Thieme

# 33. Jahrestagung der Kind-Philipp-Stiftung für pädiatrisch onkologische Forschung

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**Wissenschaftliche Leitung:**
Prof. Dr. Jan-Henning Klusmann, Martin-Luther-Univsersität Halle-Wittenberg
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## miR-128a and miR-130b determine lineage choice and leukemia-propagating cell identity in MLL-AF4+ acute leukemia

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MLL-AF4+ leukaemia is the most common leukaemia in infants and is characterised by a dismal prognosis. The failure to improve treatment outcome together with a difficulty to model this disease in mice highlight a lack of knowledge on how the disease initiates in utero. Using our previously described pre-leukaemia model as an experimental platform, we have now identified miR-128a and miR-130b as essential co-drivers of MLL-AF4+ leukaemogenesis. Their individual overexpression in mouse Mll-AF4+ LSK cells was sufficient to generate an acute leukaemia with central nervous system infiltration, a serious clinical problem of this disease. Remarkably, while the overexpression of miR-128a results in a pro-B ALL, the overexpression of miR-130b produces an acute mixed myeloid/BCP lineage phenotype. Furthermore, while miR-130b+ leukaemia is propagated by LMPPs, miR-128a+ leukaemia is maintained by a unique ckit+IL7R+ population. Both leukaemias express key signature genes associated with MLL-AF4+ leukaemia and rely on the continuous expression of the miRNA for their maintenance. Furthermore, we have discovered 2 novel tumour suppressors as downstream targets of miR-130b and miR-128a.

## Identification of the cell of origin in infant MLL-AF9 leukaemia

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MLL rearrangements are the predominant cause of acute infant leukaemias, which occur in utero within foetal HSPCs that distinctly lack additional cooperating mutations. Strikingly, MLL-AF9 causes an AML or B-ALL, but in adults almost exclusively an AML. Thus, the foetal cell of origin can explain differences between patients with respect to disease initiation, progression and outcomes. We utilised inducible MLL-AF9 expression to explore infant AML and B-ALL in foetal murine HSPCs. MLL-AF9 imparts distinct lineage, proliferation and self-renewal outputs within and between foetal and adult HSPCs. In contrast to adult HSPCs, MLL-AF9 generated significant increases in lymphoid output across foetal HSPC in CFU, even overriding CMP myeloid bias for B-ALL output, highlighting the specific nature of infant biology. HSC/MPP and LMPP gave rise to a pro-B ALL phenotype, suggesting these as cells of origin for the clinically dismal infant pro-B ALL. Fusion expression alone from E12.5 lead to a mixed-lineage AML with a median latency of 3 weeks, displaying the ability to model the human disease. Disease blasts retained lymphoid output, showing the lineage plasticity imparted by MLL-AF9.

## Small changes in the MLL-AF4 fusion protein make a difference

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We have studied the human MLL-AF4 fusion protein and compared it functionally with a murinized version, where have exchanged only the pSER domain (~120 amino acids) with murine Af4 sequences (MLL-murAF4). We have studied these MLL fusion proteins alone and in combination with the reciprocal AF4-MLL fusion proteins in a stably transfected cell line model. To our surprise, The MLL-murAF4 fusion displayed partial gain-of-funcions as well as a loss-of-function phenoytpe, e.g. by loosing its ability to show any synergism with the reciprocal AF4-MLL fusion protein at target genes. Moreover, we have investigated the target gene profile by the massive amplification of cDNA ends (MACE) technology. All findings will be discussed.

## Role of reciprocal fusions in MLL-r acute leukemia: studying t(4;11) fusion proteins

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Two fusion genes, *MLL-AF4* and *AF4-MLL*, have been cloned, stably transfected and tested by induced expression for their molecular functions (48h). DGE results were obtained by the massive amplification of cDNA ends (MACE) technology. The results of this study allow to draw quite important conclusions. Reciprocal fusion proteins deriving from the t(4;11) translocation allow to (1) ubiquitously activate chromatin which results in an increased expression of distinct sets of pseudogenes and lncRNAs, and (2) allow a functional synergism with MLL-AF4. We could also demonstrate that AF4-MLL works in a transient fashion as activated chromatin is maintained also after elimination the *AF4-MLL* fusion gene (hit & run mechanism). Thus, we have to conclude that an early presence of AF4-MLL is presumably sufficient to allow the development of a pre-leukemia state, while later the AF4-MLL fusion protein might be dispensable. This reflects the diagnostic situation in t(4;11) patients where

about 50 % of patients express both fusion genes, while the other 50 % express only the *MLL-AF4* allele, a situation associated with an even poorer outcome.

