht. **Mit Beiträgen aus diesem Projekt konnte die Qualitätskontrolle mitochondrialer *massively parallel*-Vollgenomsequenzierung etabliert und die Methodik für forensische Untersuchungen validiert werden** [2,3]. Aus der geographischen Verteilung der enorm vielfältigen mtGenome ergab sich kein spezifisches Muster, aus dem Rückschlüsse auf Datierung und Wege der Ausbreitung des Menschen im Bereich des heutigen Italiens und ganz Südeuropas hätten gezogen werden können. Solche phylogeographischen Erkenntnisse könnten in der Weiterführung des Projekts mit mehr Daten erreicht werden: ~200 weitere Proben mit dem häufigsten Kontrollregion-Haplotyp wurden von KooperationspartnerInnen zur Verfügung gestellt, sodass nunmehr ein Probenset vorliegt, das einem Screening von ~10000 ItalienerInnen entspricht. Das Projekt bietet die einmalige Möglichkeit der Untersuchung des forensisch wichtigsten mtDNA-Haplotyps im derzeit größten Probenset aus einem einzelnen Land. Es darf die Häufung gewisser Linien erwartet werden. Für diese können einzelnukleotid- basierte Schnelltests entwickelt werden, die in der forensischen Fallarbeit wertvolle Hinweise liefern, ohne den aufwändigen Weg einer kompletten mtGenom-Sequenzierung zu beschreiten. Ein Antrag auf externe Förderung durch den D. Swarovski-Förderungsfonds zur unmittelbaren Fortführung des Projekts wurde im November 2015 genehmigt. Die Beantragung weiterer externer Förderung ist beabsichtigt, um den langjährigen Forschungsschwerpunkt zur mtDNA-Haplogruppe H am Institut für Gerichtliche Medizin weiterführen zu können [1,4-6]. Es sollen zunächst die bereits vorhandenen zusätzlichen Proben (n=188) analysiert werden, in zukünftig größerem Rahmen ist an eine Ausdehnung des Projekts auf weitere südeuropäische bzw. westeurasische Länder gedacht.

Die hohe Zahl der in diesem MUI-START-Projekt ermittelten kompletten mtGenomsequenzen trägt signifikant zur Erweiterung forensischer mtDNA-Datenbanken bei: die weltweit führende mtDNA- Populationsdatenbank EMPOP [7] enthält zurzeit nur ~250 komplette mtGenome. Die Daten vergrößern nicht nur unmittelbar das Wissen um mtDNA-Phylogenie und -geographie, sondern sind auch für alle anderen wissenschaftlichen Bereiche von Nutzen, die mtDNA als Marker verwenden - wie etwa für die medizinische Genetik durch detailliertes Wissen um verifizierte, natürlich auftretende Variation. Über den Rahmen eines solchen Projektes hinaus geht die Suche nach den evolutionären Ursachen einer extrem hohen Vielfalt hinter einem identischen, nicht-codierenden Genomabschnitt.

[1] Bodner M, et al.: *Helena, the hidden beauty: resolving the most common West Eurasian mtDNA control region haplotype by massively parallel sequencing an Italian population sample*. Forensic Sci Int Genet. 2015;15:21-6.

[2] Just RS, et al.: *Full mtGenome reference data: Development and characterization of 588 forensic-quality haplotypes representing three U.S. populations.* Forensic Sci Int Genet. 2015;14:141-55.

[3] Gomes SM*, Bodner M*, et al.: *Human settlement history between Sunda and Sahul: a focus on East Timor (Timor-Leste) and its Pleistocenic mtDNA diversity*. BMC Genomics 2015;16(1):70.

[4] Brandstätter A, et al.: *Dissection of mitochondrial superhaplogroup H using coding region SNPs*. Electrophoresis 2006;27(13):2541-50.

[5] Brandstätter A, et al.: *Timing and deciphering mitochondrial DNA macro-haplogroup R0 variability in Central Europe and Middle East*. BMC Evol Biol 2008;8:191.

[6] Niederstätter H & Parson W: *Fluorescent duplex allele-specific PCR and amplicon melting for rapid homogeneous mtDNA haplogroup H screening and sensitive mixture detection*. PLoS ONE 2009;4(12): e8374.

[7] http://empop.online

## Publikationen mit MUI-START Erwähnung

Just, Rebecca S.; Scheible, Melissa K.; Fast, Spence A.; Sturk-Andreaggi, Kimberly; Röck, Alexander W.; Bush, Jocelyn M. et al. (2015): Full mtGenome reference data: Development and characterization of 588 forensic-quality haplotypes representing three U.S. populations. In: *Forensic Science International: Genetics* 14, S. 141–155. DOI: 10.1016/j.fsigen.2014.09.021.**IF: 4.604**

Gomes, Sibylle M.; Bodner, Martin; Souto, Luis; Zimmermann, Bettina; Huber, Gabriela; Strobl, Christina et al. (2015): Human settlement history between Sunda and Sahul: a focus on East Timor (Timor-Leste) and the Pleistocenic mtDNA diversity. In: *BMC Genomics* 16 (1), S. 70. DOI: 10.1186/s12864-014-1201-x. **IF: 3.986** (*geteilte Erstautorenschaft)

Bodner, Martin; Iuvaro, Alessandra; Strobl, Christina; Nagl, Simone; Huber, Gabriela; Pelotti, Susi et al. (2015): Helena, the hidden beauty: Resolving the most common West Eurasian mtDNA control region haplotype by massively parallel sequencing an Italian population sample. In: *Special Issue: DNA in Forensics 2014* 15, S. 21–26. DOI: 10.1016/j.fsigen.2014.09.012. **IF: 4.604**

Gomes, Sibylle M.; van Oven, Mannis; Souto, Luis; Morreira, Helena; Brauer, Silke; Bodner, Martin et al. (2017): Lack of gene-language correlation due to reciprocal female but directional male admixture in Austronesians and non-Austronesians of East Timor. In: *Eur J Hum Genet* 25 (2), S. 246–252. Online verfügbar unter http://dx.doi.org/10.1038/ejhg.2016.101.**IF: 4.349**

## Externe Förderung

Helena's many daughters: massively parallel sequencing provides further insights into the most common West Eurasian mtDNA control region haplotype at the highest resolution. D. Swarovski-Förderungsfonds (DSF-2015-1-1). Bewilligte Summe: € 5.000,00

Helena's many daughters - Massively parallel sequencing provides highest-resolution insights into the most common West Eurasian mtDNA control region haplotype. Tiroler Wissenschaftsfonds (TWF-2016-1-2). Bewilligte Summe: € 30.122,00

## Sonstiges

### Posters

**Bodner M**, Iuvaro A, Strobl C, Pettener D, Pelotti S, Luiselli D, Parson W: Resolving the most common mtDNA control region haplotype by massively parallel sequencing: a pilot study in an Italian population sample. DNA in Forensics 2014, Brüssel, 05/2014

**Bodner M**, Parson W: Forensic quality control tools towards better mtDNA data. 2nd MEET course in Mitochondrial Medicine, Bertinoro di Romagna, 12/2014

**Bodner M**, Iuvaro A, Strobl C, Nagl S, Huber G, Pelotti S, Pettener D, Luiselli D, Parson W: Helena, the hidden beauty: resolving the most common West Eurasian mtDNA control region haplotype by massively parallel sequencing. 9th ISABS Conference, Bol,6/2015

**Bodner M**, Strobl C, Nagl S, Xavier C, Huber G, Cardinali I, Semino O, Olivieri A, Gandini F, Achilli A, Torroni A, Parson W: Helena's many daughters: massively parallel sequencing provides further insights into the most common West Eurasian mtDNA control region haplotype. 26th World Congress, International Society of Forensic Genetics, Krakau, 09/2015

### Vorträge

**Bodner M**, Gomes SM, Souto L, Zimmermann B, Huber G, Strobl C, Röck AW, Achilli A, Olivieri A, Torroni A, Corte-Real F, Parson W: Human settlement history of East Timor: NGS insights into the Pleistocenic diversity of mtDNA haplogroup P1. DNA in Forensics 2014, Brüssel, 05/2014

## Gregor Brössner - Non-invasive measurement of brain temperature in Magnetic Resonance Imaging

*Universitätsklinik für Neurologie*

**4. Antragsperiode**

**Berichts-/Förderzeitraum:** 01.12.2013 – 30.11.2015

### Zusammenfassung

**Projektziel 1 („first level“):**

Dieser Projektabschnitt war gekennzeichnet durch den Aufbau eines Phantoms zu kontrollierten Temperaturmessung und Kalibration des gesamten Systems. Mittels einer doppelwandigen „Phantomkugel“ die mit bekannten chemischen Metaboliten (Cr, Cr2, NAA, DSS, $H_2O$) gefüllt war und von einer unterschiedlich temperierten Flüssigkeit ($H_2O$) umgeben war, wurden vorerst spektroskopische Messungen durchgeführt. Die Temperaturen wurde in dem Phantom mittels Sonden gemessen (Luxtron m3300 Biomedical Lab Kit, Luma Sense Technologies) und andererseits Spektroskopie Messungen zu unterschiedlichen Temperaturen durchgeführt. Anschließend erfolgten die Vorverarbeitung (pre-processing) der Bilddaten und danach die Erstellung von Kalibrationskurven mit einem Softwareprogram (JMRUI®).

In unseren unterschiedlichen mathematischen Modellen zeigten jeweils Cr und NAA den stabilsten Verlauf und die geringsten Standardabweichungen, den Temperaturverlauf betreffend. Insgesamt konnten somit erstmals mittels Scanner (MRT) temperaturspezifische Verlaufskurven durch temperaturbedingte Verschiebungen der Metaboliten in Relation zum „Waterpeak“ erstellt werden.

**Projektziel 2 („second level“):**

In dieser Projektphase wurden 30 gesunde Probanden rekrutiert und bei diesem vorerst mittels Infrarotthermometer die tympanale Temperatur bestimmt. Nachfolgend wurde an 3 vorab definierten ROIs (Regions of Interest) spektroskopische Messungen durchgeführt und danach die gewonnenen Spektroskopiesignale in die aus Projektziel 2 erstellten Kalibrationskurven eingesetzt und daraus die Temperatur nicht-invasiv bestimmt. Wie auch schon in kleinen Vorarbeiten aus unsere Arbeitsgruppe beschrieben (jedoch mittels invasiven Methoden) , lag die Gehirntemperatur (GH) über der Körpertemperatur. Mit dieser Methode konnte erstmals die GH zuverlässig bestimmt werden in unterschiedlichen Bereichen (graue versus weisse Substanz) des Gehirns.

**„Überraschungen“ im Projektverlauf und bei den Ergebnissen:**

Bislang konnte die Temperatur des Gehirns nur mittels invasiven Methoden (Sonden) gemessen werden. Wir sind nun im Stande mit dieser neuen Methode nicht-invasiv die GH nicht nur an einer Position, sondern an unterschiedlichen frei wählbaren Positionen zu bestimmen. Überraschenderweise war die GH nicht nur unterschiedlich zur Körpertemperatur, es zeigte sich auch ein Temperaturunterschied innerhalb eines einzelnen Probanden (z. B zwischen den beiden Hemisphären). Dies könnte auf unterschiedliche Durchblutungen zurückzuführen sein.

**Zukünftige Anwendungsgebiete:**

Mit dieser neuen Methode können wir nun erstmals nicht-invasiv die Temperatur des Gehirns bestimmen. Erstens können dadurch neue Aspekte über die Physiologie der GH gewonnen werden. Zusätzlich können bei Patienten mit fokalen Läsionen im Gehirn die keine Indikation für eine invasive Thermometrie (Sondenimplantation) haben die GH bestimmen. Hier sind sicherlich die Patientinnen mit ischämischen Insult von großem Interesse (Intraläsionell versus Penumbra versus kontralaterale Hemisphäre). Therapeutische Hypothermie (i.e. 32-25°C) wird heute in unterschiedlichen Studien bei akut-neurologischen Erkrankungen zur Neuroprotektion eingesetzt. Über die Beeinflussung der GH durch therapeutische Hypothermie ist nur wenig bekannt. Unsere neue MR-tomographische Methode soll nun helfen, die pathophysiologischen Effekte von TH bei unterschiedlichen Erkrankungen zu beleuchten.

**Projektverlauf:**

Das Projekt konnte am 01.12.2013 gestartet werden und wurde am 30.11.2015 erfolgreich beendet. Dr. Florian Frank war über den gesamten Zeitraum über diese Studie angestellt und etablierte alle einzelnen Projektteile hervorragend. Sowohl die Messungen am Phantom und die Messungen an den Gesunden Probanden wurden in Zusammenarbeit mit dem gesamten Projektteam (Dr. M. Verius, Dr. F. Frank, Dr. G. Brössner) durchgeführt.

## Publikationen mit MUI-START Erwähnung

**Abstracts:**

Verius M, Frank F, Broessner G. Non-invasive measurement of brain temperature using non-invasive Magnetic Resonance Imaging. 4th Innsbruck Berlin Targeted Temperature Management Symposium 2015

Verius M, Frank F, Broessner G. Temperature Measurement: Which site should we use? 5th International Hypothermia Symposium Edinburgh 2014

Verius M, Frank F, Broessner G. Nicht-invasive Messung der Gehirntemperatur mittels Magnetresonanztomographie. 1. Innsbrucker „Neuroimaging Colloqium“. 2014

**Papers:**

Verius M, Frank F, Broessner G. Non-invasive measurement of brain temperature using non-invasive Magnetic resonance Imaging. Journal of Magnetic Resonance (submitted)

## Externe Förderung

Ein KLIF Projekt wurde eingereicht aber leider nicht bewilligt.

## Benno Cardini - Simvastatin and tetrahydrobiopterin biosynthesis in the prevention of chronic allograft vasculopathy

*University Hospital for Visceral, Transplant and Thoracic surgery*

**4. Funding period**

**Project duration:** 01.11.2013 – 31.03.2016

### Project summary

**Introduction and aim of the project**

Solid organ transplantation represents to date the therapy of choice for end-stage organ failures. Over the last decades it has evolved from „clinical experiment“ to routine.[1]

However, to date, a large discrepancy between shortage of organ donors and the augmenting number of patients on the waiting list has led to an extension of the donor pool, with an increasing acceptance of “suboptimal/ marginal” donors, including donation after cardiocirculatory death (DCD) and organs from elderly donors. Although excellent short-term outcomes are achieved due to peri- and postoperative management, as well as improvements in immunosuppressive regimens, long-term graft as well as patient survival is by far not as satisfying .[2]

One major hurdle in increasing long-term graft survival is represented by the chronic allograft vasculopathy (CAV) characterised by a diffuse concentric narrowing of the lumen of the vessels. Despite donor characteristics (e.g. age, comorbidities, DCD etc.) and immunologic factors several alloantigen-independent factors have been shown to induce a proinflammatory state which increases immunogenicity in the long-term.[3,4]

A further important factor significantly influencing both short- as well as long-term graft survival is represented by the so-called ischemia and reperfusion injury (IRI). The lack of blood and –by that- oxygen supply during the ischemic phase (organ retrieval and preservation) as well as the reintroduction of oxygen during reperfusion induce a massive oxidative stress. This subsequently initiates a plethora of cellular and metabolic changes such as expression of proinflammatory cytokines and adhesion molecules as well as activation of endothelial cells and of the complement cascade. These factors contribute to the maintenance of an eventually chronic proinflammatory state which finally involves the whole immune system.[5,6,7,8]

In this context the nitric oxide synthase (NOS) enzyme family has been identified as crucial source for reactive oxygen species (ROS). Under physiological condition both the neuronal (nNOS) as well as the endothelial NOS (eNOS) isoforms are involved in neurotransmission as well as in regulation of the vascular homeostasis by –as dimers- maintaining and regulating the NO biosynthesis.[9,10]

To fulfill their function the presence of the essential co-factor Tetrahydrobiopterin (BH4) is required. BH4 itself –as cofactor- increases substrate affinity, stabilizes dimer formation as well as scavenges free radicals occurring during NO biosynthesis.[11,12,13]

Oxidative stress –such as in IRI- leads to a depletion of intracellular BH4, which eventually results in the so-called “uncoupling” of the NOS, with the NOS enzyme producing superoxid anions and other ROS instead of NO.[13] NOS uncoupling favours endothelial activation with

inappropriate vasoconstriction, platelet aggregation, leucocyte adhesion and smooth muscle cell proliferation, which are all hallmarks of the CAV.[9,14,15,16]

Among the huge variety of therapeutical options to prevent early and late allograft damage the inhibition of 3-Hydroxy-3-Methylglutaryl-Coenzym-A (HMG CoA)-reductase has emerged as a promising approach. HMG CoA reductase inhibitors, known as statins, are widely used for primary and secondary prevention of cardiovascular diseases. Besides their well-described lipid lowering effect, more recently other beneficial non-lipid, “pleiotropic effects” like antinflammatory and antiatherogenic effects have been described, which are still not completely understood.[17,18]

Especially interactions with the NO biosynthesis gained interest over the last years. Antoniades demonstrated that atorvastatin treatment before coronary bypass graft surgery resulted in an improved NO bioavailability and reduced arterial superoxide production in humans.[19] Furthermore, HMG-CoA reductase inhibitor treatment has also gained interest in the field of solid organ transplantation. So far, results obtained from both human as well as animal studies have shown that both short-term as well as long-term statin treatment were able to reduce the incidence of early graft damage and chronic allograft rejection.[20,21,22,23]

In previous studies of our group we were able to show the importance of a sufficient intracellular presence of the essential NOS- cofactor BH4 in a setting of solid organ transplantation in mice. In a heterotopic murine pancreas transplantation model a single donor therapy with BH4 was able to prevent from lethal graft pancreatitis. Furthermore we were able to show that BH4-mediated effects rely on its cofactor activity rather than on its antioxidative properties.

However, since at the moment BH4 treatment is indicated only for treatment of selected forms of atypical phenylketonuria, it was aim of the present supported project to investigate if a single donor therapy with 5mg /kg b.w. Simvastatin is able to (i) prevent CAV in a fully missmatched murine aortic trasplantation model by increasing BH4 bioavailability, (ii) modulate eNOS, GTPCH I and vWF mRNA expression (iii) stabilise NOS dimer formation.

**Summary of the obtained results**

The received support allowed cand med. Rebecca Eiter to perform and conclude her Master Thesis on an experimental topic at the Daniel Swarovski Research Laboratory, Dept. of Visceral, Transplant and Thoracic Surgery, Medical University Innsbruck. For this purpose a fully-missmatched heterotopic aortic transplantation model was used. Aortic grafts were retrieved from inbred male Balb/c mice, stored on ice-cold perfusion solution for 24 hours and transplanted cervically into male C57Bl/6 mice. Grafts were either reperfused for 2 hours or 28 days (were chronic allograft rejection is expected). Donors were either pretreated with a single oral dose of 5mg/kg b.w. simvastatin or received polyethyene glycol (control group).

a) Simvastatin and histopathological changes

Intima media thickness ratio was determined in H&E stained tissue to determine intimal hyperplasia, which is a hallmark of CAV. Following 24 hours cold ischemia time and 2 hours reperfusion we did not observe an intimal hyperplasia which is consistent with the recent literature. In this time period the migration of smooth muscle cells and myofibroblasts is a to short period. However, following 28 days of reperfusion a markedly lower intimal hyperplasia formation was observed in simvastatin treated grafts compared to the respective controls.

Furthermore significantly less thromboembolic events could be observed in simvastatin treated grafts compared to the control group.

b) Effects of simvastatin on BH4 bioavailability

Intragraft BH4 levels were determined by HPLC. Following 2 hours of reperfusion we observed markedly higher BH4 levels if the donors were pretreated with simvastatin compared to the PEG-treated control, however, statistical significance could not be reached. In fact PEG-treated and transplanted controls displayed markedly lower levels when compared to non-transplanted controls. This may indicate a certain consumption of BH4 in the early cold ischemic phase which is in line with our previous observation in a heterotopic pancreas transplantation model. A possible reason for the missing statistical may rely in the unequally matched group numbers (n3 vs N4). The missing samples in these groups have already been acquired and the final examination is still ongoing.

Following 28 days of reperfusion we could not find statistical differences between treated groups and the respective controls, indicating that a stabilization of the BH4 bioavailability is established following this long perfusion period.

c) Effects of simvastatin on eNOS monomer-dimer formation

eNOS dimer and monomer formation was evaluated by Western Blot. Herein we could not confirm the hypothesis that simvastatin influences eNOS monomer formation. In fact following 2h of reperfusion monomer formation was observed in treated as well as in the control group. Hence dimer formation was significantly impaired by the cold ischemia time. However, following 28 days of reperfusion significantly higher dimerization was detected when compared to the 2 hours reperfused group. However, simvastatin treatment did not show any advantageous effects. We assume that following this long reperfusion period eNOS stabilization occurs either by regeneration of the endothelium or by migration of endothelial cells from the adjacent vessels. This hypothesis could eventually be verified using a different sex of mouse as recipient and with determination of mitochondrial DNA.

d) Effects of simvastatin on mRNA expression

Following 2 hours of reperfusion we could not confirm the hypothesis that simvastatin leads to an increased BH4 biosynthesis by an upregulation of GCH 1 mRNA. In fact only low levels of GCH1 as well as eNOS mRNA were detectable in grafts reperfused for 2 hours. However, simvastatin treatment and 28 days of reperfusion resulted in a markedly lower expression of vWF mRNA when compared to the respective PEG treated control. vWF is an important biomarker to assess and discriminate acute versus chronic endothelial cell injury. Our data indicate that simvastatin indeed has an effect on vWF expression and therefore a certain anti-inflammatory aspect in its mode of action may be derived. However, the main key mechanism of action cannot be explained so far.

In conclusion in this supported project we could not confirm the hypothesis, that a single oral application of the donor leads to an increased GTPCH-1 expression and hence to an increased BH4- biosynthesis.

However the reduced neointima formation and less thromboembolic events as well as the higher intragraft BH4 levels and the decreased vWF mRNA expression in simvastatin treated

grafts display a certain protective effect of the single donor therapy in the setting of transplantation which will be investigated in future projects.

We faced several problems and limitations which have to and will be addressed in further projects:

(i): Regarding dosages we adopted the treatment protocol of Tuuminen et al. We cannot rule out, that in the aortic transplantation model this dosage may be to low due to rheological factors. Hence an increase in dosages as well as verification in a solid organ model (e.g. heart transplatation model) is planned.

(ii): So far we cannot say if simvastatin reaches the „region of interest“. For this purpose an HMG-CoA reductase activity assay has been established by Dr. David Bernhard and the samples will be evaluated, adding a further hint to our hypothesis.

**References**


1. Linden P (2009) History of solid organ transplantation and organ donation. Crit Care Clin 25: 165-184, ix.

2. Lodhi SA, Lamb KE, Meier-Kriesche HU (2011) Solid organ allograft survival improvement in the United States: the long-term does not mirror the dramatic short-term success. Am J Transplant 11: 1226-1235.

3. Atkinson C, Floerchinger B, Qiao F, Casey S, Williamson T, et al. (2013) Donor Brain Death Exacerbates Complement-Dependent Ischemia Reperfusion Injury in Transplanted Hearts. Circulation.

4. Fellmer P, Pascher A, Kahl A, Ulrich F, Lanzenberger K, et al. (2010) Influence of donor- and recipient-specific factors on the postoperative course after combined pancreas-kidney transplantation. Langenbecks Arch Surg 395: 19-25.

5. de Groot H (2005) [Injury to visceral organs by ischemia and reperfusion. Processes in pathogenetic networks]. Zentralbl Chir 130: 202-212.

6. Kaminski K, Bonda T, Korecki J, Musial W (2002) Oxidative stress and neutrophil activation--the two keystones of ischemia/reperfusion injury. Int J Cardiol 86: 41-59.

7. Carden D, Granger D (2000) Pathophysiology of ischaemia-reperfusion injury. J Pathol 190: 255-266.

8. Guarrera JV (2012) Assist devices: machine preservation of extended criteria donors. Liver Transpl 18 Suppl 2: S31-33.

9. Alp N, Channon K (2004) Regulation of endothelial nitric oxide synthase by tetrahydrobiopterin in vascular disease. Arterioscler Thromb Vasc Biol 24: 413-420.

10. Melikian N, Seddon MD, Casadei B, Chowienczyk PJ, Shah AM (2009) Neuronal nitric oxide synthase and human vascular regulation. Trends Cardiovasc Med 19: 256-262.

11. Werner ER, Blau N, Thöny B (2011) Tetrahydrobiopterin: biochemistry and pathophysiology. Biochem J 438: 397-414.

12. Berka V, Yeh H, Gao D, Kiran F, Tsai A (2004) Redox function of tetrahydrobiopterin and effect of L-arginine on oxygen binding in endothelial nitric oxide synthase. Biochemistry 43: 13137-13148.

13. Katusic Z, d'Uscio L, Nath K (2009) Vascular protection by tetrahydrobiopterin: progress and therapeutic prospects. Trends Pharmacol Sci 30: 48-54.

14. Fukuda Y, Teragawa H, Matsuda K, Yamagata T, Matsuura H, et al. (2002) Tetrahydrobiopterin restores endothelial function of coronary arteries in patients with hypercholesterolaemia. Heart 87: 264-269.

15. Naseem K (2005) The role of nitric oxide in cardiovascular diseases. Mol Aspects Med 26: 33-65.

16. Moens AL, Kietadisorn R, Lin JY, Kass D (2011) Targeting endothelial and myocardial dysfunction with tetrahydrobiopterin. J Mol Cell Cardiol 51: 559-563.

17. Landmesser U, Bahlmann F, Mueller M, Spiekermann S, Kirchhoff N, et al. (2005) Simvastatin versus ezetimibe: pleiotropic and lipid-lowering effects on endothelial function in humans. Circulation 111: 2356-2363.

18. Fichtlscherer S, Schmidt-Lucke C, Bojunga S, Rössig L, Heeschen C, et al. (2006) Differential effects of short-term lipid lowering with ezetimibe and statins on endothelial function in patients with CAD: clinical evidence for 'pleiotropic' functions of statin therapy. Eur Heart J 27: 1182-1190.

19. Antoniades C, Bakogiannis C, Leeson P, Guzik TJ, Zhang MH, et al. (2011) Rapid, direct effects of statin treatment on arterial redox state and nitric oxide bioavailability in human atherosclerosis via tetrahydrobiopterin-mediated endothelial nitric oxide synthase coupling. Circulation 124: 335-345.

20. Kobashigawa JA, Moriguchi JD, Laks H, Wener L, Hage A, et al. (2005) Ten-year follow-up of a randomized trial of pravastatin in heart transplant patients. J Heart Lung Transplant 24: 1736-1740.

21. Wenke K, Meiser B, Thiery J, Nagel D, von Scheidt W, et al. (2003) Simvastatin initiated early after heart transplantation: 8-year prospective experience. Circulation 107: 93-97.

22. Tuuminen R, Syrjälä S, Krebs R, Keränen MA, Koli K, et al. (2011) Donor simvastatin treatment abolishes rat cardiac allograft ischemia/reperfusion injury and chronic rejection through microvascular protection. Circulation 124: 1138-1150.

23. Gracia-Sancho J, García-Calderó H, Hide D, Marrone G, Guixé-Muntet S, et al. (2013) Simvastatin Maintains Function and Viability of Steatotic Rat Livers Procured for Transplantation. J Hepatol.

## Publications issued from this project

The support of the MUI-Start is mentioned in a submitted publication with the title „Mouse Model for Pancreas Transplantation Using a Modified Cuff Technique" (submitted to JOVE, actual state: under review)

## External funding

The impact of simvastatin on the ischemia reperfusion injury in the murine heart transplantation model. (TWF-2016-1-6). Amount granted: € 32.069,00.

## Miscellaneous

The results of the supported project were presented at national congresses (39. Seminar „ Österreichische Gesellschaft für Chirurgische Forschung“, Wagrain, and 57. Östereichischer Chirurgenkongress, Salzburg).

Parts of the results of this project were drawn bei cand.med Rebecca Eiter for her Master Thesis submitted in accordance with the requirement for the Degree „Dr. med univ“ ( Title of the master thesis: Simvastatin and the prevention of chronic transplant vasculopathy)

## Cedric De Smet - LAMTOR commands adipogenesis: a novel lipid sensor in the endosomal membrane

*Division of Cell biology*

**4. Funding period**

**Project duration:** 01.08.2013-01.08.2015

**Project summary**

The final report of Cedric De Smet contains sensitive unpublished data that cannot be made available at the moment.

## Elke Griesmaier-Falkner - Neuroprotective potential of sigma-1 receptor agonists in in vitro models of neonatal brain injury

*Pediatrics II*

**4. Funding period**

**Project duration**: 01.08.2013 – 31.07.2015

**Project summary**

Newborn brain injury is a relevant problem. One important factor that contributes to newborn brain injury is excitotoxicity. Several studies have demonstrated the crucial role of excitatory amino acids and sustained the administration of anti-excitotoxic substances with the aim of neuroprotection. During the last years, N-methyl-D-aspartate (NMDA) receptor antagonists have been favoured as therapeutic strategies, but they finally failed to live up to the expectations and showed unwanted side effects by triggering apoptotic neurodegeneration in the healthy developing newborn rodent brain.

We focus on therapeutic strategies, which after sufficient data acquisition in the experimental setting, could be rapidly transferred to a clinical study. Substances acting as sigma-1 receptor ligands were shown to be protective in adult models of brain injury and are undergoing clinical trials in adult neurological diseases. Several properties of sigma-1 receptor agonists may bring about neuroprotective effects also in newborn brain injury: they inhibit ischemia-induced presynaptic glutamate release, attenuate postsynaptic glutamate- evoked calcium influx, modulate neuronal responses to NMDA receptor stimulation and attenuate glutamate- and NMDA-induced nitric oxide synthase activation. These mechanisms have been shown to be involved in the pathophysiologic cascade of newborn brain injury and therefore are a highly attractive target for neuroprotection. We previously showed that sigma-1 receptor agonists are protective in in vivo models of excitotoxic newborn brain injury (*Griesmaier et al. Exp Neurol 2012; Wegleiter et al. Exp Neurol 2014; Posod et al. Neuroscience 2014*).

The aim of this study supported by the MUI-START was to investigate the neuroprotective potential of the selective sigma-1 receptor agonist PRE-084 (2-(4-morpholinethyl)1-phenylcyclohexane-carboxylate) in vitro.

**Important findings:**

In newborn brain injury, both white and gray matter structures are affected. Neurons, oligodendrocytes and their precursors are known to be particularly vulnerable to noxic stimuli. We used neuronal cell types (HT-22) and oligodendroglial cell types (OLN-93), after induction of a more immature phenotype corresponding to pre-oligodendrocytes (pre-OL), as model systems for neonatal brain injury. Cells were pre-treated with PRE-084 in three dosages (1, 10 and 100 µM) before glutamate was applied. Cell viability in OLN-93 and HT- 22 cells was subsequently assessed by means of a colorimetric assay (CCK-8, Dojindo). In HT-22 cells FACS analysis employing annexin V and propidium iodide (PI) staining was also performed. Pre-treatment with PRE-084 did not increase cell viability after glutamate exposure, neither in pre-myelinating oligodendroglial (pre-OL cell viability: glutamate control 21.2% versus PRE-084 1µM 18.8%, PRE-084 10µM 21.0%, PRE-084 100µM 19.6%, n=5, $p>0.05$) nor in neuronal cell types (HT-22 cell viability: glutamate control 24.8% versus PRE-084 1µM 18.4%, PRE-084 10µM 19.8%, PRE-084 100µM 20.9%, n=4, $p>0.05$). Following

glutamate treatment, HT-22 cells were stained with Annexin V-FITC and PI and apoptosis was measured by flow cytometry (glutamate control 46.2% vs. PRE-084 1 µM 50.9%, PRE-084 10 µM 55.9%, PRE-084 100 µM 59.8%, n=3, $p>0.05$). PRE-084 did not decrease apoptosis after glutamate-induced injury in HT-22 cells.

Based on previous studies, we also performed experiments in primary cell culture. Primary hippocampal neurons were treated with PRE-084 and subsequently analyzed for cell death using PI and calcein-AM staining. PRE-084 significantly reduced cell death (percentage of cell death: glutamate control 43.1% versus PRE-084 10µM 23.8%, PRE-084 100µM 26%, n=3, $p<0.05$).

In the aftermath of insults to the developing brain, microglial cell activation potentially furthers neuronal and oligodendroglial damage. Thus, the second specific objective was to evaluate whether PRE-084 reduces microglial cell activation following excitotoxic or inflammatory stimulation which is detrimental to the developing brain. In this study we used mouse primary hippocampal neurons and primary microglia. After pre-stimulation of microglia with PRE-084 cells were treated with either lipopolysaccharide, glutamate or phosphate buffered saline (PBS). After a recovery period the supernatant (microglia -conditioned medium) from each well was collected for co-culture experiments with hippocampal neurons. Neuronal cell viability was assessed after co-culture with microglia-conditioned media using PI and calcein- AM staining. To assess formation of free radicals/reactive oxygen species by microglial cells as a functional outcome parameter, CM-$H_2$DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) solution was added to glutamate- or PBS-treated microglial cells. Reactive oxygen species formation was measured by means of fluorescence microscopy. Preliminary data show a reduction of neuronal cell death in vitro after application of PRE-084 pre-stimulated microglia-conditioned media to primary hippocampal neurons. First results also show that PRE-084 reduces reactive oxygen species formation in microglia cells. However, this needs to be confirmed in additional experiments.

## Publications issued from this project

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## External funding

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## 4. Funding period

**Project duration:** 01.08.2013 – 28.02.2015

## Project summary

### Background and objective

B cell development, which generates the diversity of B cell antigen receptor (BCR) specificities that are a hallmark of the adaptive immune system, takes place in several consecutive stages[1]. Each of these steps is tightly controlled at several checkpoints, as defective development can give rise to autoimmunity, immunodeficiency or tumorigenesis. Traditionally, research has mainly focused on the role of protein-coding genes in this checkpoint control, however, recent studies have revealed that also long non-coding RNAs (lncRNAs) play a significant role in orchestrating and fine-tuning transcriptional programs both in health and disease[2,3].

In haematopoiesis, lncRNAs have been identified and described in diverse cellular lineages, such as in myeloid cells, NK cells or T lymphocytes, but their role in B lymphocytes, a main cellular component of the adaptive immune system, has not been addressed thus far[4,5]. When this research project was initiated, little was known about which lncRNAs are expressed by B cells throughout their development, whether certain lncRNAs are tightly linked to a defined developmental stage, or whether lncRNAs qualitatively influence lymphocyte maturation and function.

Thus, the objective of this work was to acquire a comprehensive understanding of how lncRNAs shape and regulate B cell development. In particular, we aimed to characterize the B cell-specific lncRNA transcriptome and to functionally analyze a subset of lncRNAs expressed throughout B cell-development in more detail.

### Results

To identify the total transcriptome including lncRNAs in developing murine B cells, large-scale RNA sequencing (RNAseq) of different developmental B cell stages was performed in collaboration with the Core Facility Deep-Sequencing at the Division of Genomics and RNomics, Medical University of Innsbruck. In particular, five different stages of B cells were sorted and pooled from 4 mice based on the surface expression of distinct markers (**Figure 1**). From the bone marrow, we collected pro-B cells (defined by $B220^+$ $\mu HC^-$ $ckit^+$), large pre-B cells ($B220^+$ $\mu HC^-$ $CD25^+$ $FSC^{high}$), small pre-B cells ($B220^+$ $\mu HC^-$ $CD25^+$ $FSC^{low}$) and immature B cells ($B220^+$ $\mu HC^+$ $\square HC^-$), whereas only mature B cells with a signaling-competent BCR ($B220^+$) were retrieved from the spleen. Splenic B cells were either left untreated or further stimulated with an anti-CD40 antibody for 48 h to mimic T cell-dependent activation.

Following RNA preparation and library preparation, RNAseq on the Ion Proton platform generated about 80 million reads per stage, most of which passed the quality threshold and could be aligned to the reference mouse genome sequence (UCSC). From the mapped reads, transcripts were assembled using cufflinks, followed by quantification of annotated transcripts based on FPKM-values (Fragments per Kilobase of Exon per Million Fragments Mapped) normalized to the total number of mappable read counts. At this stage, data quality was

validated by comparing the transcriptional pattern of selected genes as predicted by the RNAseq analysis with qPCR data from independent sets of cDNAs (**Figure 2**). We found a high correlation between both methods, indicating that the RNAseq, the consecutive read mapping and the transcript assembly generated data of high quality. Hence, the RNAseq data were further analyzed with respect to long non-coding transcripts.

In short, we subjected all transcripts (annotated and non-annotated) derived from read mapping and transcript assembly to a bioinformatics pipeline aimed to identify and select lncRNA candidates for a thorough investigation (**Figure 3**). LncRNAs *per se* are a diverse set of non-coding RNAs that can broadly be categorized based on their genomic location with respect to coding genes. As such, lncRNAs can be transcribed from introns of coding genes, can overlap coding exons or can be transcribed in a divergent manner from the same promoter as a coding genes. Long intergenic non-coding RNAs (lincRNAs), on the other hand, form independent genetic units that do not overlap with coding genes and are transcribed from their own promoter. Since most of the functional lncRNAs that have been described so far fall into this category[6], we decided to focus only on these lincRNAs in this study. In consequence, we removed all transcripts that overlapped with any annotated genes from the pool as a first step, followed by subtraction of transcripts predicted to encode proteins as assessed by different computational algorithms[7]. This gave rise to a list of about 2000 putative novel lincRNAs expressed at each individual B cell developmental stage. In an attempt to identify functionally relevant lincRNAs, this gene list was further narrowed down by additional selection criteria. In particular, we mainly focused on lincRNA genes encoding multi-exonic transcripts of more than 1 kb length that showed a differential expression pattern throughout B cell development as predicted by RNAseq.

About 10 lincRNAs of the resulting group of functional candidates were manually chosen for detailed analysis, an example of which is depicted in **figure 4**. This lincRNA, termed lincRNA-A_AS due to its antisense orientation to the annotated coding gene A, was spliced from a precursor to give rise to a transcript of about 1.5 kb. Predicted 5'- and 3'-ends as well as the splice junctions of lincRNA-A_AS were confirmed by rapid amplification of cDNA ends (RACE) and cDNA sequencing, providing strong evidence that this transcript is not an artifact of our analysis pipeline. Notably, we found expression of lincRNA-A_AS within the B cell pool to be restricted to mature B cells. Furthermore, lincRNA-A_AS was neither detectable in mature $CD4^+$ nor in $CD8^+$ T cells, suggesting a B cell-specific function.

To evaluate whether any of our retrieved lincRNAs is indeed implicated in B cell development and possibly immune function, we took use of a classical loss-of-function approach. In particular, we designed and generated retrovirally encoded small hairpin RNAs (shRNAs) in order to specifically knock-down distinct lincRNAs *in vitro*. Some of these knockdown constructs indeed provoked an effect on cellular proliferation, differentiation and signaling (preliminary experiments, data not shown) when expressed in B cell precursors of different developmental stages, suggesting that at least a subset of lincRNAs identified in this study is of functional relevance. However, it turned out that shRNAs are probably not the best technique for our loss-of-function approach (see below), so more experiments are needed to confirm these initial observations and to identify the mechanisms underlying lincRNA function.

**Difficulties we faced during the study**

During this project, we had to cope with two major difficulties. First, it turned out that the bioinformatics analysis of the RNAseq data – especially in the context of lncRNA identification – was more complex than initially estimated. We therefore teamed up with two groups of bioinformatics experts that supported our analysis. Although the initial analysis has been finished, we still work on the data at the moment to retrieve even more lincRNAs of potential interest.

Second, we realized that the use of shRNAs to knock-down lincRNA candidates of interest, although widely used in the field[2], only provides relatively poor results in our experimental system. Knockdown efficiencies for the majority of the tested shRNAs were rather low, that is, individual lincRNA transcripts were only marginally reduced. Moreover, we observed severe off-target effects for the majority of shRNAs. Both phenomena, poor efficiency and massive off-targeting, make it difficult to establish strong links between a lincRNA knockdown and an observed biological effect.

In consequence, we have established and adopted a new technique, CRISPR/Cas9-mediated genome editing, for lincRNA knockout in our B cell lines. In contrast to conventional CRISPR/Cas9-approaches on coding genes, lincRNA function cannot be abrogated by the insertion of Indel-mutations, since they do not contain an open reading frame that can be disrupted. We therefore developed tools that allow us to insert larger genomic deletions, thereby completely abolishing lincRNA expression and thus function. Initial results in this context have been so promising that we now plan to use this system to systematically knock-out several lincRNA candidates *in vitro* to assess their functional relevance.

*Conclusion and Outlook*

In this project, we have used RNAseq and bioinformatics tools to describe and characterize the lincRNA transcriptome in murine B cell development. Our analysis gave rise to some interesting candidate genes that we have started to investigate in more detail. Preliminary data suggest that at least some of these lincRNA genes are functionally relevant in lymphocyte development and possibly also immune function, warranting a thorough investigation.

**Figure 1**

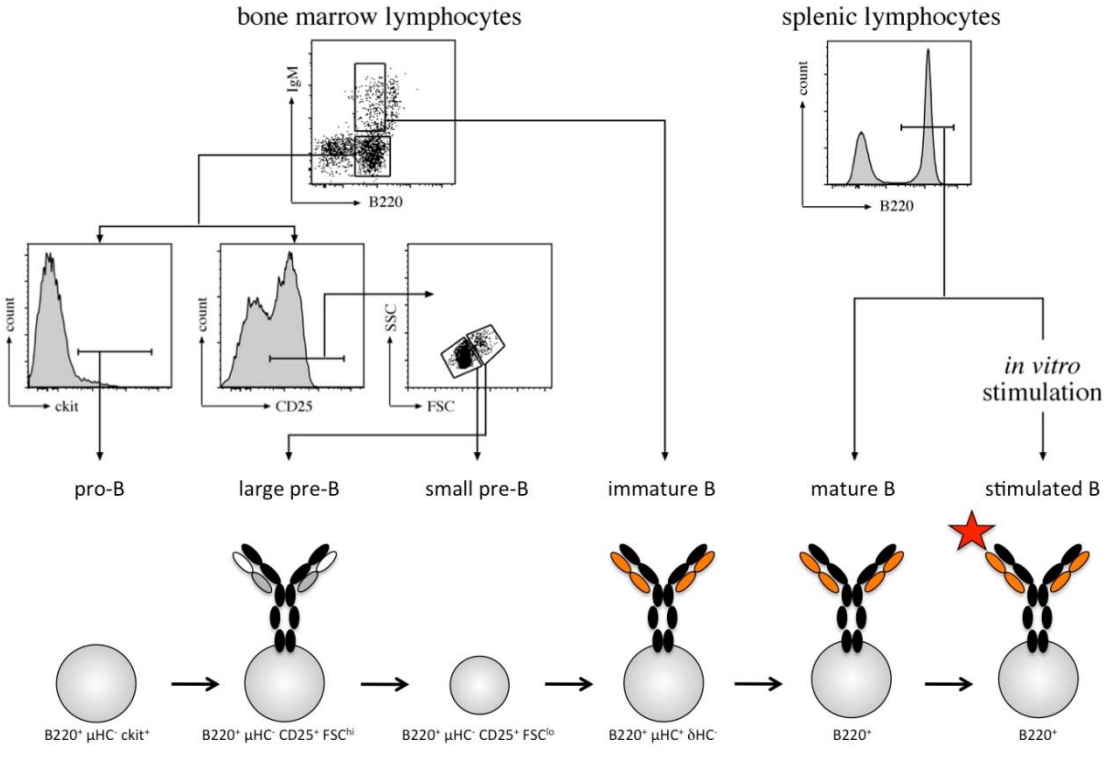


**Figure 1:** Sorting scheme for the different developmental B cell stages

B lymphocytes were sorted from the bone marrow and spleen according to their expression of distinct cell surface markers. The transcriptome of each sorted populations was analyzed by RNA sequencing.