## The role of reciprocal fusions in MLL-r acute leukemia: studying t(6;11) fusion proteins

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We have identified at DCAL two different recombination breakpoints in t (6;11) leukemia patients: those with breakpoints in the major breakpoint cluster region of MLL (introns 9-11; associated with AML), while the other cases displayed breakpoints in the minor breakpoint cluster region (introns 21-23), associated with T-ALL. All 4 fusion genes (*MLL-AF6, AF6-MLL, exMLL-AF6, AF6-shMLL*) have been cloned, were stably transfected and tested by induced expression (48h) for their molecular functions. Here, we present our DGE results obtained by the „massive amplification of cDNA ends" technology. Our results indicate that the reciprocal fusion protein AF6-MLL allows to (1) ubiquitously activate chromatin which caused an increased expression of distinct sets of pseudogenes and lncRNAs, and (2) allows a functional synergism with the molecular counterpart MLL-AF6. The exchange of the PHD/BD domain from the der(6) to the der(11) fusion protein in the second set of fusion proteins changed the functional properties of the exMLL-AF6 fusion protein, as a T-cell specific program became activated. This gain-of-function was accompanied by a loss of synergizing with the AF6-shMLL fusion protein.

## Deciphering STAT3 dependency in paediatric acute lymphoblastic leukemia

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Although there have been tremendous advances in the successful treatment of paediatric ALL, this disease contributes to half of all leukaemia deaths in children. Development of new, broad range therapies is urgently needed. In this project we focused our attention on STAT3 in B-ALL, investigating the STAT3 regulated pathways. Global gene expression changes in B-ALL REH cells following pharmacological inhibition and shRNA-mediated silencing of STAT3 highlighted induction of TP53 target genes, without significant changes in TP53 mRNA. Indeed, STAT3 inhibition led to increased TP53 protein levels and to increased expression of TP53-target genes. Loss of TP53 expression in CRISPR/Cas9-generated TP53-/- REH cells resulted significant attenuation of REH sensitivity to STAT3 inhibition. We have also discovered that susceptibility to STAT3 inhibition is much broader in patient-derived xenograft (PDX) B-ALL subtypes than previously noted in cell lines, and that it correlates with TP53 status. Our results indicate a functional link between STAT3 and TP53 in B-ALL. STAT3 inhibition coupled with TP53 induction could represent a novel therapeutic strategy in B-ALL.

## CD79a/CD79b Promote CNS-Involvement and Leukemic Engraftment in Pediatric B-cell Precursor Acute Lymphoblastic Leukemia

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**Aim** To detect novel markers for specific detection and eradication of B-cell precursor acute lymphoblastic leukemia (BCP-ALL) cells in the CNS. Methods: RNA-sequencing with patient derived xenograft (PDX) BCP-ALL cells recovered from CNS versus bone marrow (BM) of NSG-mice was conducted. In vivo engraftment of BCP-ALL cells after knockdown (KD) and antibody (AB)-targeting of candidate molecules CD79a/b was investigated. CD79a/b levels were measured in patient samples and associated with clinical parameters. Results: CD79a/b were significantly upregulated in PDX cells recovered from the CNS versus BM. KD of CD79a in PDX cells resulted in diminished in vivo engraftment in spleen and BM and most markedly in the CNS. Furthermore, absence of CD79a and CD79b in BCR-ABL transformed murine B cells hampered leukemic engraftment. CD79b-AB-therapy significantly reduced CNS-infiltration in xenograft mice. High CD79a/b expression was associated with CNS-infiltration and high CD79a was linked to CNS-relapse in BCP-ALL patients. Conclusion: We suggest CD79a and CD79b as novel markers and potential therapeutic targets for the identification and treatment of CNS-involvement in BCP-ALL.

## Efficient healthcare for children with cancer using telemedicine in intensive chemotherapy phases

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**Background** Children with cancer are at risk for life-threatening infections due to chemotherapy-induced immunodeficiency during therapy. Initial symptoms can be subtle and require professional knowledge in pediatric oncology resulting in very frequent clinical visits. This affects family structures and causes high costs. Aim: Our aim is to show an equivalence of telemedical support compared to on-site visits in intensive chemotherapy phases. Methods: A monocentric randomized crossover study will start in October 2020 in our Pediatric Oncology Department in Kiel. Defined clinical visits will be replaced by telemedicine appointments. Technical devices will be used in order to record heart rate, oxygen saturation and body temperature at home. The safety of telemedicine will be assessed by measuring number and severity of complications and the "time-to-antibiotic" in neutropenic fever. Patient satisfaction and economic aspects will also be evaluated. Conclusion: Safety and successful implementation of telemedicine in these most vulnerable patients will allow an expansion of telemedicine to other high-risk populations and to medical interventions (e. g. therapy at home) in oncology.