## Figure 2

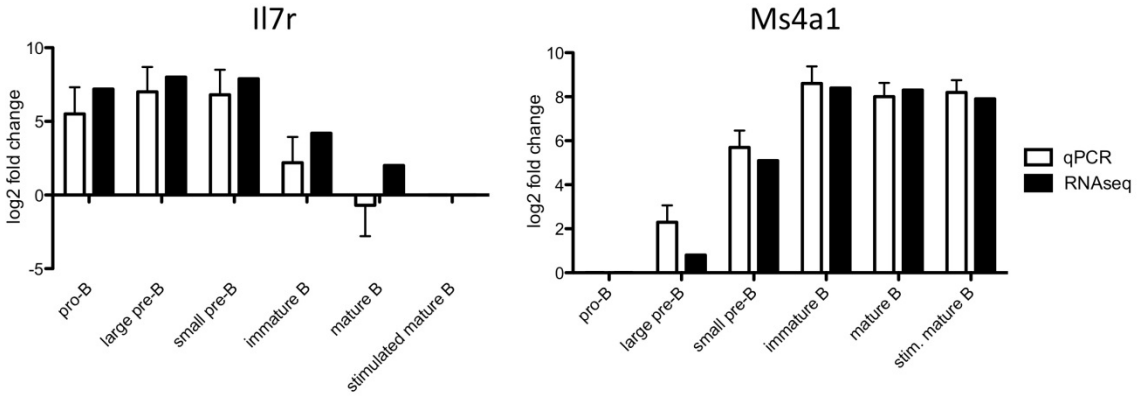



**Figure 2:** High correlation between RNAseq and qPCR data

In analogy to the RNAseq experiment, B cell populations sorted from 4 mice were analyzed for the expression of selected genes by qPCR (only a subset of the tested genes is shown here). In comparison with the expression values as predicted by RNAseq, qPCR analysis revealed nearly identical expression patterns for the IL-7 receptor alpha chain (Il7R; turned off in latter B cell stages) and Ms4a1 (turned on during B cell development), indicating a high correlation between both methods. Data were normalized to stimulated mature B cells (Il7R) and pro-B cells (Ms4a1), respectively, and log2-transformed.

## Figure 3

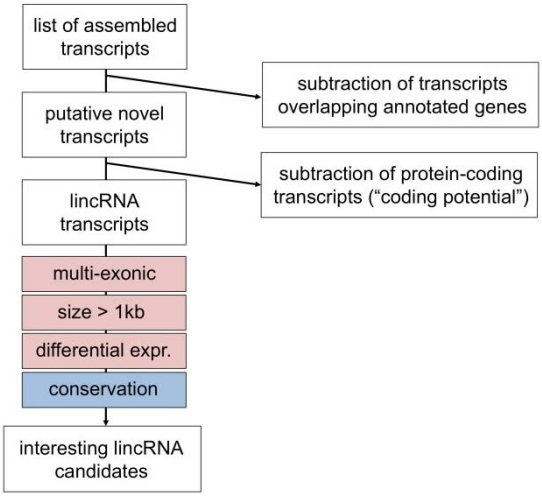


**Figure 3:** Overview of the bioinformatics pipeline used for lncRNA identification

**Figure 4**

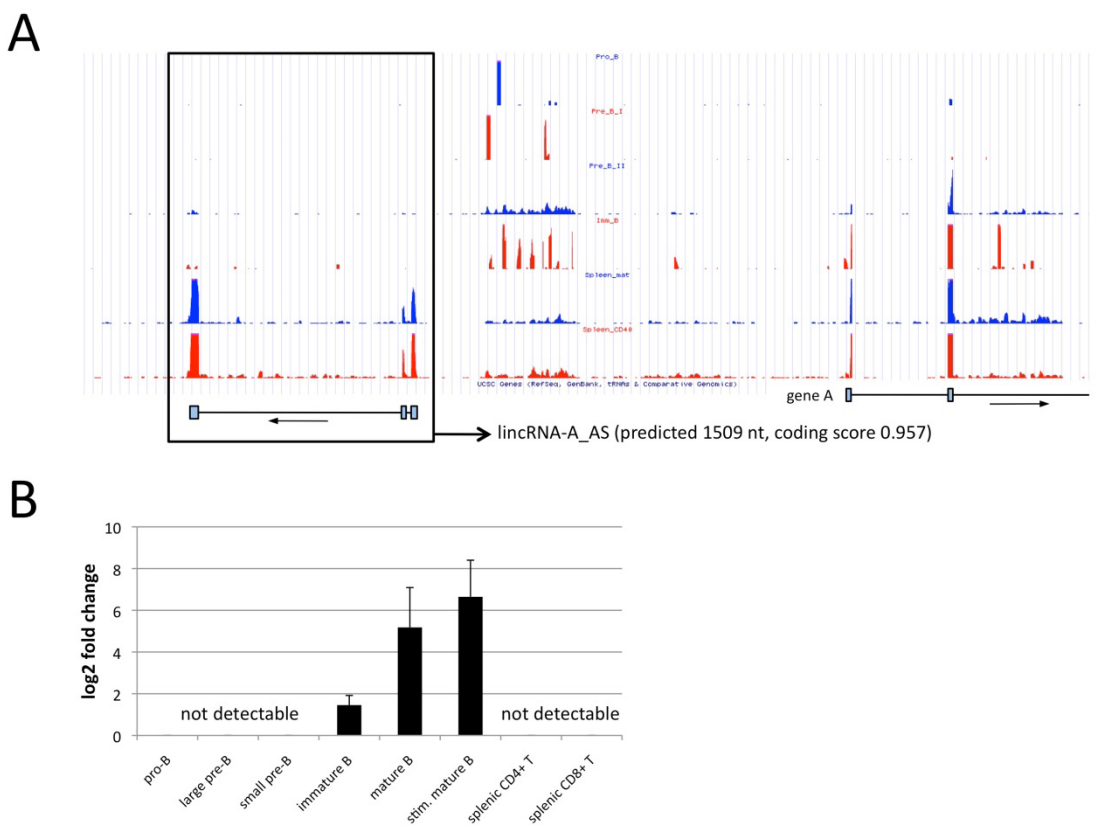



**Figure 4** Expression of lincRNA-A_AS is restricted to mature B cells

A. Mapped reads identify three exons encoding lincRNA-A_AS in the proximity of gene A. Lanes (from top to bottom) correspond to pro-B, large and small pre-B, immature B and mature B (non-stimulated and stimulated) cells, respectively. Individual exons and transcript orientation are marked by boxes and arrows. With a score of 0.957 (scores below 0.8 are considered as evidence for a coding transcript), lincRNA-A_AS is most likely non-coding. B. Quantitative PCR confirms the restricted expression of lincRNA-A_AS to mature B cells. Note that RNAseq-independent cDNAs were used as PCR templates.

## References


1. Herzog, S., Reth, M. & Jumaa, H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. *Nat. Rev. Immunol.* **9,** 195–205 (2009).

2. Guttman, M. *et al.* lincRNAs act in the circuitry controlling pluripotency and differentiation. *Nature* **477,** 295–300 (2011).

3. Rinn, J. L. & Chang, H. Y. Genome Regulation by Long Noncoding RNAs. *Annu. Rev. Biochem.* **81,** 145–166 (2012).

4. Hu, G. *et al.* Expression and regulation of intergenic long noncoding RNAs during T cell development and differentiation. *Nat. Immunol.* (2013). doi:10.1038/ni.2712

5. Carpenter, S. *et al.* A long noncoding RNA mediates both activation and repression of

immune response genes. *Science* **341,** 789–792 (2013).

6. Cabili, M. N. *et al.* Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses. *Genes & Development* **25,** 1915–1927 (2011).

7. Sun, K. *et al.* iSeeRNA: identification of long intergenic non-coding RNA transcripts from transcriptome sequencing data. *BMC Genomics* **14 Suppl 2,** S7 (2013).

## Publications issued from this project

Knackmuss, U.; Lindner, S. E.; Aneichyk, T.; Kotkamp, B.; Knust, Z.; Villunger, A.; Herzog, S. (2015): MAP3K11 is a tumor suppressor targeted by the oncomiR miR-125b in early B cells. In: *Cell Death and Differentiation* 23 (2), S. 242–252. DOI: 10.1038/cdd.2015.87.

## External funding

Systematic analysis of the miR-26 family in lymphocyte development and cancer (TWF-2014-1-17). Amount granted: € 26.500,00.

Molecular regulation of the oncogenic miR-17-92 cluster (FWF P 30194). Amount granted: € 297.304,89.

Unraveling miR-15 function in health and disease (FWF P 30196). Amount granted: € 364.019,25.

## Michiel Langeslag - FABRYpain: Understanding pain in Fabry disease

*Division of Physiology*

### 4. Funding period

**Project duration**: 01.08.2013 – 31.08.2015

### Project summary

Fabry disease is a life-limiting genetic metabolic disorder caused by a deficiency of lysosomal alpha-galactosidase A activity. A recent screening study in Austria discovered Fabry associated mutations in 1 out of 4000 births. The mutations lead to progressive accumulation of glycolipids in numerous cell types including neurons. First symptoms include chronic burning pain with attacks of excruciating pain and sensory losses. Enzyme replacement therapy improves the severity of the systemic disease, however is insufficient to treat the persisting pain.

We have used mice deficient of alpha-Galactosidase A ($Gla^{-/0}$) that serves as a genetic model of Fabry disease to investigate Fabry related functional changes in nociceptive neurons. We have investigated thermal and mechanical nociception and locomotive performance in juvenile to adult stages in age-matched $Gla^{-/0}$ and wildtype mice. The Fabry-diseased juvenile mice only showed mechanical allodynia. Surprisingly, the adult Fabry-diseased mice displayed mechanical and thermal hyposensitivity. No differences in locomotion and behavior were found between wild-type and Fabry-diseased mice in all ages investigated. In contrast to the behavioral data, free nerve endings of nociceptive C-fibers in the skin of adult Fabry-mice did not show any alterations when they were challenged by mechanical and thermal stimuli compared to age-matched wild-types. Cultured sensory neurons served as a model for electrophysiological recordings that were specifically designed to reveal functional alterations of Gla depletion. In accordance to the skin nerve recordings, we did not find a difference between heat-activated currents in cultured $Gla^{-/0}$ and wildtype nociceptors. The loss of heat perception in $Gla^{-/0}$ mice contradicts with the unchanged heat-sensitivity of $Gla^{-/0}$ nociceptors in culture, which implies another mechanism than altered properties of heat-sensitive ion channels in $Gla^{-/0}$ mice.

The evoked action potentials recorded from juvenile and adult $Gla^{-/0}$ nociceptive neurons were indistinguishable from the evoked action potentials of age-matched controls. However, the frequency of action potential firing of $Gla^{-/0}$ nociceptors from adult mice is significantly increased. Analysis of current-voltage relations of peak-inward currents and sustained-outward currents revealed significant changes in $Gla^{-/0}$ nociceptors. The increased hyperexcitability of $Gla^{-/0}$ nociceptors from adult mice could be caused by changed ionic conductances observed in these nociceptors. The results collected within this project suggest that electrophysiological signatures were different in Gla-deficient neurons and these could be a cause of spontaneous pain attacks and altered nociception observed in Fabry patients.

A manuscript that describes the alterations in the pain pathway underlying deficits in pain processing associated with FD that have been collected with support from the MUI-Start grant are being prepared for publication in Neuron or similarly high ranked journal. Furthermore, based on our findings I have applied for a stand-alone grant at the Fonds zur Förderung der wissenschaftlichen Forschung (FWF). Therefore the “summarized” data (marked in yellow) is not eligible for publication in any form at this moment.

## Publications issued from this project

Manuscript for publication will be prepared soon.

## External funding

Applied for a stand-alone FWF grant (P 28989-B26). Unfortunately the application was rejected.

## Hannes Neuwirt - Complement system and MAPK signaling in calcineurin-inhibitor induced nephrotoxicity

*University Hospital for Internal Medicine IV*

**4. Funding period**

**Project duration:** 01.10.2013 – 30.09.2015

**Project summary:**

The current project consisted of the following **aims:**

Aim 1. Investigate expression of complement components after CsA and FK506 treatment.

Aim 2. Evaluate MAPK-involvement in complement system regulation in vitro.

Aim 3. Investigate the role of SOCS-3 in MAPK regulation in vitro.

Aim 4. Investigate complement activation after CsA/FK506 treatment under conditions with altered MAPK signaling in vitro.

**Background**: The gap between organ demand and supply for transplantation is getting wider. One way to improve the situation is to prolong allograft survival. One entity that significantly contributes to renal allograft loss is calcineurin inhibitor (CNI) nephrotoxicity (CIN). Various mechanisms are discussed to play a role in CIN pathogenesis, one of which is complement mediated injury.

**Purpose**: To investigate the impact of CNIs on MAPK signaling, complement regulators and complement activation.

**Methods / Results**: We have performed experiments utilizing two proximal tubule cell lines of human origin, HK2 and hTERT-RPTCs. CyclosporinA (CyA) and tacrolimus (FK506, 10 µM each) treatment induced phosphorylation of MAPK1/-2 in both cell lines (Figure 1). This was associated with a significant decrease in protein levels of suppressor of cytokine signaling (SOCS)-3 (Figure 2). Since we have shown recently SOCS-3 acts as a negative regulator of MAPK1/-2 signaling, one might hypothesize that SOCS-3 downregulation enhances MAPK activation.

In order to screen for alterations in the complement system we performed qPCR- arrays assessing 15 different complement factors and –regulators (Figure 3). We found that most complement factors were upregulated upon treatment, whereas complement inhibitors DAF (CD55), MCP (CD46) and also SOCS-3 were significantly downregulated. The latter result was confirmed by Western blotting (Figure 4).

Figure 1

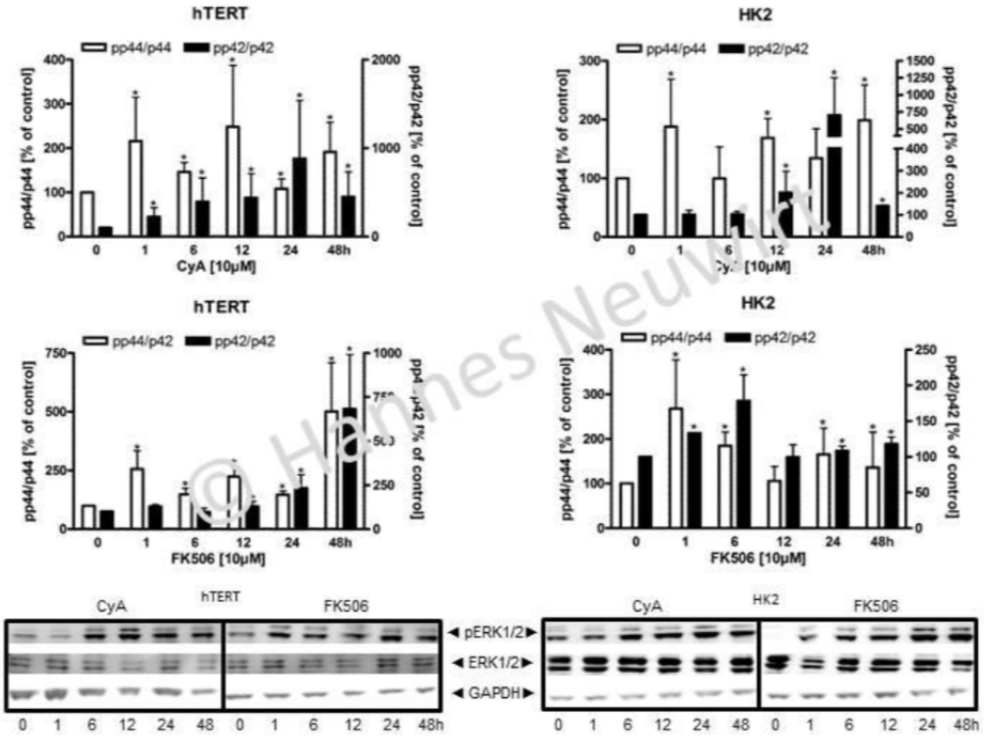





Figure 2

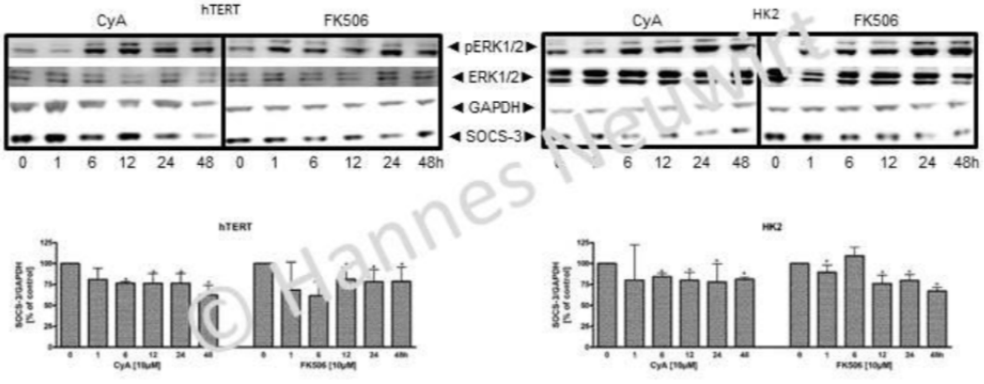


Figure 3

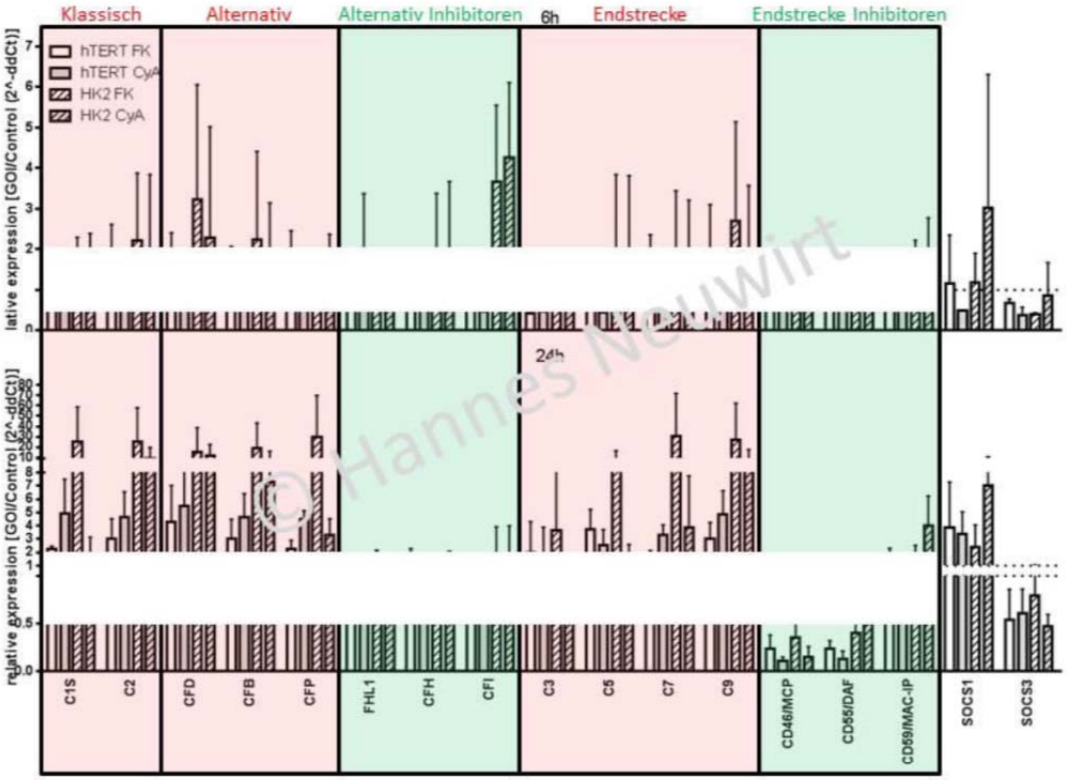
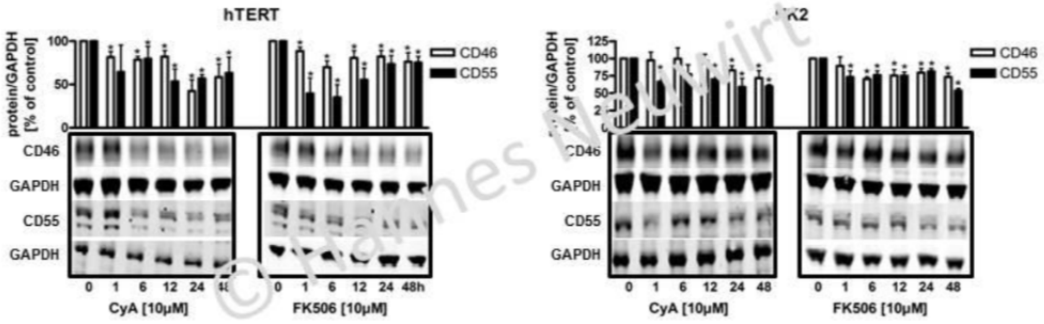


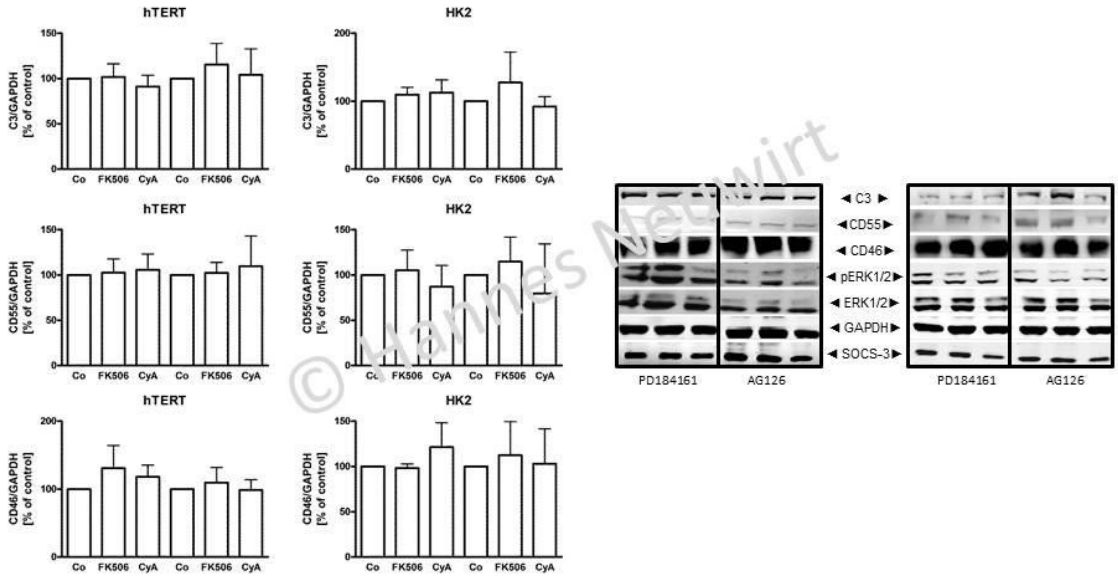

Figure 4




Next we wanted to establish a connection between CNI-induced MAPK phosphorylation and complement system. For this purpose, cells were treated with PD184161 and AG126, two selective inhibitors of MEK1/-2 and MAPK1/-2, respectively. Under these conditions

MAPKs were not phosphorylated. Moreover, expression of C3 (which is consumed during complement activation), DAF, MCP and SOCS-3 were not altered (Figure 5). Next we overexpressed and knocked down SOCS-3 via transient transfection with an overexpression plasmid (pBIG2i) and siRNA. SOCS-3 overexpression ameliorated CNI effects on MAPK and complement compared to vehicle treated cells, whereas knock-down of SOCS-3 enhanced the regulation (phosphorylation of MAPK and down-regulation of DAF, MCP and C3). Of note, SOCS-3 knock-down alone yielded an equal effect as CNI-treatment (Figure 6). Hence, we believe that a part of CIN may be attributed to dysregulated complement system activation by the SOCS-3-MAPK1/-2 signaling complex, which induces down regulation of complement regulators DAF/MCP.

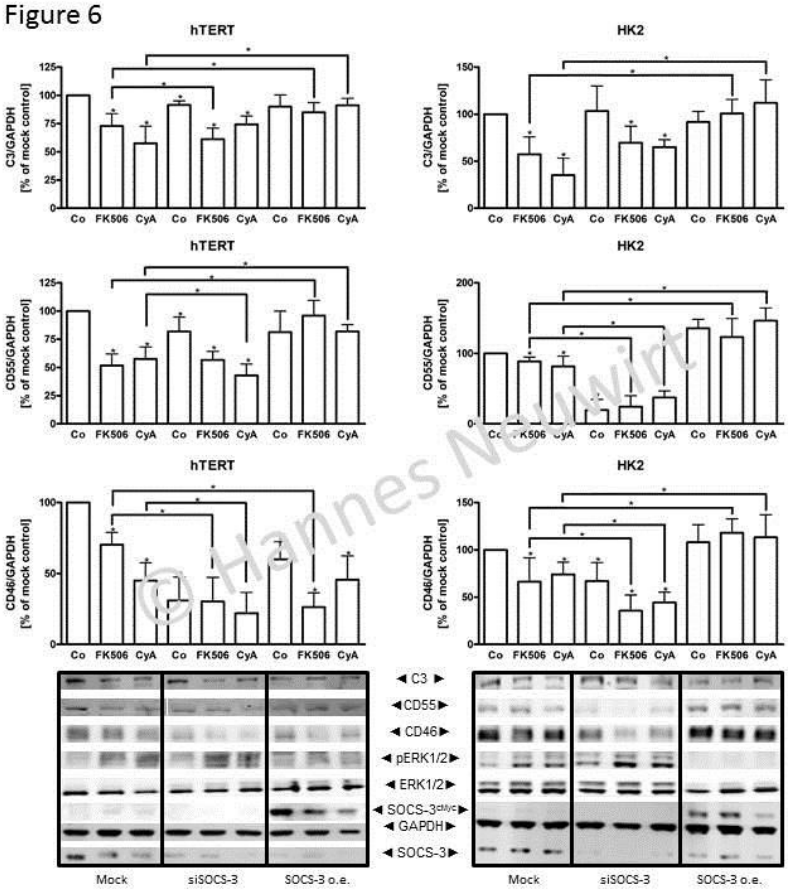
Figure 5






Finally, we found a significant upregulation of TCC (terminal complement complex) after CNI-treatment, as measured by ELISA. Cellular proliferation, as assessed by $^{3}$H-thymidine incorporation, was about 20% less in cells treated with CyA and FK506 in normal human serum (NHS, containing complement) compared to heat-inactivated normal human serum (HIS, complement factors denatured by heat). Thus, reduced cell growth could be attributed to complement system induced cell death (Figure 7).

Figure 6

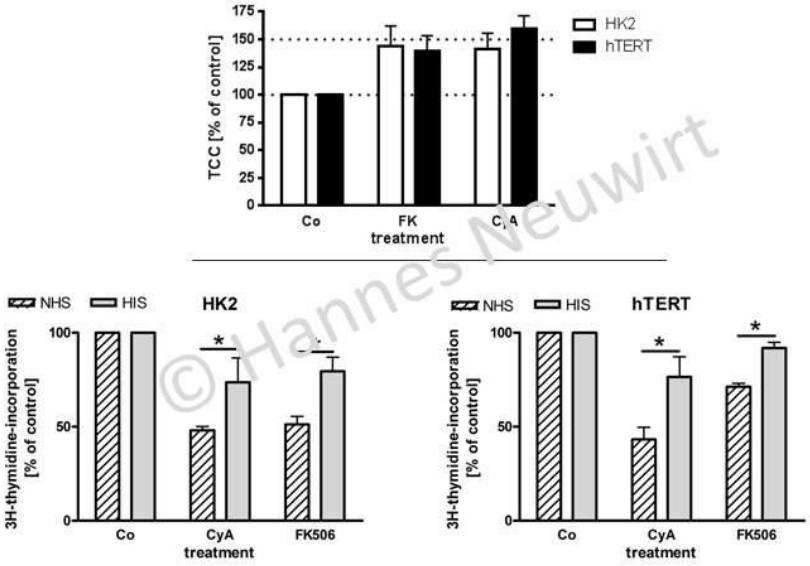





**Conclusion:** CNI-induced MAPK1/-2 phosphorylation is modulated by SOCS-3 (aim 1-3) and causes downregulation of complement inhibitors (aim 1&4) yielding to complement activation and growth inhibition in human proximal tubule cells (aim 1&4). This might be one additional pathomechanism of CIN development.

The applicant was not able to find PhD students to work on this project, possibly due to the limited amount of payment (8000,- per year). Hence, the experiments were executed by the applicant with help of two diploma students (Beatrix Löschenberger, Lea Niess), who were employed part time (geringfügig beschäftigt). This is the reason why we did not exploit the budget and did not perform $^{51}$Cr-release-assays (as proposed in aim 4) due to reason of time.

Figure 7






## Publications issued from this project

One publication is under revision for the Journal of Immunology

## External funding

A proposal entitled: “Suppressors of cytokine signaling -3 and -1 in regulation of STAT-driven renal carcinogenesis” was submitted to anniversary fund from National Bank of Austria. Unfortunately this proposal was not funded.

## Christa Pfeifhofer-Obermair - PKCtheta is essential for protective immunity against infection with *Salmonella enterica serovar Typhimurium*

*Division of Cell Genetics /Internal Medicine II*

**4. Funding period**

**Project duration:** 01.08.2013 – 30.08.2015

### Project summary

*Salmonella enterica* serovar *typhimurium* (*S. typhimurium*) is a Gram negative, facultative intracellular bacterium, which invades and multiplicates within mononuclear phagocytic cells in liver, spleen, lymph nodes, and Peyer`s Plaques. *S. typhimurium* causes severe gastrointestinal disorders in humans and typhoid fever with systemic infections in mice. Macrophages, as one of the first barriers of the innate immune system, try to rapidly control *S. typhimurium*, however, these bacteria can evade immune control by macrophages and even multiply within these cells by mechanisms which are insufficiently understood so far.

The Protein Kinase C family is a serine-threonine kinase family required for full T cell activation as well as for adaptive immune responses. Wild type mice and mice deficient for PKC were infected with *Salmonella enterica* serovar *typhimurium*. Survival, serum cytokines, and colony forming units in liver and spleen were analyzed. As an *in vitro* model we either infected bone marrow derived macrophages with *S. typhimurium* or stimulated them with lipopolysaccharide (LPS) + interferon-gamma. The capacity to kill the bacteria after infection was analyzed by measuring colony forming units, and gene expression and protein levels of pro- and anti-inflammatory cytokines were analyzed. PKC-deficient mice fail to mount appropriate innate immune responses determined by a markedly decreased survival combined with a significantly enhanced number of bacteria in spleen and liver when compared to wildtype mice. This is paralleled by a significant increase in interleukin-10 serum levels in PKC-deficient mice which was also confirmed *in vitro* when challenging bone marrow derived macrophages with *S. typhimurium* or LPS + interferon-gamma. Our data indicate that PKC are important to mount an appropriate immune response in macrophages challenged with Gram negative bacteria *Salmonella enterica* serovar *typhimurium* by regulating the production of the anti-inflammatory cytokine interleukin-10. The elucidation of underlying molecular mechanisms are in the focus of our current research.

### Publications issued from this project

Pfeifhofer-Obermair, Christa; Albrecht-Schgoer, Karin; Peer, Sebastian; Nairz, Manfred; Siegmund, Kerstin; Klepsch, Victoria et al. (2016): Role of PKCtheta in macrophage-mediated immune response to Salmonella typhimurium infection in mice. In: *Cell Communication and Signaling: CCS* 14, S. 14. DOI: 10.1186/s12964-016-0137-y.

### External funding

PKCtheta exerts a necessary role in *Il10* gene suppression in inflammatory macrophages during host-protective bacterial immunity"; FWF-Projekt; 2 Mal abgelehnt; wird neu überarbeitet.

## Oliver Schmidt - The functional characterization of golgi tethering factors in a stress response to defective membrane protein degradation

*Division of Cell Biology*

### 4. Funding period

**Project duration:** 01.08.2013 – 31.07.2015

### Project summary

Failure of protein degradation and subsequent accumulation or misfolding of proteins leads to deleterious cellular phenotypes, causing a wide range of diseases including cancer and neurodegeneration. When membrane proteins at the cell surface become activated or damaged, they are selectively internalized, and transported into lysosomes for degradation. This process critically involves the sorting of membrane proteins into small (25 nm) intralumenal vesicles on specialized endosomes termed multivesicular bodies by the ESCRT machinery (endosomal sorting complexes required for transport).

The MUI START project was based on a genome-wide synthetic genetic array screen identifying genes required for survival of yeast cells under conditions of chronic membrane protein accumulation in ESCRT mutants. These genes were cumulatively hypothesized to be involved in an 'endosomal stress response' mitigating the deleterious effects of membrane protein accumulation. We identified a large number of genes encoding for proteins involved in membrane trafficking, including multiple subunits of two membrane tethering complexes: the ¬conserved ¬oligomeric golgi (COG) complex and the golgi-associated retrograde protein (GARP) complex. These tethering complexes mediate fusion of retrograde vesicles in subcompartments of the golgi apparatus, and of retrograde transport vesicles trafficking from endosomes/multivesicular bodies to the trans golgi network, respectively. We hypothesized an involvement of these complexes in ESCRT-dependent endosomal sorting, and especially in the formation of 'class E' compartments, which constitute aberrant endosomal membrane structures serving as an intracellular 'sink' for non-degradable membrane proteins in ESCRT mutants. Phenotypic analysis of deletion mutants of the COG complex subunits Cog5 and Cog6, however, revealed that this complex did not influence endosomal sorting of a typical ESCRT substrate in general. In addition, 'class E' compartment formation was indistinguishable in ESCRT/COG double mutants, refuting our initial hypothesis. We found that ESCRT and COG double mutants had a synthetic growth defect under nutrient limiting growth conditions. The COG complex is known to be required for autophagy (Yen et al., 2010), a catabolic process activated specifically during nutrient starvation to non-specifically degrade cellular material. Interestingly, we recently demonstrated an important catabolic function of specific ESCRT-dependent membrane protein degradation along the multivesicular body pathway during early nitrogen starvation, which happens prior to and is essential for the development of a strong autophagic response (Müller, Schmidt et al., 2015). We observed a similar synthetic growth and survival defect of ESCRT mutants with core components of the autophagy machinery under nutrient limitation. We therefore assume that the genetic interaction of ESCRTs with the COG complex, which is not observed during growth on rich medium, might be caused by involvement of the latter in autophagosome formation during autophagy.

In case of the GARP complex mediating endosome to golgi trafficking we observed a different situation. GARP mutants showed reduced growth and severe aberrations of lysosomal morphology (Conibear and Stevens, 1999). GARP/ESCRT double mutants showed a prominent synthetic growth defect at all tested growth conditions, and were unable to concentrate membrane proteins in 'class E' compartments. We are currently aiming at consolidating these findings for a first publication and for the application of an FWF stand–alone project. We plan to apply for follow-up funding to characterize additional mutants identified in the screen to better understand the molecular mechanism regulating an endosomal stress response.

Part of the results of this project is not included in this final report because they are still not published and this is a highly competitive research field.

## Publications issued from this project

Müller, Martin*; Schmidt, Oliver*; Angelova, Mihaela; Faserl, Klaus; Weys, Sabine; Kremser, Leopold et al. (2015): The coordinated action of the MVB pathway and autophagy ensures cell survival during starvation. In: *eLife* 4, S. e07736. DOI: 10.7554/eLife.07736. (*equal contribution)

## External funding

Eine Förderung durch den Tiroler Wissenschaftsfond zur Weiterführung des Projekts wurde zum 1. Januar 2015 bewilligt.

Die MUI START-Förderung ermöglichte zudem einen Antrag auf Weiterförderung im Rahmen eines 'EMBO advanced fellowships'. Dieser wurde 2014 zunächst positiv beurteilt, in der finalen Auswahlrunde jedoch nicht bewilligt.

Antrag für ein FWF stand-alone Projekt ist in Vorbereitung, jedoch noch nicht erfolgt.

## Ramon Tasan - Characterization of Neurokinin B Neurons in the Amygdala and their role in Anxiety and Fear

*Institute of Pharmacology*

**4. Funding period**

**Project duration:** 01.08.2013 – 31.08.2015

### Project summary

Anxiety disorders constitute a major burden for the society and are characterized by dys-regulation of anxiety and fear. Among the different brain areas involved in the encoding and modulation of fear memories, the amygdala plays an exceptionally important role. Intrinsic and extrinsic amygdala connections are predominantly mediated by glutamate and GABA, but the resulting behavioral response is fundamentally shaped by various neuromodulators, including different neuropeptides. Despite extensive investigations, the role of many of these neuropeptides is still poorly understood. Among the neuropeptide family of tachykinins, substance P is promoting anxiety-related behavior, whereas the role of neurokinin B (NKB) that is abundantly expressed in different nuclei of the extended amygdala is not clear yet.

**Methods**

We aimed for a detailed characterization of NKB-expressing neurons in the extended amygdala by combining several complementary approaches that focus on the function of NKB neurons in the basolateral amygdala (BLA), the central amygdala (CEA) and in the bed nucleus of the stria terminalis (BNST):

**Structural approach**

Immunohistochemistry and *in situ* hybridization were combined with genetic neuronal tract tracing to elucidate the localization, neurochemical content and the projection targets of NKB-expressing neurons in the adult mouse brain.

**Functional approach**

We combined *ex vivo* electrophysiology in amygdala slices with viral vector injections and optogenetics to elucidate the role of NKB and NKB-expressing neurons in microcircuitries of the extended amygdala.

**Behavioral relevance for fear processing**

To investigate the behavioral relevance of NKB and NKB-expressing neurons in fear conditioning and fear extinction we combined local injections of viral vectors and permanent silencing or chemogenetic activation of defined neuronal populations.

**Results of the structural approach**

*(1) Immunohistochemical characterization of amygdala NKB neurons*

The amygdala complex consists of several different nuclei with highly specialized functions. Thus, fear-related signaling is entering *via* the lateral nucleus and the BLA and then transmitted to the central nucleus (CEA) as major output nucleus. The CEA connects directly

or indirectly *via* the BNST to downstream brain areas that are relevant for the expression of a coordinated fear response. These brain regions include different hypothalamic nuclei, midbrain areas, such as the periaqueductal grey, as well as different brain stem nuclei. On a neuronal level, fear-related stimuli are transmitted via glutamatergic principal neurons, while a diversity of inhibitory GABA-ergic neurons are controlling and suppressing excitatory activity. Thus in general, inhibition of GABA-ergic neurons results in an increased fear response. Interestingly, NKB was confined to GABA-ergic neurons in the BLA, CEA and in the BNST. According to the neurochemical content, GABA neurons may be subdivided into several distinct subclasses. In the BLA, NKB was expressed in GABA neurons that also expressed calretinin (CR) and vasoactive intestinal peptide (VIP). In the CEA, NKB was additionally detected in cortocotropin (CRF) and somatostatin neurons.

*(2) Genetic neuronal tract tracing of amygdala NKB neurons*

GABA neurons are well known for their role as local interneurons, in particular those that also express neuropeptides. Interestingly, our preliminary data demonstrate that NKB neurons in the CEA and specifically in the BNST are also GABA-ergic projection neurons that connect to hypothalamic nuclei, to midbrain areas and even to more distant projection targets of the brainstem. We mapped in detailed the different projections of amygdala BNST neurons throughout the mouse brain and we demonstrated the functionality of these projections by combining *in vivo* DREADD (designer-receptor activated by designer drugs)-mediated neuronal activation of specific NKB neurons with *ex vivo* immediate early gene labeling.

**Results of the functional approach**

*(1) Electrophysiological characterization of NKB-expressing BLA neurons*

To examine the functional properties of NKB-expressing neurons in the BLA, we bilaterally infused a Cre-inducible recombinant adeno-associated viral (rAAV) vector expressing the excitatory opsin ChETA and an eYFP reporter (rAAV-ChETA-eYFP) into the BLA of Tac2-Cre mice. This gave us the possibility to specifically record *ex vivo* from NKB neurons and to excite these neurons by light of a specific wavelength. Whole-cell patch clamp recordings were obtained from eYFP-negative neurons and eYFP-positive (=NKB-expressing) BLA neurons. Glutamatergic principal neurons (pyramidal-like) and inhibitory GABAergic interneurons are the main cell types of the BLA and are to some degree distinguishable based upon their distinct action potential characteristics. Depolarizing current pulses generated action potentials with “regular” spike-firing patterns with a range of frequency adaptation in 83% of eYFP-negative neurons and 36% of eYFP-positive neurons. These characteristics are considered hallmarks of BLA pyramidal-like neurons, however subtypes of BLA interneurons may also exhibit regular spiking. 64% of eYFP-positive neurons exhibited fast-spiking action potentials without accommodation, shorter action potential duration and larger afterhyperpolarizations typical of BLA inhibitory interneurons. Thus, NKB-expressing BLA neurons can be divided into fast-spiking and regular-spiking neurons based on their firing properties.

*(2) NKB-expressing BLA neurons receive excitatory input from the thalamus and cortex and provide local inhibition*

Coordinated synaptic input from the cortex and thalamus to the BLA is essential for associative fear learning, but how these pathways modify the activity of NKB-expressing neurons is unknown. To determine whether NKB-expressing neurons receive excitatory input from the cortex and thalamus, we obtained whole-cell voltage-clamp recordings from eYFP-positive BLA neurons (=NKB neurons in TAC2-Cre mice) and evoked excitatory postsynaptic currents (evEPSCs) by electrical stimulation of the cortical and thalamic pathways. In all recorded neurons, cortical and thalamic stimulation resulted in mono- or polysynaptic EPSCs, demonstrating that cortical and thalamic inputs provide excitatory input to NKB-expressing neurons. Polysynaptic EPSCs were often observed and may indicate that NKB-expressing neurons also receive excitatory input from glutamatergic BLA neurons activated by cortical and thalamic stimulation.

Local interneurons are an important source of inhibition within the BLA and have an essential role in modulating synaptic plasticity and fear learning. To determine whether NKB-expressing neurons provide inhibition within the BLA we utilized the optogenetic opsin ChETA. In a preliminary experiment we performed whole-cell current-clamp recordings demonstrating that light pulses delivered through the microscope objective (470 nm, 1-50 ms) induced action potentials in all recorded ChETA-eYFP-positive neurons. We next performed whole-cell voltage-clamp recordings from eYFP-negative BLA neurons and evoked neurotransmitter release from NKB-expressing neurons using brief light pulses (470 nm, 1 ms). Picrotoxin-sensitive inhibitory postsynaptic currents (evIPSCs) were observed in 41% of eYFP-negative BLA neurons with an average latency of 4.6 ± 0.5 ms, providing strong evidence that NKB-expressing neurons provide local inhibition within the BLA.

*(3) NKB increases inhibition in the BLA*

Since NKB-expressing neurons provide GABAergic inhibition in the BLA, it is likely that they also release NKB locally. How NKB influences synaptic transmission in the BLA has not been previously described. We thus recorded spontaneous inhibitory postsynaptic currents (sIPSCs) in BLA neurons in the absence and presence of NKB. Bath application of NKB (200 nM) increased the frequency of sIPSCs but did not alter the amplitude of events. The increase in sIPSC frequency was not blocked by pre-incubation with either the NK1 receptor antagonist L732,138 (10 μM) or the NK3 receptor antagonist SB222200 (5 μM). However, co-application of the NK1 and NK3 receptor antagonists blocked the increase in sIPSC frequency caused by NKB. In support of this finding, previous studies have shown that both the NK1 and NK3 receptor depolarize neurons and increase action potential firing. To further elucidate how NKB increases the frequency of sIPSCs, we recorded miniature IPSCs (mIPSCs) in the presence of the voltage-gated Na+ channel blocker, Tetrodotoxin (1 μM). mIPSCs in BLA neurons were unaltered by NKB. Taken together these results indicate that NKB increases GABAergic signaling in the BLA through an action potential-dependent mechanism.

**Results regarding the behavioral relevance of NKB neurons for fear processing**

Considering the above-mentioned characterization of amygdala NKB neurons on a structural and functional level, we aimed at correlating these results with behavioral consequences of NKB neuron activation during fear conditioning and extinction. Specific silencing of NKB neurons in the BLA by injection of a rAAV vector expressing tetanus-light chain (rAAV-TeLC) in TAC2-Cre mice, did not alter the acquisition or extinction of conditioned fear. However, it

promoted the ability to differentiate between fear-related and unrelated stimuli. This is an important finding, since generalization of fear is a hallmark of human anxiety disorders and frequently associated with posttraumatic stress disorders (PTSD). In contrast to NKB neurons, suppressing the activity of all subgroups of BLA GABA neurons enhanced the generalization of fear. These data indicate that an NKB-expressing subpopulation of GABA neurons in the BLA has an opposite function to GABA neurons in general and raises the question of how these neurons are integrated in the local neuronal network of the BLA-

In the CEA, silencing of NKB neurons reduced the expression of conditioned fear. Regarding the BNST, we activated NKB neurons specifically before fear testing by DREADD technology and consecutive fear extinction. As expected, activation of NKB neurons in the BNST enhanced the expression of a conditioned fear memory during fear recall. Unexpectedly, however, the extinction of cued fear was accelerated. This, at first sight contradicting results, may have several reasons that we plan to explore in a follow-up investigation. Firstly, NKB is frequently co-localized with CRF, another anxiogenic neuropeptide. The corticotropin system is known to enhance the expression of fear, however, application of corticotropin also promotes fear extinction in rodents and humans. A similar function may be proposed for the co-localized NKB. Secondly, we demonstrated that BNST NKB neurons display a highly diversified projection pattern throughout the mouse brain and different populations of BNST NKB neurons with different projection targets may generate highly specific behavioral patterns. Clearly, further studies are warranted to elucidate the function of NKB neurons in the extended amygdala.

**Conclusions**

Together our data indicate that NKB-expressing neurons constitute a specific GABAergic population with highly specified functions. Activation of NKB neurons in the CEA and BNST increases fear expression. Activation of NKB neurons in the BLA, on the other hand, results in a generalization of fear. In the BLA, NKB, released from CR/VIP neurons, increases GABAergic signaling through an action potential-dependent mechanism *via* both, NK1 and NK3 receptors. Further studies will pinpoint the exact position of NKB neurons in the neuronal circuitry of fear and fear extinction. It is, however, already evident form our data that targeting the NKB system, for instance by blocking both, NK3 and also NK1 receptors, will suppress the expression of fear and inhibit fear generalization, both hallmarks of PTSD.

## Publications issued from this project

Two publications are in preparation and will be submitted.

Also a diploma thesis (Stefan Pauly, MUI) was conducted in the frame of the project.

## External funding

Role of neurokinin B-expressing neurons in the bed nucleus of the stria terminalis (FWF P 29952). Amount granted € 399.441,00.

## Luca Fava - Caspase-2 in cell death induced by polyploidization

*Division of Developmental Immunology*

**5. Funding period**

**Project duration:** 01.10.2014 – 30.09.2016

### Project summary

The project proposal I submitted in March 2014 was built on the preliminary observation that HeLa cells exposed to agents inhibiting cytokinesis triggered cell death in a Caspase-2 dependent fashion. Given the fact that the physiological function of Caspase-2 and its relationship with other proteases of the Caspase family was hotly debated, I proposed to exploit the strict dependency on Caspase-2 within the above mentioned cell death paradigm to better define the epistatic relationship between Caspase-2 and other apoptotic regulators. Initially I aimed to test whether cell types different to HeLa behaved similarly when exposed to cytokinesis inhibitors. Strikingly, a broad range of cell lines that retained functionality of the p53 pathway showed signs of Caspase-2 activation in response to cytokinesis failure with no measurable apoptosis (addressing original Aim 2). Rather, Caspase-2 activation appeared to trigger a selective cleavage of MDM2, a known negative regulator of p53 (addressing Aim 4). The series of experiments that I carried out delineated therefore a novel pathway in which Caspase-2 acts as a p53 activator in response to cytokinesis failure.

While the fact that cytokinesis failure can trigger p53 activation and subsequent cell cycle arrest was known since the 1970s, neither the upstream activators nor the signals arising in polyploid cells leading to p53 activation were known. Caspase-2 activation in response to cytokinesis failure appeared dependent on the protein PIDD1 and RAIDD, that are the members of the so-called PIDDosome. Thus, we could show that the PIDDosome defines a p53-activating pathway in response to cytokinesis failure that is distinct from the DNA damage response and other means of perturbing cell division. Interestingly, the PIDDosome appeared as a necessary activator of p53 in hepatocytes in the polyploidization process occurring during liver organogenesis. This work did not only show that the contribution of the PIDDosome upstream of p53 in response to cytokinesis failure is conserved across species (Aim 3), but also led to the description of the first discernible phenotype observed in PIDDosome deficient mouse models, i.e. an increased polyploidization of liver hepatocytes comparable to the one observed in p53 deficient animals.

Finally, I aimed to identify the molecular cues that are responsible for PIDDosome activation following cytokinesis failure and focused on increase ploidy as opposed to increase centrosome numbers (Aim 1). On one hand increasing ploidy in the absence of extra centrosome did not trigger PIDDosome activation, but on the other hand increasing the number of centrosomes in the absence of increased ploidy was sufficient to trigger PIDDosome activation. While extra centrosomes can alter cell physiology by several means, the facts that PIDD1 localizes to extra centrosomes and that perturbing this localization impinges on pathway activation are consistent with the notion that the PIDDosome directly responds to the presence of extra centrosomes.

All the data described above have been incorporated into a recent publication, ***(Fava et al., 2017)***. In the near future I will establish my research group at the Centre for Integrative

Biology of the University of Trento, where I will focus on the mechanistic aspects of the signalling connecting extra centrosomes with PIDDosome activation.

### Publications issued from this project

Fava, L. L., Schuler, F., Sladky, V., Haschka, M. D., Soratroi, C., Eiterer, L., Demetz, E., Weiss, G., Geley, S., Nigg, E. A. and Villunger, A. (2017). The PIDDosome activates p53 in response to supernumerary centrosomes. *Genes & Development*, *31*(1), 34–45. http://doi.org/10.1101/gad.289728.116

### External funding

I have been recently notified the award of a research grant of 1 Million US Dollars by the Giovanni Armenise-Harvard foundation for the coming five years, titled “How do cells count their centrosomes? A mechanistic study”. This will serve as a basis for establishing my own laboratory at Centre for Integrative Biology of the University of Trento.

http://www.armeniseharvard.org/

http://www.cibio.unitn.it/

### Miscellaneous

In the last two years, I have attended two international congresses presenting the data related to the project:

- EMBO Workshop: Chromosome Segregation & aneuploidy (Galway, Ireland, June 2016, poster presentation)
- EMBO Workshop: Cell Death, Inflammation and Cancer (Obergurgl, Austria, January 2017, oral presentation)

I also supervised one Master Thesis related to the MUI-start project (Lisa Eiterer, MSc, who co-authored our publication).

## Gabriele von Gleissenthall - Tryptophan and kynurenine metabolism in alcohol dependent patients in acute and medium-term withdrawal

*University Hospital for Psychiatry I*

**5. Funding period**

**Project Duration:** 01.10.2014 – 30.09.2016

### Project summary

**Framework / aim of the current project:**

**Background:**

Chronic alcohol dependence constitutes a major disease burden in modern society. Although current treatments show a considerable success, the relapse rate in chronic alcohol dependent patients remains high. In order to efficiently treat alcohol dependency, it is vital to understand the underlying complex mechanisms of recovery during alcohol withdrawal. Recent research including our own study has suggested that tryptophan and kynurenine metabolism is profoundly disturbed during alcohol withdrawal both by immune-associated and cortisol-related mechanisms. Kynurenine production has been shown to be intensified during the alcohol withdrawal and to correlate with some alcohol-associated neuropsychiatric symptoms such as affective symptoms and sleep disturbances. The catabolism of kynurenine during alcohol withdrawal and in particular the role of its catabolites, which are substantially neuroactive (whether toxic or neuroprotective) are still unclear.

This study aims to investigate the dynamics of tryptophan metabolism and kynurenine catabolism in alcohol dependent patients during acute and medium-term alcohol withdrawal.

**Methods and Results:**

Thirty patients (23 male, 7 female) were enrolled, three male subjects had to be excluded (substance relapse or deliberate dropouts). The mean age was 44 years (range 25-62a), the mean alcohol consumption per day 154 g. The self-reporting alcohol consumption (Audit - Alcohol Use Disorders Identification Test) showed a mean score of 30 (score 0 - 40 points). Heavy alcohol consumption seems to involve moderate depressive symptoms. We observed moderately depressive symptoms at the beginning of the alcohol withdrawal (Beck depression inventory, BDI = 24). During the withdrawal these symptoms attenuated (BDI score 17 at day 14 and BDI: 9 after four weeks).

Tryptophan (TRP) decreased in the first three measurement points, than returned quite to the start point. Kynurenin (KYN) increased significant at timepoint 5. For kynurenic Acid (KA) and quinolinic acid (QA) there were no significant changes during the whole study period. Cortisol increased till measurement point 3. Neopterin was slightly increased but showed no significant changes during the study period.

We found indolamin 2-3 dioxygenase (IDO) activation at time point 4 and 5 as measured by correlation between neopterin and KYN/TRP ratio. There was no significant evidence for thryptophan 2,3-dioxygenase (TDO) activation as measured by means of correlation between the cortisol levels and KYN/TRP ratio. Neither neopterin vs. KA/ KYN ratio nor

cortisol vs. QA/ KYN ratio showed significant correlations. At measurement point 5 we found significant correlation between neopterin and the QA/ KYN ratio.

**Key findings:**

The activation of the kynurenin pathway through IDO activation during the acute alcohol withdrawal, as measured by correlation between neopterin and KYN/ TRP ratio. Cortisol levels did not correlate with the KYN/TRP ratio, meaning that IDO activation plays a more important role than TDO activity. The production of QA also seems to be induced by immune activation (neopterin versus QA/KYN ratio).

**Conclusion:**

Immune related enzymatic activation seems to play primary role in the intensified kynurenin production during acute alcohol withdrawal. Furthermore, the quinolinic acid arm of the kynurenin catabolism seems to be more activated by immune signals than the kynurenic acid arm.

**Short outlook:**

The potential effects of the quinolic acid pathway activation during the alcohol withdrawal may have relevant impact on outcome in alcohol dependent patients during the post withdrawal period. Given the eminent demand on new treatments for alcohol disease, these specific aspects of alcohol withdrawal warrant detailed investigation.

## Publications issued from this project

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## External funding

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## Johanna Gostner - Formaldehyde metabolism – on the role of formaldehyde in inflammation

*Division of Medical Biochemistry*

**Project duration**: 01.08.2014 – 31.12.2016

### Project summary

Formaldehyde (HCHO) is an important metabolic intermediate that is present in all kingdoms, it is an essential intermediate in the biosynthesis of purines, thymidine and certain amino acids [Neuberger, 1981]. In cellular metabolism, it is produced endogenously from serine, glycine, methionine and due to demethylation (both N-demethylation, O-demethylation and S-demethylation) [Kalasz, 2003]. In human blood, concentrations of formaldehyde range from 10 to 100 µM [Heck et al., 1982]. In aqueous systems, formaldehyde is almost exclusively hydrated (geminal diol). Free formaldehyde is an electrophile and can bind amine, sulfhydryl, and hydroxyl groups in proteins and DNA, forming adducts and crosslinks. Within the cell, formaldehyde is detoxified either by formation of hydroxymethylglutathione (HMG), which is oxidized by alcohol dehydrogenase 5 (ADH5) and subsequently hydrolysed to give formate, or it can be directly oxidized to formate via mitochondrial aldehyde dehydrogenase (ALDH2) [Kimbell et al., 2001]. Formate then enters the one-carbon pool and is consumed in different biochemical pathways.

Production of elevated levels of formaldehyde has been associated with different pathologies such as cancer [Tong et al., 2010] or neurodegeneration [Tupule et al., 2013]. Released formaldehyde can be destructive for the microenvironment, a property that might favour metastasis but could also be of relevance for pathogen defence. It is suggested that cells protect themselves by upregulation of enzymes involved in formaldehyde breakdown. Additionally, formaldehyde is a strong reducing agent and may be able to modulate immune responses by generating a reductive milieu. This project aimed to get a deeper insight into the endogenous formaldehyde metabolism, whereby an improved analytical method for the detection of formaldehyde in biological samples has been developed and the regulation of formaldehyde metabolism and associated cellular processes has been investigated in different cell types, either stimulated with pro-inflammatory mediators or treated with formaldehydogenic compounds.

### Main results and conclusions

Formaldehyde induced effects are strongly concentration (and time) dependent [Kaden et al., 2010], ranging from highly toxic and carcinogenic to irritant properties. It is suggested that formaldehyde toxicity begins when the cells´ metabolic capacity to catabolize formaldehyde is saturated [Gonzalez-Suarez et al., 2014]. In a first step, we were interested to determine the concentration range in which different cell types respond to the presence of formaldehyde. Both freshly isolated peripheral blood mononuclear cells (PBMC) and the myelomonocytic cell line THP-1 were used to investigate the effects on immune cells. The inflammation-inducible metabolic pathway of tryptophan degradation by the enzyme indoleamine 2,3-dioxygenase (IDO-1) was used as readout, as it is known that this immunoregulatory pathway is highly susceptible to modulation by chemicals [Gostner et al., 2015]. The estimated half maximal inhibitory concentrations for IDO-1 activity ($IC50_{IDO}$) were 51 and >200 µM in unstimulated and mitogen stimulated PBMC, respectively. IDO-1

suppression occurred at concentrations that preceded cellular toxicity, in particular in cells stimulated with pro-inflammatory mediators, indicating that this pathway could also be used as a sensitive readout for immunotoxicity [Gostner et al., 2016]. A similar, even more sensitive response pattern was observed in THP-1 cells stimulated or not with lipopolysaccharide (LPS). The cell viability was affected at lower treatment concentration in these cells resulting in a calculated $IC50_{viab}$ at 53 µM formaldehyde after 48h of treatment, whereby for PBMC a two times higher concentration was necessary for the same effect. For both cell cultures, upon stimulation the $IC50_{viab}$ was slightly higher. Other cell types, such as A549 lung adenocarcinoma cells, Hep G2 liver carcinoma cells, SH-SY5Y neuroblastoma cells were less sensitive e.g. the $IC50_{viab}$ after 48h formaldehyde treatment were 740 µM, 387µM and 187 µM, respectively.

To summarize, the formaldehyde concentrations at which cellular responses are initiated are highly dependent on the cell type, however, interestingly for immune cells these concentrations are in the range of concentrations measured in human serum. The results point towards a differential regulation of the formaldehyde detoxification machinery in the different cell types and also in dependence of the immunological and oxidative milieu.

In a second step, expression changes of genes involved in formaldehyde metabolism were investigated in stimulated and unstimulated immune cells. Stimulation of PBMC led to a shift of formaldehyde catabolic routes indicated by an upregulation of ADH5 and downstream S-formylglutathione hydrolase (ESD), while ALDH2 was down-regulated. In addition, enzymes involved in one carbon metabolism were upregulated such as e.g. methylenetetrahydrofolate dehydrogenase (MTHFD1 and 2, MTHFD1L). In addition, homocysteine formation was significantly increased in mitogen stimulated PBMCs. Interestingly, none of the above mentioned enzymes was regulated in THP-1 cells upon LPS treatment, while upon GM-CSF treatment ALDH2 was slightly but significantly downregulated and MTHFD2 was upregulated. Another myelomonocytic cell line, MUTZ-3, did not regulate enzymes involved in formaldehyde detoxification neither upon LPS nor IL-4/GM-CSF stimulation. In other cell types, such as A549 cells upregulation of ADH 5 could be triggered by formaldehyde-generating compounds aspartame and methanol but not with inflammatory stimuli.

A differential regulation of formaldehyde metabolic enzymes under inflammatory conditions, seems to require the T cell/macrophage interplay and/or to be associated with increased proliferation rates.

An important aspect of this project was to develop a reliable detection method of formaldehyde in biological samples. To date, only few studies exist, where formaldehyde concentrations have been measured in biological specimens such as cell culture medium, plasma or urine. No analytical method has yet been applied in a larger extent for clinical investigations. Common techniques to measure formaldehyde concentrations for industrial applications include both integrated active and passive methods. Most frequent detection methods are based on trapping of formaldehyde on a sorbent impregnated with 2,4-dinitrophenylhydrazine (2,4-DNPH) followed by high-performance liquid chromatography and ultraviolet detection at 360 nm (Deng et al., 1999).

We set up an liquid chromatography–mass spectrometry (LC-MS)-based method to measure formaldehyde after derivatization with 2,4-DNPH. Briefly, an Agilent 1260 LC

system was coupled to an Amazon SL Iontrap MS. After chromatographical separation, the analyte was then detected with a mass of 209 m/z. The method was calibrated in the range of 1.7 to 134 µM formaldehyde. Formaldehyde concentrations of about 10 µM were measured in serum pools of healthy human donors, concentrations measured in urine and in cell culture medium were in the lower micromolar range. Most importantly, for future experiments, the sample collection method will have to be optimized as in the best case derivatization of the highly volatile metabolite has to be performed immediately.

For future broader investigations of the fate of endogenous formaldehyde as an amino acids modifying agent, an additional approach has been explored with collaboration partners from the Human Proteome Organization (HUPO). Formylation of amino acids has not only been reported for f-Met in bacteria, but does also play role in mitochondrial protein synthesis (Hinttala, et al., 2015). Moreover, there are emerging data that formylation is a more frequently observed secondary modification of proteins as being reported e.g. for histone proteins, in analogy to acetylation (JJiang, et al., 2007). By using a modified search algorithm which was previously used for acetylation, several hits of formylated petides were found in an enriched mitochondrial HUPO test data set. This proof of principle approach confirmed that formylation can be detected and peptide formylation is a relatively frequent event. This preliminary setup will be applied to perform a broader testing on PRIDE (PRoteomics IDEntifications database) to identify new formylation targets.

**Summary and outlook**

The developed methods and collected data in this project provide a basis for a larger proposal focusing on formaldehyde metabolism in inflammation. Both inflammatory conditions and the presence of formaldehyde-releasing agents can modulate the expression of metabolic enzymes. However, changes are highly dependent on the expression level and the inducibility of the enzymatic detoxification machinery in the individual cell types. In immune cells, the PBMC model seems to favour flexible responses. It remains to be elucidated whether upon stimulation overall concentrations of formaldehyde increase or if there is a shift in the catabolic route only. Despite its importance as a carbon source for several biochemical pathways after its oxidation to formate, we postulate a signaling function of formaldehyde by exerting biphasic effects: The reductive capacity of formaldehyde may favor a reductive milieu and thus suppress Th1-type related immunobiochemical pathways such as IDO-1 activity on the one hand, while on the other hand its electrophilic properties may contribute to the activation of stress signalling pathways. In addition, we established and validated an LC-MS method to determine the amount of formaldehyde in biological samples after derivatization with dinitrophenylhydrazine. Future investigation will clarify whether the amount of formaldehyde itself will be affected under the different treatment conditions.

**References:**

Deng Y and Yu PH, J. Chromatogr. Sci.1999, 37: 317–322.
Gostner JM, et al. Sci Rep. 2016, 6:37842.
Gostner JM, et al. Toxicol. Lett. 2016, 258:S143.
Gostner JM, et al. Immunol Lett. 2015, 168(2):285-92.
Heck HD, et al. Biomed Mass Spectrom. 1982, 9(8):347-53.
Hinttala R, et al. Hum Mol Genet. 2015, 24(14):4103-13.
Jiang T, et al. Proc Natl Acad Sci U S A. 2007, 104(1):60-5.

Kaden DA, et al. WHO Guidelines for Indoor Air Quality. World Health Organization, 2010.
Gonzalez-Suarez, et al. Chem. Res. Toxicol. 2014, 27, 367-376.
Kalász H. Mini Rev Med Chem. 2000, 3(3):175-92.
Kimbell JS, et al. Toxicol. Scie. 2001, 64:111–121.
Liesivuori J and Savolainen H. Pharmacol. Toxicol. 1991, 69: 157–163.
Neuberger A. T. Amino acid metabolism and sulphur metabolism. Elsevier, 1981: 257–303.
Tong Z, et al. PLoS One. 2010, 5(4):e10234.
Tulpule K, et. al. J Neurochem. 2013, 125(2):260-72.
Uotila L and Koivusalo M. Adv. Exp. Med. Biol. 1997, 414: 365–371.

## Publications issued from this project

Gostner JM, Zeisler J, Alam MT, Gruber P, Fuchs D, Becker K, Neubert K, Kleinhappl M, Martini S, Überall F. Cellular reactions to long-term volatile organic compound (VOC) exposures. Sci Rep. 2016 Dec 1;6:37842. doi: 10.1038/srep37842. PMID: 27905399

## External funding

LRI-ECO36 (Long-Range Research Initiative), 2016, rejected

FFG Bridge P11567668, rejected, re-submitted March 2017.

## Miscellaneous

### Congress presentations

Fagundes dos Santos P, Stonig M, Warter A, Geisler S, Fuchs D, Gostner JM Immunotoxicity of Formaldehyde and Formaldehydogenic Compounds. 36th International Winter-Workshop for Clinical, Chemical and Biochemical Aspects of Pteridines and Related Topics, February 21.-24.2017, Innsbruck, Austria.

Gostner JM, Geisler S, Becker K, Stonig M, Warter A, Fuchs D. IDO-1 mediated tryptophan catabolism – a MIE für formaldehyde mediated immunotoxicity. Eurotox 2016, 52nd Congress of the European Societies of Toxicology, 04.-07. September 2016, Seville, Spain.

### Master/bachelor theses

Immunosuppressive activity of formaldehyde, Marlies Stonig, bachelor thesis in chemistry, 2016, Leopold-Franzens University Innsbruck

Determination of formaldehyde in biological samples using LC-MS, Alexander Warter, BSc., master thesis in biology, 2016/2017, University of Salzburg

## Mario Gründlinger - Peroxisomal import pathways and their role in *A. fumigatus*' virulence and adaptation

*Division of Molecular Biology*

### 5. Funding period

**Project duration:** 01.10.2014 – 30.09.2015

**Project summary:**

*Aspergillus fumigatus* is the most common airborne fungal pathogen causing life-threatening infections in immunocompromised patients. Improvement of antifungal treatment requires a detailed understanding of the fungal physiology (Latge 1999). Peroxisomes are highly dynamic and versatile organelles , which harbor several fungal pathways that are absent in vertebrates (e.g. biosynthesis of siderophores and biotin, glyoxylate cycle) (Grundlinger et al. 2013)(Tanabe et al. 2011)(Hynes et al. 2008).Peroxisomal protein import is mediated either by a C-terminal peroxisomal target signal 1 (PTS1) or the less common N-terminal PTS2. In all eukaryotes, PTS1-mediated import depends on the receptor Pex5. In humans, PTS2-mediated import is mediated by a complex of Pex7 and a long isoform of Pex5; in *Saccharomyces cerevisiae*, by the receptor Pex7 and the co-receptors Pex18 and Pex21, while in most other fungi it appears to be mediated by Pex7 and the co-receptor Pex20 (Kiel et al. 2006). However, functional studies on Pex20 are limited.

Here we investigated peroxisomal protein import pathways in *A. fumigatus* by genetic inactivation of the Pex5 and Pex20 homologs PexE and PexT, respectively. We confirmed that, PexT is indeed required for PTS2- but not PTS1-protein import into peroxisomes. Deficiency in PexE or PexT impaired siderophore biosynthesis and consequently growth in the presence of an iron chelator. Deficiency in PexE and to a lesser degree PexT, impaired ß-oxidation and virulence in a mouse model for pulmonary aspergillosis. Deficiency in PexE, but not PexT, impaired biotin biosynthesis, growth at 50°C, growth on acetate, conidiation, resistance against the detergent SDS and most likely production of various secondary metabolites. Moreover, we found that cytosolic, but not peroxisomal, overexpression of the mevalonate-consuming siderophore-biosynthetic enzyme SidI caused increased sensitivity to ergosterol biosynthesis inhibitors, most likely via depleting the mevalonate pool, which is important for siderophore as well as ergosterol biosynthesis. These data provide a rational for the spatial separation of ergosterol and siderophore biosynthesis. This is the first comprehensive study of the peroxins PexE and PexT, crucial for peroxisomal protein import, in an opportunistic human fungal pathogen.

### Short general introduction

Microbodies, the original term for peroxisomes, were discovered in 1954 by Johannes Rhodin (Rhodin 1954). In 1966 De Duve and Baudhuin were the first who isolated peroxisomes from rat liver. Biochemical studies led to the findings that several $H_2O_2$ producing enzymes as well as the $H_2O_2$ degrading catalase colocalize in this compartment. Therefore, De Duve proposed the functional term "peroxisomes" instead of "microbodies" as a morphological description (De Duve C 1966). Today it's known, that peroxisomes are multifunctional organelles and harbour various biochemical pathways (Islinger et al. 2012).

In contrast to mitochondria, peroxisomes are devoid of DNA and protein synthesis machinery. Therefore, all peroxisomal proteins are encoded by nuclear genes and have to be posttranscriptionally transported into the peroxisomal lumen(Schrader & Fahimi 2008). The so-called Peroxins (Pex) are proteins essential for peroxisomal biogenesis, proliferation, the import of peroxisomal membrane proteins (PMP) as well for the import of peroxisomal matrix proteins into this organelle (Kiel et al. 2006). In the proposed peroxisomal import pathways in most fungi, the proteins with a C-terminal peroxisomal targeting signal (PTS1) or N-terminal peroxisomal targeting signal 2 (PTS2) are guided to the peroxisomal lumen via their corresponding soluble receptors PexE and PexG, respectively. PTS2 import requires additionally an accessory protein, in most fungi Pex20(T), while in the yeast S. cerevisiae Pex18/Pex21 (Kiel et al. 2006).

**Project outcome and retrospective process of the project**

In order to describe the overall outcome and retrospective process of the project, it will be structured in the initial work programs (WP1-3). This assures a comparison of the earlier planned aims and project schedule of the original MUI-START application with the actually achieved aims and deviations.

**Work program (WP) 1:** For functional characterisation of PexE (AFUA_8g05240) and PexT (AFUA_4G06100) the generation of *A. fumigatus* null mutant strains was fully successful. As recipient, the *A. fumigatus akuA::loxP* strain derived from ATCC46645, (AfS77, termed wt here), largely lacking non-homologous recombination, was used (Hartmann et al. 2010)(Krappmann et al. 2006). For strain creation the bipartite marker technique was used (Yasmin et al. 2012). Because of delayed project starting point the initially proposed “golden gate” cloning technique (Terfruchte et al. 2014) was rejected and we retained to approved methods in the local lab. Transformants were checked by southern blot analysis to confirm single integration of the knout-out marker construct in the right gene locus. Complementation of the deleted strain was carried out by reintegration of intact *pexE* or *pexT* alleles to assure monitoring of gene deletion- specific effects. Although fungal transformation worked well in the first place, isolation and knockout confirmation of transformants is still one of the most time and work consuming process. Additionally, culturing and maintain the Δ*pexE* strain was demanding, due to its defect in conidial development.

The proposed role of PexT as a co-receptor for PTS2 import was confirmed by visualisation of a PTS2 protein fused to eGFP. The PTS2 GFP-fusionprotein delocalized from peroxisomes to the cytoplasm in the pexT deletion background (Δ*pexT),* whereas it stayed exclusively peroxisomal in Δ*pexE.* The PTS2 protein for GFP tagging was the siderophore biosynthetic enzyme SidI (Grundlinger et al. 2013). However, due to the strain recipient AfS77, the SidI-GFP fusion construct had to be reconstructed. Instead of the withdrawn “golden gate” technique as mentioned above, the “seamless cloning” system from Thermo Fisher Scientific was used for one pot reaction of multiple DNA construct assembly. With the

help of the PhD-student Matthias Misslinger this system is now well established in the PI’s lab, which speeds up cloning work.

Deliverables: fully characterised *A. fumigatus* mutants blocked in PTS1- or PTS2-import

(Δ*pexE and* Δ*pexT)* with their corresponding complemented strains. Strains were subsequently on hand for peroxisomal protein localization in live cell studies, by introducing GFP-tagged version of the siderophore biosynthetic enzyme SidI harbouring a canonical PTS2.

**WP 2:** Functional analysis of *A. fumigatus* mutant strains. Growth of *A. fumigatus* wt and mutant strains were tested under different growth conditions (liquid and solid cultures). The main characteristics were the expected biotin auxotrophy and decreased conidiation of Δ*pexE,* and as well the decreased growth of Δ*pexE and* Δ*pexT* under ß-oxidation conditions.

Abrogation of peroxisomal import pathways also nearly blocked siderophore biosynthesis. Furthermore PTS1 import is crucial for conidiation and a bunch of secondary metabolites, which was surprising, because none of the identified compounds have been associated with peroxisomes yet. This is of special interest, because filamentous fungi are producer of secondary metabolites with indispensable therapeutically benefits e.g. ß-lactam antibiotics (Bartoszewska et al. 2011). It has also be considered, that peroxisomes are involved in fungal toxin biosynthesis, which is an agricultural, economic, and medical issue worldwide (Maggio-Hall et al. 2005)(Imazaki et al. 2010). Strains were also extensively tested on different carbon sources, limitation conditions and external stressors e.g. oxidative stress, heavy metals and hypoxia.

Unexpectedly, delocalization of the overexpressed siderophore biosynthetic enzyme SidI to the cytoplasm decreased growth of the Δ*pexTSidI-Gfp* strain under minimal medium conditions and increased susceptibility to statins and azoles. This is most likely, because cytoplasmic SidI depleted the mevalonate pool, essential for ergosterol biosynthesis. Both pathways, ergosterol and siderophore biosynthesis share the metabolite mevalonate (Yasmin et al. 2012). This might give explanation, why the fungal cell compartmentalized siderophore biosynthesis to peroxisomes. This prevents disturbing interference of the two pathways.

Deliverables: detailed phenotyping of mutants abrogated either in PTS1 or PTS2 import. New insights of the role of peroxisomes in secondary metabolism in general and findings regarding cell compartmentalization.

**WP 3:** Assessment of relative virulence of *A. fumigatus* wt, and *pexE* or *pexT* deletion conidia. For an initial virulence testing the greater wax moth *Galleria sp*.(Fallon et al. 2012) was infected with *A. fumigatus* wt and isogenic deletion strains. This was performed in cooperation with Dr. Ulrike Binder (Department of Hygiene, Microbiology and Social Medicine). Surprisingly, abrogation of the PTS1 or PTS2 pathway in *A. fumigatus* had no effect on virulence in the *Galleria* model. The test run was not conclusive enough, because negative and positive controls were slightly inconsistent, most likely to a bad batch of larvae. However, shortly after the initial testing the complemented strains were already made and we proceeded for the murine infection model. Virulence testing using murine model of invasive aspergillosis was performed in the lab of Prof. Brakhage in Jena, instead of Dr. Elaine Bignell's lab (Imperial College London, UK). Both strains impaired either in PTS1 or PTS2 were highly attenuated in virulence in the model tested, showing that PexE and PexT are crucial for full virulence of *A. fumigatus*. Notably, several known e.g. biotin biosynthesis but also still uncharacterized pathways unique to fungi might be impaired in these two mutants, which elevate further potential new targets for diagnostic and fungal treatment, beside PexT.

Furthermore, it has been previously shown, that genes of peroxisomal pathways and peroxisomal proteins are activated, when *A. fumigatus* is exposed upon human neutrophils (Sugui et al. 2008). This underlines again the significance of peroxisomes in pathogenicity.

Deliverables: Virulence data, where we could show that PTS1 and PTS2 import are crucial for virulence of *A. fumigatus*.

All planned work programs (WP) of the suggested study were fully implemented and the goals were achieved within 15 months of project duration.

**Collaborations**

The great majority of the study was performed at the Division of Molecular Biology/ Biocenter, Innsbruck Medical University. The virulence study with the *Galleria* model was realized with the help of Dr. Ulrike Binder. The virulence study with the murine model of invasive aspergillosis was kindly done by Dr. Thorsten Heinekamp in the research group of Dr. Prof. Axel Brakhage in Jena. HPLC analysis was first done by Dr. Gerald Brosch and later on repeated by Dr. Kirstin Scherlach to identify several secondary metabolites. Dr. Martin Hermann helped us for the live cell imaging at the confocal microscope. Prof. Dr. Hubertus Haas enabled me to work in his lab, supported me with fruitful discussions for planning experiments setups and will contribute to the manuscript.

**References**

Bartoszewska, M. et al., 2011. The significance of peroxisomes in secondary metabolite biosynthesis in filamentous fungi. *Biotechnology letters*, 33(10), pp.1921–31.

De Duve C, B.P., 1966. Peroxisomes (microbodies and related particles). *Physiol Rev.*, 46,pp.323–357.

Fallon, J., Kelly, J. & Kavanagh, K., 2012. Galleria mellonella as a model for fungal pathogenicity testing. *Methods in molecular biology*, 845, pp.469–485.

Grundlinger, M. et al., 2013. Fungal siderophore biosynthesis is partially localized in peroxisomes. *Molecular microbiology*, 88(5), pp.862–875.

Hartmann, T. et al., 2010. Validation of a self-excising marker in the human pathogen Aspergillus fumigatus by employing the beta-rec/six site-specific recombination system. *Applied and environmental microbiology*, 76(18), pp.6313–7.

Hynes, M.J. et al., 2008. Genetic analysis of the role of peroxisomes in the utilization of acetate and fatty acids in Aspergillus nidulans. *Genetics*, 178(3), pp.1355–1369.

Imazaki, A. et al., 2010. Contribution of peroxisomes to secondary metabolism and pathogenicity in the fungal plant pathogen Alternaria alternata. *Eukaryotic cell*, 9(5), pp.682–694.

Islinger, M. et al., 2012. The peroxisome: an update on mysteries. *Histochemistry and cell biology*, 137(5), pp.547–574.

Kiel, J.A., Veenhuis, M. & van der Klei, I.J., 2006. PEX genes in fungal genomes: common, rare or redundant. *Traffic*, 7(10), pp.1291–1303.

Krappmann, S., Sasse, C. & Braus, G.H., 2006. Gene targeting in Aspergillus fumigatus by homologous recombination is facilitated in a nonhomologous end- joining-deficient genetic background. *Eukaryotic cell*, 5(1), pp.212–5.

Latge, J.P., 1999. Aspergillus fumigatus and aspergillosis. *Clinical microbiology reviews*, 12(2), pp.310–350.

Maggio-Hall, L.A., Wilson, R.A. & Keller, N.P., 2005. Fundamental contribution of beta-oxidation to polyketide mycotoxin production in planta. *Molecular plant-microbe interactions : MPMI*, 18(8), pp.783–93.

Rhodin, J., 1954. Correlation of ultrastructural organization and function in normal experimentally changed convoluted tubule cells of the mouse kidney. *Ph.D. thesis. Stockholm- Aktiebolaget Godvil.*

Schrader, M. & Fahimi, H.D., 2008. The peroxisome: still a mysterious organelle. *Histochemistry and cell biology*, 129(4), pp.421–440.

Sugui, J.A. et al., 2008. Genes differentially expressed in conidia and hyphae of Aspergillus fumigatus upon exposure to human neutrophils. *PloS one*, 3(7), p.e2655.

Tanabe, Y. et al., 2011. Peroxisomes are involved in biotin biosynthesis in Aspergillus and Arabidopsis. *The Journal of biological chemistry*, 286(35), pp.30455–30461.

Terfruchte, M. et al., 2014. Establishing a versatile Golden Gate cloning system for genetic engineering in fungi. *Fungal genetics and biology : FG & B*, 62, pp.1–10.

Yasmin, S. et al., 2012. Mevalonate governs interdependency of ergosterol and siderophore biosyntheses in the fungal pathogen Aspergillus fumigatus. *Proceedings of the National Academy of Sciences of the United States of America*, 109(8), pp.E497–504.

## Publications issued from this project

A manuscript is in preparation for publication in a suitable peer reviewed journal.

## External funding

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## Daniela Kuzdas-Wood - Cardiovascular phenotyping of a transgenic mouse model for multiple system atrophy

*University Hospital for Neurology*

**5. Funding period**

**Project Duration:** 01.10.2014 - 28.02.2015

### Project summary

**Introduction & Background:**

Multiple system atrophy is a rapidly progressive, fatal neurodegenerative disease presenting with autonomic failure (including prominent cardiovascular dysfunction with neurogenic orthostatic hypotension and nocturnal hypertension), Parkinsonism and cerebellar ataxia in different combinations and severity. The morphological hallmark of MSA are alpha-Synuclein (α-Syn) positive inclusions in the cytoplasm of oligodendrocytes, so-called (oligodendro-) glial cytoplasmic inclusions (GCIs). In the past decade, focus has moved towards characterization of autonomic features that often occur before the onset of motor-symptoms and are therefore also referred to as pre-motor features. These autonomic symptoms involve a broad range including cardiovascular, urogenital, respiratory and sudomotor domains. Over the last decade transgenic MSA models have been developed to provide a testbed for preclinical studies aimed at elucidating the pathogenesis and developing novel therapies. Our work has shown that the PLP-α-Syn mouse model replicates GCIs, MSA like neurodegeneration and motor features similar to Parkinsonism and ataxia occurring in patients. We recently identified autonomic brainstem and spinal cord pathology in PLP-α-Syn mice. Further we showed reduced heart rate variability that is characteristic for the human disease. The focus of the present study was to extend the cardiovascular phenotyping approach by investigating whether abnormalities of blood pressure regulation occur in the PLP-α-Syn mouse model of MSA.

**Our preliminary cardiac characterization of the tg PLP-α-Syn mouse model**

Following up the results of a previous study showing reduced heart rate variability analysis in the tg PLP-α-Syn mouse model, invasive blood pressure measurements have been performed in the same mouse model as an attempt of cardiovascular phenotyping.

Measurement of systolic and diastolic blood pressure showed major differences between values of tg and wt animals in this pilot study. Both parameters were markedly increased in five month old tg MSA mice indicating disturbed cardiovascular regulation. Figure 1 visualizes the systolic blood pressure values over a time course of 64 hours. Despite these obvious differences, we cannot yet conclude statistical differences due to a small study size (n=2). Data blotted are means.

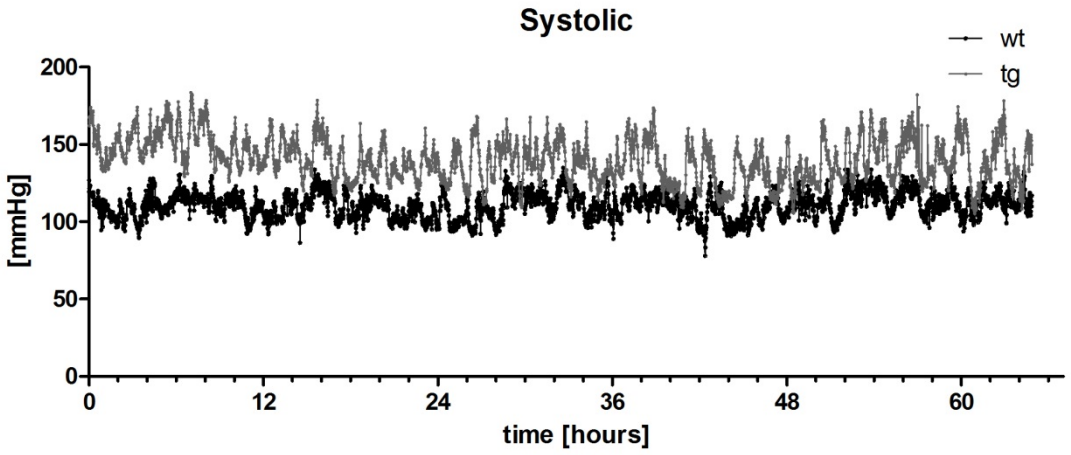


**Figure 1 Systolic blood pressure**

The systolic blood pressure measurements throughout the 64 hour recording period were elevated in tg animals (grey) at all time points. However, the overall fluctuations seem to be comparable. No statistical analysis has been performed yet due to a small study size (n=2). Data blotted are means.

**Major aim and objective**

In MSA patients, BP regulation is disturbed which deficit can be investigated with so-called tilt-tests. 24-hour BP measurement studies have shown that MSA patients present a so-called non-dipping behavior during night, which means that they lack the drop of BP during the sleep-cycle (Gilman *et al.*, 2008; Jecmenica-Lukic *et al.*, 2012).

Since neurogenic BP dysregulation including daytime NOH and nighttime hypertension are hallmark features of central autonomic failure in MSA, the aim of the current study was to further characterize the central versus peripheral origin of BP dysregulation in the PLP-α-Syn MSA mice.

- **Objective 1:**

The main aim of Objective 1 was to confirm the results of the pilot study with a more meaningful study size (pilot study only used two wt vs two tg animals). However, the results of the pilot study showing elevated BP parameters in the tg group, could not be repeated. For this reason, the project was preliminary terminated. The mouse model has already been well-characterized and many autonomic regions histologically quantified, therefore, without functional differences, there was no objective to continue the in-depth histological analysis.

- **Objective 2:**

The aim of Objective 2 was to identify the underlying mechanism of the pathological BP alterations that were observed in the pilot study. Since no differences were detected in the BP parameters in Objective 1, the investigation of parasympathetic vs. sympathetic autonomic nervous system was obsolete.

## Perspectives

Most likely, the differences in BP parameters detected in the preliminary analysis (n=2) were artifacts. We aim to perform all recordings under the same conditions, however, some environmental factors cannot be excluded. Due to unexpected, unavoidable changes, different surgeons had to perform the surgeries for the preliminary and actual study. They do use standardized protocols, however, a certain human-factor cannot be ruled out.

For future analysis, use of older animals with a more phenotype and potential severe cardiovascular deficits might be considered, however, the PLP-α-Syn animals are rather sensitive to anesthesia in old-age, which could pose further difficulties.

## Conclusion

The results of the preliminary study could not be repeated, however, the PLP-α-Syn animal model remains a valuable testbed for MSA-reasearch. Simultaneously to this study, cardiovascular phenotyping has been performed in the MBP-α-Syn mouse model of MSA and also did not display differences in BP parameters (Tank *et al*., 2014).

## References


Abele, M., Klockgether, T. and Wullner, U. (2004) Spectral analysis of heart rate variability in multiple system atrophy and unexplained sporadic ataxia. J Neurol 251**,** 894-895.

Azuma, T., Uemichi, T., Funauchi, M., Nagai, Y. and Matsubara, T. (2002) Ambulatory blood pressure monitoring in patients with spinocerebellar degeneration. Acta neurologica Scandinavica 106**,** 213-217.

Benarroch, E.E. (1993) The central autonomic network: functional organization, dysfunction, and perspective. Mayo Clinic proceedings. Mayo Clinic 68**,** 988-1001.

Benarroch, E.E., Smithson, I.L., Low, P.A. and Parisi, J.E. (1998) Depletion of catecholaminergic neurons of the rostral ventrolateral medulla in multiple systems atrophy with autonomic failure. Ann Neurol 43**,** 156-163.

Benarroch, E.E. (2002) New findings on the neuropathology of multiple system atrophy. Auton Neurosci 96**,** 59-62.

Benarroch, E.E. (2003) Brainstem in multiple system atrophy: clinicopathological correlations. Cell Mol Neurobiol 23**,** 519-526.

Benarroch, E.E., Schmeichel, A.M. and Parisi, J.E. (2003) Preservation of branchimotor neurons of the nucleus ambiguus in multiple system atrophy. Neurology 60**,** 115-117.

Benarroch, E.E., Schmeichel, A.M., Sandroni, P., Low, P.A. and Parisi, J.E. (2006) Involvement of vagal autonomic nuclei in multiple system atrophy and Lewy body disease. Neurology 66**,** 378-383.

da Costa-Goncalves, A.C., Tank, J., Plehm, R., Diedrich, A., Todiras, M., Gollasch, M., Heuser, A., Wellner, M., Bader, M., Jordan, J., Luft, F.C. and Gross, V. (2008) Role of the multidomain protein spinophilin in blood pressure and cardiac function regulation. Hypertension 52**,** 702-707.

Furushima, H., Shimohata, T., Nakayama, H., Ozawa, T., Chinushi, M., Aizawa, Y. and Nishizawa, M. (2012) Significance and usefulness of heart rate variability in patients with multiple system atrophy. Mov Disord 27**,** 570-574.

Gilman, S., Wenning, G.K., Low, P.A., Brooks, D.J., Mathias, C.J., Trojanowski, J.Q., Wood, N.W., Colosimo, C., Durr, A., Fowler, C.J., Kaufmann, H., Klockgether, T., Lees, A., Poewe, W., Quinn, N., Revesz, T., Robertson, D., Sandroni, P., Seppi, K. and Vidailhet, M. (2008) Second consensus statement on the diagnosis of multiple system atrophy. Neurology 71**,** 670-676.

Goldstein, D.S. and Sharabi, Y. (2009) Neurogenic orthostatic hypotension: a pathophysiological approach. Circulation 119**,** 139-146.

Jecmenica-Lukic, M., Poewe, W., Tolosa, E. and Wenning, G.K. (2012) Premotor signs and symptoms of multiple system atrophy. Lancet Neurol 11**,** 361-368.

Kahle, P.J., Neumann, M., Ozmen, L., Muller, V., Jacobsen, H., Spooren, W., Fuss, B., Mallon, B., Macklin, W.B., Fujiwara, H., Hasegawa, M., Iwatsubo, T., Kretzschmar, H.A. and Haass, C. (2002) Hyperphosphorylation and insolubility of alpha-synuclein in transgenic mouse oligodendrocytes. EMBO Rep 3**,** 583-588.

Kato, S., Oda, M., Hayashi, H., Shimizu, T., Hayashi, M., Kawata, A. and Tanabe, H. (1995) Decrease of medullary catecholaminergic neurons in multiple system atrophy and Parkinson's disease and their preservation in amyotrophic lateral sclerosis. J Neurol Sci 132**,** 216-221.

Kiyono, K., Hayano, J., Kwak, S., Watanabe, E. and Yamamoto, Y. (2012) Non-gaussianity of low frequency heart rate variability and sympathetic activation: lack of increases in multiple system atrophy and Parkinson disease. Frontiers in physiology 3**,** 34.

Kuzdas, D., Stemberger, S., Gaburro, S., Stefanova, N., Singewald, N. and Wenning, G.K. (2013) Oligodendroglial alpha-synucleinopathy and MSA-like cardiovascular autonomic failure: experimental evidence. Exp Neurol 247**,** 531-536.

Ozawa, T. (2007) Morphological substrate of autonomic failure and neurohormonal dysfunction in multiple system atrophy: impact on determining phenotype spectrum. Acta Neuropathol 114**,** 201-211.

Papp, M.I., Kahn, J.E. and Lantos, P.L. (1989) Glial cytoplasmic inclusions in the CNS of patients with multiple system atrophy (striatonigral degeneration, olivopontocerebellar atrophy and Shy-Drager syndrome). J Neurol Sci 94**,** 79-100.

Papp, M.I. and Lantos, P.L. (1994) The distribution of oligodendroglial inclusions in multiple system atrophy and its relevance to clinical symptomatology. Brain 117 ( Pt 2)**,** 235-243.

Sheward, W.J., Naylor, E., Knowles-Barley, S., Armstrong, J.D., Brooker, G.A., Seckl, J.R., Turek, F.W., Holmes, M.C., Zee, P.C. and Harmar, A.J. (2010) Circadian control of mouse heart rate and blood pressure by the suprachiasmatic nuclei: behavioral effects are more significant than direct outputs. PLoS One 5**,** e9783.

Spillantini, M.G., Crowther, R.A., Jakes, R., Cairns, N.J., Lantos, P.L. and Goedert, M. (1998) Filamentous alpha-synuclein inclusions link multiple system atrophy with Parkinson's disease and dementia with Lewy bodies. Neurosci Lett 251, 205-208.

Stefanova, N., Reindl, M., Neumann, M., Haass, C., Poewe, W., Kahle, P.J. and Wenning, G.K. (2005) Oxidative stress in transgenic mice with oligodendroglial alpha-synuclein overexpression replicates the characteristic neuropathology of multiple system atrophy. Am J Pathol 166, 869-876.

Stefanova, N., Reindl, M., Neumann, M., Kahle, P.J., Poewe, W. and Wenning, G.K. (2007) Microglial activation mediates neurodegeneration related to oligodendroglial alpha-synucleinopathy: implications for multiple system atrophy. Mov Disord 22, 2196-2203.

Stefanova, N., Poewe, W. and Wenning, G.K. (2008) Rasagiline is neuroprotective in a transgenic model of multiple system atrophy. Exp Neurol 210, 421-427.

Stefanova, N., Bucke, P., Duerr, S. and Wenning, G.K. (2009a) Multiple system atrophy: an update. Lancet Neurol 8, 1172-1178.

Stefanova, N., Hainzer, M., Stemberger, S., Couillard-Despres, S., Aigner, L., Poewe, W. and Wenning, G.K. (2009b) Striatal transplantation for multiple system atrophy--are grafts affected by alpha-synucleinopathy? Exp Neurol 219, 368-371.

Stefanova, N., Georgievska, B., Eriksson, H., Poewe, W. and Wenning, G.K. (2012a) Myeloperoxidase inhibition ameliorates multiple system atrophy-like degeneration in a transgenic mouse model. Neurotoxicity research 21, 393-404.

Stefanova, N., Kaufmann, W.A., Humpel, C., Poewe, W. and Wenning, G.K. (2012b) Systemic proteasome inhibition triggers neurodegeneration in a transgenic mouse model expressing human alpha-synuclein under oligodendrocyte promoter: implications for multiple system atrophy. Acta Neuropathol.

Stemberger, S., Poewe, W., Wenning, G.K. and Stefanova, N. (2010) Targeted overexpression of human alpha-synuclein in oligodendroglia induces lesions linked to MSA-like progressive autonomic failure. Exp Neurol 224, 459-464.

Stemberger, S., Jamnig, A., Stefanova, N., Lepperdinger, G., Reindl, M. and Wenning, G.K. (2011) Mesenchymal stem cells in a transgenic mouse model of multiple system atrophy: immunomodulation and neuroprotection. PLoS One 6, e19808.

Tank, J., da Costa-Goncalves, AC., Kamer, I., Qadri, F., Ubhi, K., Rockenstein, E., Diedrich, E., Masliah, E., Gross, V., Jordan, J. (2014) Preserved functional autonomic phenotype in adult mice overexpressing moderate levels of human alpha-synuclein in oligodendrocytes. Physiol Rep. 2014

Trojanowski, J.Q. and Revesz, T. (2007) Proposed neuropathological criteria for the post mortem diagnosis of multiple system atrophy. Neuropathol Appl Neurobiol 33, 615-620.

Wenning, G.K. and Stefanova, N. (2009) Recent developments in multiple system atrophy. J Neurol 256, 1791-1808.

Yazawa, I., Giasson, B.I., Sasaki, R., Zhang, B., Joyce, S., Uryu, K., Trojanowski, J.Q. and Lee, V.M. (2005) Mouse model of multiple system atrophy alpha-synuclein expression in oligodendrocytes causes glial and neuronal degeneration. Neuron 45**,** 847-859.

Ziemssen, T. and Reichmann, H. (2010) Treatment of dysautonomia in extrapyramidal disorders. Therapeutic advances in neurological disorders 3**,** 53-67.

Zuscik, M.J., Sands, S., Ross, S.A., Waugh, D.J., Gaivin, R.J., Morilak, D. and Perez, D.M. (2000) Overexpression of the alpha1B-adrenergic receptor causes apoptotic neurodegeneration: multiple system atrophy. Nat Med 6**,** 1388-1394.

## Publications issued from this project

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## External funding

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## Lourdes Rocamora-Reverte - Glucocorticoid production in the thymus and its influence on T cell development

*Division of Developmental Immunology*

**5. Funding period**

**Project duration:** 01.10.14 – 31.12.16

**Project summary:**

**Background**

Glucocorticoids (GC) are steroid hormones which take part in a feedback mechanism in the immune system shutting down inflammatory responses. Mechanistically, GC are thought to inhibit T-cell receptor (TCR) signaling of those thymocytes that would otherwise be negatively selected, suggesting a crucial role for GC in shaping the TCR repertoire.

GC are not only synthesized by the adrenal glands, there is a large body of evidence that GC are *de novo* synthesized in other organs including brain, intestinal track and thymus. However, *de novo* GC synthesis in mouse thymus is a matter of controversy. The presence of key enzymes (StAR, CYP11A1, 3□-HSD, CYP11B1) for *de novo* GC synthesis has been extensively described in thymic epithelial cells (TEC) as was the production of the active GC corticosterone. On the other hand, some studies show the ability of thymocytes to synthesize *de novo* GC, and there is no agreement on whether the expression of GC-synthesizing enzymes depends on T-cell activation.

Of note, corticosterone can also be produced from the inactive metabolite 11-dehydrocorticosterone (11-DHC) via the reductase activity of 11□HSD1, which is expressed by murine peripheral T-cells and, as recently shown, by thymocytes.

**Aims**

In order to clarify the role of thymocytes in synthesizing and/or activating GC and to assess whether T-cell development is affected by this, we centered our research on the study of the enzymes involved in GC synthesis in TEC and T cells, with special focus on the two main *de novo* GC synthesizing enzymes, CYP11A1 and CYP11B1, as well as the GC-activating enzyme 11□HSD1.

**Results**

Analysis of the expression of the GC pathway enzymes showed that the first of the synthesizing enzymes, CYP11A1, and the activating enzyme 11□HSD1 are expressed in different T-cell subsets in thymus and spleen, as well as in TEC. Surprisingly, we did not find any detectable expression level of the final *de novo* synthesis enzyme CYP11B1 neither in T cells nor in TEC.

Using an *in vitro* T-cell development system, we studied GC effects at different T-cell maturation stages on wild type (WT) as well as on glucocorticoid receptor (GR)-deficient T-cells. We observed differential GC effects on WT-thymocytes, being CD4+CD8+ cells the most susceptible subset to high concentrations of GC which impaired their progress in the maturation process; whereas GR-deficient T-cells remained resistant to this treatment. In

untreated cultures, GR-deficient T-cells developed in a similar fashion than WT T-cells did. If thymocytes would be able to produce sufficient amount of *de novo* GC *in vitro*, we would expect enhanced cellular survival and increased cell numbers in cultures with GR-deficient T cells under basal conditions, which was not the case.

One of the most prominent effects of GC is to induce apoptosis in T-cells. In order to analyze 11□HSD1 activity we administered the inactive corticosterone 11-DHC to thymocytes and peripheral T-cells and checked for cell death. In both, T-cell development system and total thymocytes cultures, we observed increased apoptosis levels when cells were treated with 11-DHC. This effect was also observed on peripheral T-cells, either CD4+ or CD8+ sorted splenocytes. In any case, GC-driven cell death could be prevented via activation of the TCR. Importantly, GR-deficient T-cells were not affected by 11-DHC treatment.

To independently confirm the conversion of 11-DHC into corticosterone, we analyzed the supernatants of cell cultures that were incubated overnight with 11-DHC using a corticosterone ELISA immunoassay. As expected, we found a clear conversion of the inactive precursor 11-DHC into corticosterone by WT and GR-deficient T-cells, and this production was not blocked or decreased by TCR activation.

**Conclusions**

These results propose that GC may modulate T-cell development affecting thymocytes in a different way depending on their maturation status. The absence of CYP11B1 expression suggests that if GC are needed for T cell selection and development they may not be synthesized using the *de novo* synthesis pathway but rather generated by the conversion of inactive 11-DHC into active corticosterone by the action of the enzyme 11□HSD1 which is expressed throughout T cell development. The presence of functional 11□HSD1 enables T-cells to generate GC autonomously and provides them with an intrinsic means to control T-cell development, selection and function.

**Short Outlook**

These studies have been performed using *in vitro* systems which do not completely reflect reality. A mouse model in which T-cell were deficient for 11□HSD1 would unravel the importance of 11-DHC as the main GC available freely diffusing in blood.

## Publications issued from this project

The results of this project have been summarized in a manuscript that, at the moment, is under revision for publication.

## External funding

Role of Glucocorticoides on B cell development and function (TWF-2016-1-23). Amount granted: € 37.390,00.

## Miscellaneous

**Congress presentations:**

- ***11th EFIS-EJI Tatra Immunology Conference, Slovakia (2014).*** "*GC production in the thymus and its influence on T cell development."* Lourdes Rocamora-Reverte, Jan G. Wiegers

- **4th European Congress of Immunology, Vienna, Austria (2015).** "Glucocorticoid synthesis in the thymus." L. Rocamora-Reverte, J. G. Wiegers

- **ÖGAI Annual Meeting, Innsbruck, Austria (2016).** "T-cell derived glucocorticoids: a conversion process from an inactive precursor" L. Rocamora-Reverte, A. Villunger, J. G. Wiegers

## Natalia Schiefermeier - microRNAs in axonal regeneration: Regulation of mir-138 and mir-21 by gp130 signalling in peripheral nerve injury and recovery

*Division of Physiology*

**5. Funding period**

**Project duration:** 01.10.14 – 30.04.2015

**Project summary:**

In the proposed project we wanted to explore the biological significance of mir-21 or mir-138 microRNAs after axon injury and during regeneration; and their mutual interaction with gp130 signalling. Further we planned study validated miRNA target genes that are involved in the STAT3/JAK/PTEN pathway.

Following goals were achieved:

**Assessment of mir-21 and mir-138 regulation by gp130 in DRG neuronal cultures**

Using qRT-PCR levels of mir-21 and mir-138 expression in SNS-gp130-/- vs. control DRG cultures were compared and the time-course of regulation in vitro was assessed. miRNA expression 24, 48 and 72 h after plating was measured. These experiments were repeated three times.

For the primary sensory neuronal culture, lumbar and thoracic DRG were dissected from adult mice as previously published (Agarwal, N., et al., Nat Neurosci, 2007; Obreja, O., et al., FASEB J, 2002). The neurons were further plated on cover slips coated with poly-l-lysine and laminin (10 µg/ml) and cultured in TNB™ medium at 37 °C in 5 % CO2 for 20- 48 h.

For analysis of miRNA expression levels, total RNA was isolated from primary cultures using TRI Reagent (Andratsch, M., et al., J Neurosci, 2009). The quantity of RNA was analyzed using a Nanodrop 2000 (Thermo Scientific). Reverse transcription to cDNA was performed using small RNA-specific, stem-loop RT primers from the TaqMan® Small RNA Assays and the TaqMan® MicroRNA Reverse Transcription Kit according to manufacturer´s instructions. cDNA samples were analyzed for expression of target miRNA by quantitative real-time Taqman® PCR using TaqMan® Small RNA Assays (all Applied Biosystems). Reactions were performed in a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems) using the 7500 Fast Real-Time PCR System (Applied Biosystems) for thermal cycling and real-time fluorescence measurements. Relative fold changes in miRNA levels were calculated by the ΔΔCT-method using snoRNA55 as a reference standard. The range for the target gene relative to gp130fl/fl samples as calibrator was calculated by 2-ΔΔCT (Livak, K.J. and T.D. Schmittgen, Methods, 2001).

Results of these experiments are shown on Figure 1 (A,B). Expression of mir138 was gradually decreased during the time-course and no significant difference was observed in SNS-gp130-/- neuronal cultures as compared to the control gp130 flox/flox cells. In opposite, expression of mir21 was strongly upregulated in time-course (Figure 1 B) and mir21 was less increased in SNS-gp130-/- neuronal cultures when compared to the control gp130 flox/flox DRG cells.

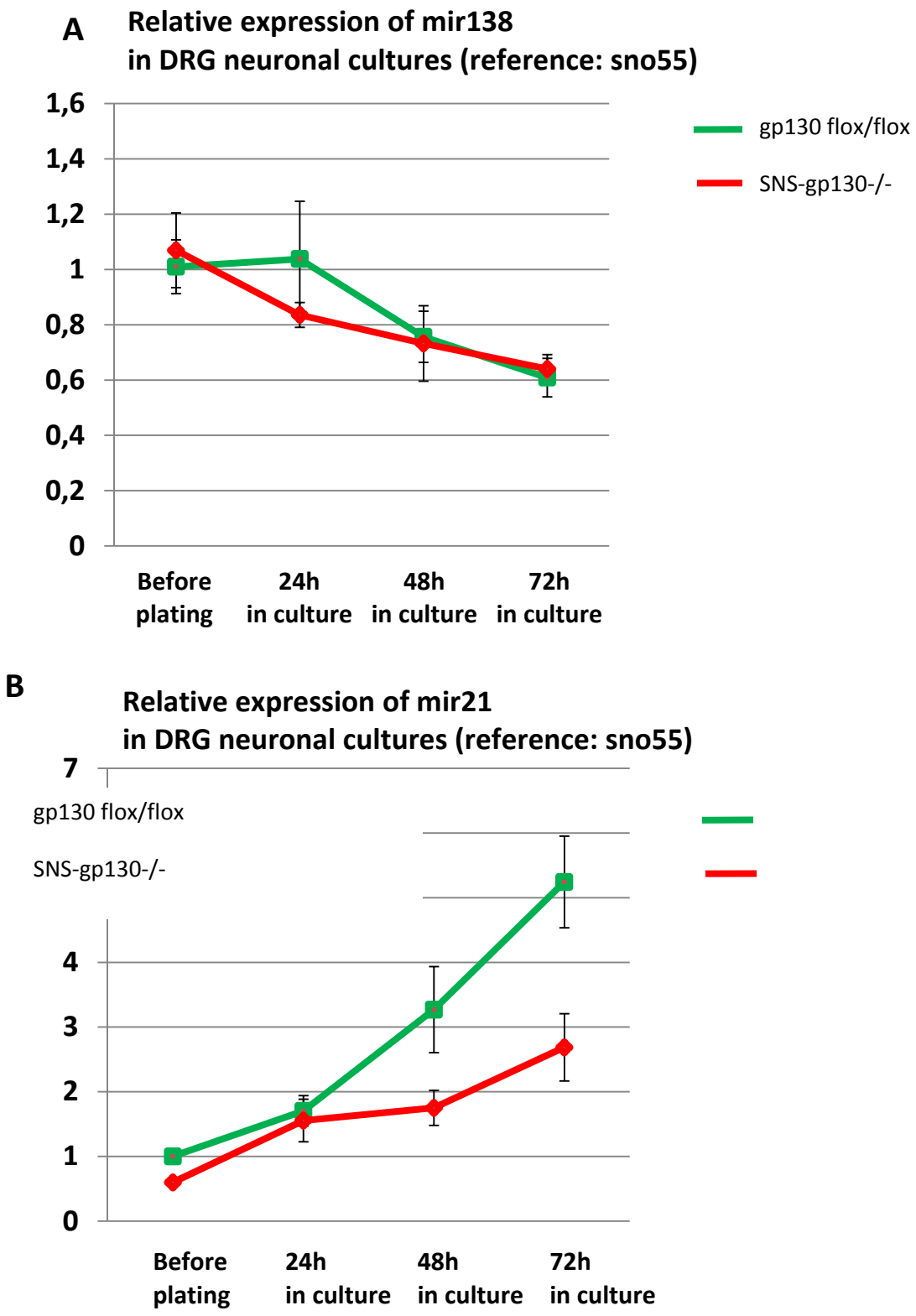



Figure 1. Relative expression of mir138 (A) and mir21 (B) in DRG neuronal cultures during given time-course was measured using quantitative real-time Taqman® PCR. The value of mir138 (in A) or mir21 (in B) expression in gp130 flox/flox neuronal cultures before plating was taken as 1 and used for normalization. Data are shown as mean ± standard error of the mean.

**Regulation of mir-21 and mir-138 expression by gp130 during axon regeneration *in vivo***

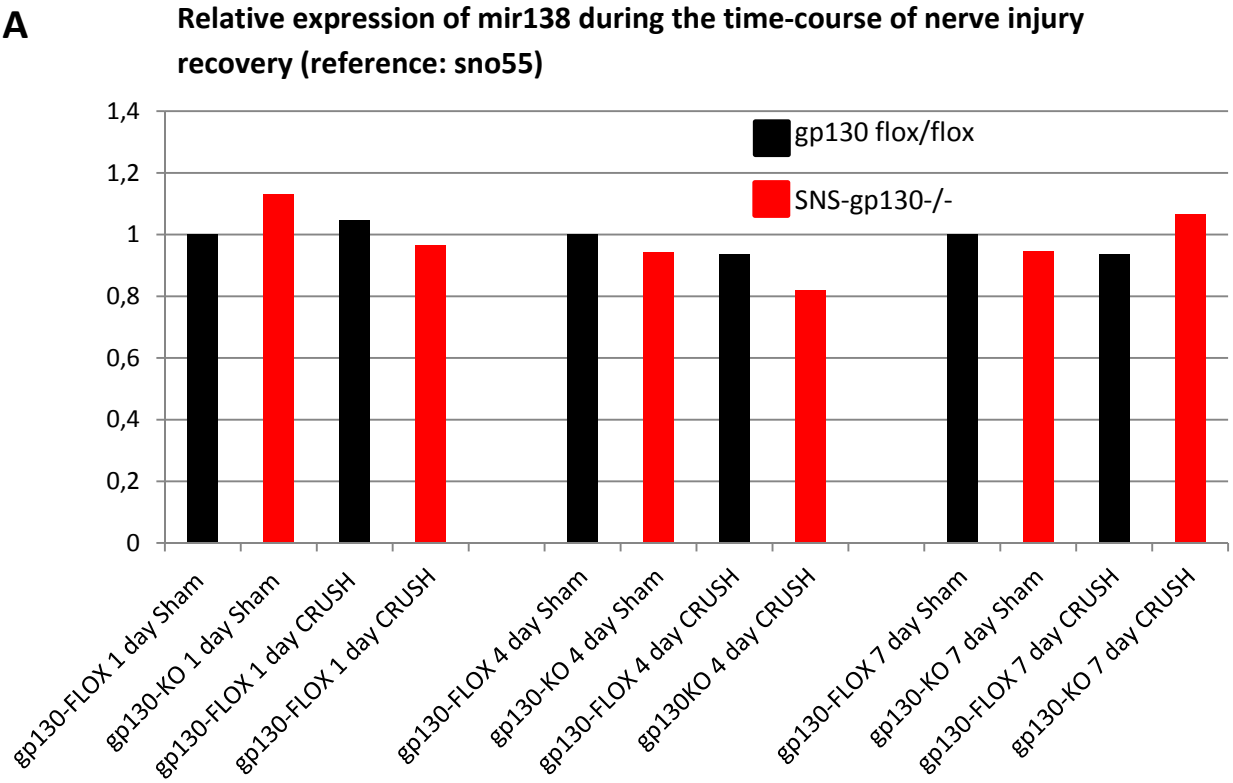
We used sciatic nerve injury model established in our laboratory and stem loop primer miRNA qRT-PCR technique to compare levels of mir-21 and mir-138 expression in SNS-gp130-/- vs control (SNS-gp130fl/fl and wild type) mice before sciatic nerve injury and during the recovery time.

Control gp130fl/fl and SNS-gp130-/- mice, age of 8- 12 weeks were used in all experiments. Mice were briefly anesthetized and the sciatic nerve was crushed perpendicular, at mid-thigh level, for 1 min using a semi-electric forceps applying a standard force of 19±1.8 N (modified Bioseb Rodent pincher RP-1, Chaville Cedex, France). Sham mice underwent similar procedure, however nerve was left intact. Mice were further scarified 1h, 4 and 7 days after the injury, DRG explants were frozen and further used for the protein and RNA isolations. For each group of animals 3 to 5 mice were used.

Results exposed on Figure 2 (A,B) show preliminary data with measures of one animal pro group. All values were normalized to values obtained in Sham mice. Expression of mir138 (Figure 2A) did not undergo significant changes during the after crush recovery time-course and no significant difference was observed in SNS-gp130-/- as compared to the control gp130 flox/flox mice.

Expression of mir21 was gradually upregulated in the control gp130 flox/flox mice during the recovery time-course (Figure 2 B, marked with #). In SNS-gp130-/- mice, mir21 was increased 4 days after injury and decreased after 7 days (Figure 2B, marked with §).

**A**



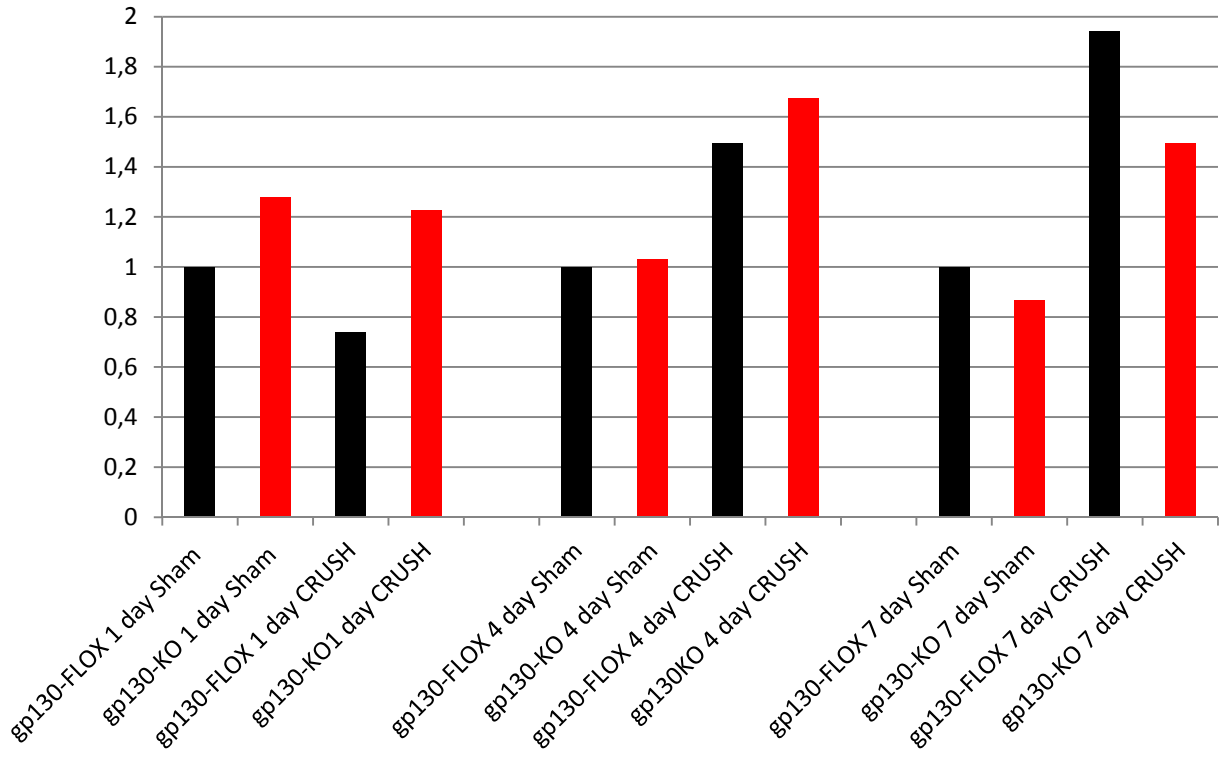



Figure 2. Relative expression of mir138 (A) and mir21 (B) in DRG explants during given time-course of recovery after nerve crush injury was measured using quantitative real-time Taqman® PCR. The value of mir138 (in A) or mir21 (in B) expression in Sham gp130 flox/flox mice was taken as 1 and used for normalization. Data are shown as mean values. Measures of one animal pro group are shown (therefore no standard deviation).

Conclusion. In order to make conclusions of shown above experiments, further experiments/repetitions should be performed and amount of investigated animals should be increased to at least 5 animals pro group.

Current report contains preliminary data on the project **"2014-05-012 microRNAs in axonal regeneration: Regulation of mir-138 and mir-21 by gp130 signaling in peripheral nerve injury and recovery"** that was closed on 30.04.2015 due to a new job position (outside of the Medical University of Innsbruck) of project applicant Dr.rer.nat. Natalia Schiefermeier.

## Publications issued from this project

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## External funding

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# 9 Overview on the output of completed MUI-START projects (Stand December 2016)

| Principal Investigator | Project title | Project ended | Publications | Funding acquired |
|---|---|---|---|---|
| **Wegene Borena** | Metabolic Syndrome and Cancer | 29.02.2012 | 1 | MFF |
| **Rohit Arora/ Erhart** | Pressure distribution in malunited intraarticular distal radius fractures | 13.03.2012 | 1 | __ |
| **Natascha Kleiter** | Orphan Receptor NR2F6 as barrier against Th17-dependent autoimmunity - composite analysis of NR2F6-selective signal transduction in Th17CD4+ T cells | 21.04.2012 | 1 | 2 FWF |
| **Christian Ploner** | Cell death mechanisms in adipogenesis | 30.06.2012 | --- | Land Tirol |
| **Andreas Ploner** | A non-coding RNA Expression Library for the functional Analysis of Proliferation and Differentiatoin of Human Adipocyte-derived Stem Cells | 31.12.2012 | --- | --- |
| **Markus Theurl** | Therapeutic angiogenesis by the neuropeptide catestatin | 13.02.2013 | --- | FWF |
| **Galina Apostolova** | Role of Satb2 in postmitotic neuronal plasticity | 28.02.2013 | 1 | FWF |
| **Anelia Dietmann** | Pathopyhsiology of Global Cerebral Edema in Aneurysmal Subarachnoid Hemorrhage - A Microdialysis Study | 28.02.2013 | --- | --- |
| **Claudia Manzl** | A new Role of Caspase-2: partial rescue of p53 X-linked embryonic lethality by caspase-2 null mutation | 28.02.2013 | 2 | --- |
| **Peter Lackner** | Brain metabolic changes during the acute phase of murine cerebral malaria. In depth characterization of cell death mechanisms, glutamate associated excitotoxicity and potential treatment strategies. | 31.03.2013 | 2 | Land Tirol |
| **Rober Öllinger** | The heme Oxygenase-1 system and long-term organ graft survival-"tolerance" | 31.03.2013 | --- | --- |
| **Markus Schretl** | PhoG - a transcription factor regulating adaptation to limitation and stresses in A. fumigatus | 31.03.2013 | 1 | --- |
| **Katharina Cima** | Drug Target Analysis in the Pulmonary Orphan Disease Pulmonary Arterial Hypertension | 14.04.2013 | --- | --- |
| **Birgit Frauscher** | Assessment of normative values of motor activity during sleep: A video-polysomnographic study in a | 21.04.2013 | 3 | FWF+ÖNB |

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|---|---|---|---|---|
| | representative tyrolean population sample | | | |
| **Martin Puhr** | Identification of molecular mechanism responsible for docetaxel resistance in prostate cancer cell lines | 30.06.2013 | 4 | FWF |
| **Ruper Oberhuber** | The Role of Endothelial Nitric Oxide Synthase in Ischemia - Reperfusion Injury and Chronic Allograft Vasculopathy | 21.07.2013 | 4 | TWF+ÖNB |
| **Nina Clementi** | Molecular Characterization of Ribosomal E-Site Functions | 14.08.2013 | 1 | --- |
| **Theresa Hautz** | Targeting IL-1b in Composite Tissue Allotransplantation: Investigation of its local Effect on Skin Rejection | 19.10.2013 | --- | --- |
| **Janine Kimpel** | Vesicular Stomatitis Virus Pseudotyped with the Glycoprotein of the Lymphocytic Choriomeningitis Virus as an HIV Vaccine Vector | 31.10.2013 | 1 | --- |
| **Martina Stichelberger** | Effects of Vasopressin on Migration and Oxygen Free Radical Release of Human Leukocytes | 31.12.2013 | --- | --- |
| **Selma Tuzlak** | Role of the prosurvival BCl-2 family protein A1 in T cell homeostasis and autoimmune disease | 31.12.2013 | --- | ÖAW |
| **Michael Blatzer** | BoIA - a transcriptional regulator required for stress adaptation in A. fumigatus? | 31.07.2014 | --- | TWF |
| **Judith Hagenbuchner** | Regulation of glycolysis and mitochondrial respiration by BIRC5/Survivin in neuronal tumor cells | 31.07.2014 | 4 | ÖNB decision pending |
| **Manfred Nairz** | Effects of the Erythropoietin-analogue ARA290 on the course of chemically-induced colitis | 31.07.2014 | 4 | FWF-Schrödinger |
| **Joachim Schmutzhard** | The involvement of the inner ear in sepsis syndrome in the cecal ligation puncture (CLP) mouse model | 31.07.2014 | 2 | --- |
| **Rober Sucher** | Targeted immunomodulation by use of biologics to minimize/avoid maintenance immunosuppression in vascularized composite tissue alltografts | 31.07.2014 | --- | --- |
| **James Wood** | Dopaminergic modulation at synapses of the medial central amygdala | 31.10.2014 | --- | FWF Meitner |

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| **Ulrike Binder** | Galleria mellonella as a host model to study invasive fungal infections due to Mucorales | 31.12.2014 | 3 | FWF decision pending |
| **Daniela Kuzdas-Wood** | Cardiovascular phenotyping of a transgenic mouse model for multiple system atrophy | 14.02.2015 | --- | --- |
| **Sebastian Herzog** | Regulation of B cell development by long non-coding RNAs | 28.02.2015 | 1 | 2 FWF+TWF |
| **Gert Klug** | Circulating fetuin-A and arterial stiffness after acute ST-segment elevation myocardial infarction | 31.03.2015 | 5 | --- |
| **Natalia Schiefermeier** | micro RNAs in axonal regeneration: Regulation of mir-138 and mir-21 by gp130 signalling in peripheral nerve injury and recovery | 31.03.2015 | --- | --- |
| **Kerstin Bellaire-Siegmund** | Coronin 1A – potential modulator of the TGF-ß signaling pathway influencing Treg/Th17 balance | 31.07.2015 | 2 | --- |
| **Martin Bodner** | Helena, the hidden beauty - Molecular dissection of West Eurasia's most common mitochondrial DNA haplogroup H at the highest resolution | 31.07.2015 | 4 | TWF+ DSF |
| **Cedric De Smet** | Endosomal biogenesis: a delicate interplay between proteins and lipids | 31.07.2015 | --- | --- |
| **Elke Griesmaier-Falkner** | Neuroprotective potential of sigma-1 receptor agonists in in vitro models of neonatal brain injury | 31.07.2015 | --- | --- |
| **Oliver Schmidt** | The functional characterization of golgi tethering factors in a stress response to defective membrane protein degradation | 31.07.2015 | 1 | TWF |
| **Ramon Tasan** | Characterization of Neurokinin B Neurons in the amygdala and their Role in anxiety and fear | 31.07.2015 | --- | FWF |
| **Michael Langeslag** | FABRYpain: Understanding pain in FABRY disease | 31.08.2015 | --- | --- |
| **Christa Pfeifhofer-Obermair** | PKC0 is essential for protective immunity against infection with Salmonella enterica serovar Typhimurium | 31.08.2015 | 1 | FWF decision pending |
| **Hannes Neuwirt** | Complement system and MAPK signaling in calcineurin-inhibitor induced nephrotoxicity | 30.09.2015 | --- | --- |

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| **Gregor Brössner** | Non-invasive measurement of brain temperature in Magnetic Resonance Imaging | 30.11.2015 | --- | --- |
| **Benno Cardini** | Simvastatin and tetrahydrobiopterin biosynthesis in the prevention of chronic allograft vasculopathy | 31.03.2016 | --- | TWF |
| **Mario Gründlinger** | Peroxisomal import pathways and their role in A.fumigatus virulence and adaptation | 31.03.2016 | --- | --- |
| **Luca Fava** | Caspase-2 in cell death induced by polyploidization | 30.09.2016 | 1 | Armenise-Harvard Foundation |
| **Gabriele von Gleissenthall** | Tryptophan and kynurenine metabolism in alcohol dependent patients in acute and medium-term withdrawal | 30.09.2016 | --- | --- |
| **Johanna Gostner** | Formaldehyde metabolism - on the role of formaldehyde in inflammation - | 31.12.2016 | 2 | FFG decision pending |
| **Lourdes Rocamora Reverte** | GC production in the thymus and its influence on T cell development | 31.12.2016 | --- | TWF |

# 10 Contact data - PIs of MUI-START projects presented in this report

**Dr.rer.nat Kerstin Bellaire-Siegmund**
Sektion für Zellgenetik
Schöpfstraße 41; 6020 Innsbruck
E-Mail: Kerstin.Siegmund@i-med.ac.at

**Martin Bodner, PhD.**
Institut für Gerichtliche Medizin
Müllerstraße 44; 6020 Innsbruck
E-Mail: Martin.Bodner@i-med.ac.at

**Assoz. Prof. Priv.-Doz. Dr.med.univ. Gregor Brössner**
Universitätsklinik für Neurologie
Anichstraße 35; 6020 Innsbruck
E-Mail: Gregor.Broessner@i-med.ac.at

**Dr.med.univ. Benno Cardini**
Universitätsklinik für Visceral-, Transplantations- und Thoraxchirurgie
Anichstraße 35; 6020 Innsbruck
E-mail: Benno.Cardini@i-med.ac.at

**Cedric Hubert De Smet PhD.**
Sektion für Zellbiologie
Innrain 80/82; 6020 Innsbruck
E-Mail: Cedric.De-Smet@i-med.ac.at

**Mag.Dr.phil. Luca Fava**
Sektion für Entwicklungsimmunologie
Innrain 80-82; 6020 Innsbruck
E-Mail: Luca.Fava@i-med.ac.at

**Dr.med.univ. Gabriele von Gleissenthall**
Universitätsklinik für Psychiatrie I
Anichstraße 35; 6020 Innsbruck
E-Mail: Gabriele.Gleissenthall@i-med.ac.at

**Assoz. Prof. Priv.-Doz. Dr.med.univ. Elke Griesmaier-Falkner PhD**
Universitätsklinik für Pädiatrie II
Anichstraße 35; 6020 Innsbruck
E-Mail: Elke.Griesmaier@i-med.ac.at

**Mag. Biol. Dr. Johanna Gostner**
Sektion für Medizinische Biochemie
Innrain 80/82; 6020 Innsbruck
E-mail: johanna.gostner@i-med.ac.at

**Mario Gründlinger PhD**
E-Mail: mario.gruendlinger@gmx.at

**Daniela Kuzdas-Wood, PhD**
E-Mail: daniela.kuzdaswood@gmia.i-med.ac.at

**Priv.-Doz. Michiel Langeslag PhD**
Sektion für Physiologie
Schöpfstraße 41; 6020 Innsbruck
E-Mail: Michiel.Langeslag@i-med.ac.at

**Assoz. Prof. Dr.med.univ. Hannes Neuwirt PhD**
Universitätsklinik für Innere Medizin IV
Anichstraße, 35; 6020 Innsbruck
E-Mail: Hannes.Neuwirt@i-med.ac.at

**Dr.rer.nat. Christa Pfeifhofer-Obermair**
Universitätsklinik für Innere Medizin II
Anichstraße, 35; 6020 Innsbruck
E-Mail: Christa.Pfeifhofer@i-med.ac.at

**Lourdes Rocamora Reverte PhD**
Sektion für Entwicklungsimmunologie
Innrain 80-82; 6020 Innsbruck
E-Mail: Rocamora.Lourdes@i-med.ac.at

**Natalia Schiefermaier-Mach**
Wissenschaftliche Mitarbeiterin AZW Tirol Kliniken
Innrain 98, 6020 Innsbruck

**Assistenzprofessor Dr.rer.nat Oliver Schmidt**
Sektion für Zellbiologie
Innrain 80/82; 6020 Innsbruck
E-Mail: Oliver.Schmidt@i-med.ac.at

**Assoz. Prof. Dr.med.univ. Ramon Osman Tasan PhD**
Institut für Pharmakologie
Peter-Mayr-Straße 1a
E-Mail: Ramon.Tasan@i-med.ac.at