## cf-DNA and EVs: Biomarkers for early detection of second primary malignancies in patients with heritable retinoblastoma

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Patients with heritable retinoblastoma, a tumour predisposition syndrome caused by heritable pathogenic variants of the RB1 gene, have a high risk to develop a Second Primary Malignancy (SPM). We develop a non-invasive test for early detection of SPMs in Rb-survivors. The test exploits the fact that

most SPMs are initiated by RB1 allele loss that results in an allelic imbalance. We extract EVs and cfDNA from blood samples from children with Rb and Rb-survivors with and without SPM. DNA released by tumour cells is expected to result in a skewed ratio of RB1 alleles in cfDNA. The allelic ratio at the RB1 locus is detected by analysis of linked SNP variants in heterozygous individuals. EVs are analyzed to identify EV characteristics of diagnostic relevance. Quantification of cfDNA by Droplet Digital PCR showed that the analysis of only one SNP may not be sufficient for sensitive detection of skewed allelic ratios. Parallel analysis of multiple SNPs by Deep Amplicon NGS will increase the power to detect skewed ratios. In view of the goal to offer a lifelong screening for early detection of SPMs analytical validity is of paramount importance.

## Efficacy of Allicin on DMBA-Induced BRCA-1 Defective Breast Carcinoma in Wistar Rats

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Carcinoma of the breast is a type of malignant growth which is developed from the tissue of the breast. The study was aiming to assess the effectiveness of (allicin) on dimethylbenz[a]anthracene (DMBA)-induced mammary carcinomas. There were no significant differences in the weight of the experimental animals. DMBA only, Allicin only or the combination of Allicin and DMBA low concentration group showed that tumor is present in the mammary rats, while group A, D, E and F showed no breast tumor at the end of the study. There is existence of proliferation as regards ductal epithelial cells as well as the tissue reaction within mammalian concerning the animal induced with DMBA only. DNA band which was secured in the midst of gel electrophoresis propose that DMBA instigated impairment which occurred at certain degree. This study has shown that allicin could be used as a chemopreventive agent against DMBA mammary carcinoma at the lowest concentration of allicin.

## Immunotherapeutic co-targeting of CD38 and CD47 in T-cell acute lymphoblastic leukemia (T-ALL)

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**Aim** Elevated surface expression of CD38 and the "don´t-eat-me" protein CD47 have been described in T-ALL, making both targets attractive candidates for antibody therapy. Methods: The CD38 antibody daratumumab (Dara) and a CD47 antibody (IgG2σ modified Hu5F9-G4-clone) were examined for antibody-dependent effector mechanisms in T-ALL cell lines and patient-derived xenograft (PDX) samples. Furthermore, NSG mice injected with heterogenous T-ALL PDX cells were treated with Dara and a CD47 antibody with and without chemotherapy. Results: Dara caused enhanced phagocytosis in antibody dependent cellular phagocytosis (ADCP) assays. Using eight different PDX samples in a preclinical phase II-like setting, Dara-monotherapy resulted in minimal residual disease negativity in 50 % of the cases and substantial survival prolongation in xenograft mice. To further improve ADCP, Dara was combined with a CD47 antibody. Thereby, phagocytosis in T-ALL cell lines and in PDX cells was enhanced, which is subject of current in vivo investigations. Conclusion: Dara represents a promising novel approach in antibody-based immunotherapy for T-ALL patients, especially when combined with CD47 blockade.

## Therapeutic stratification of acute leukemia using high throughput drug screening

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The functional patterns from high-throughput drug sensitivity vs. resistant profiles of different diagnostics leukemic samples and at the same time their comprehensive integration to their genetics and transcriptomics profiles can be helpful in better stratification of the existing therapies and finding the novel and overlooked biomarkers. Therefore we map the dependencies on various signalling pathways with a library of (n = 175) compounds, which includes chemotherapeutics in the GPOH recommended treatment protocols for childhood cancer, inhibitors in early/late clinical phase or waiting to enter clinical trials. The automation process of drug and cell dispensing minimize the probability of human induced errors and allows working with limited biopsy or Primografts patient material. Synergistic combinations of drugs are later explored in a combination screen with promising hits. Using this platform (> 100 samples), we have stratified and identified novel Myc related biomarkers using BCR-ABL1+ and T-ALL samples. In another instance we have also employed the platform to measure for individual acute/long term toxicity to chemotherapy in childhood cancer based on genetic predisposition.

## Whole-genome CRISPR screen as a tool to identify mechanism of relapse and potential targets for novel drug combinations

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Steroid resistance and relapse is one of the major clinical challenges in ALL high-risk cases. Here we performed genome-wide CRISPR screens in t(17;19) patient material, obtained at both diagnosis and relapse stage. PDX samples were generated and transduced with CRISPR(ko) library. To investigate the mechanism of steroid resistance, PDX cells were treated with dexamethasone in both ex vivo and in vivo conditions. We have identified NR3C1 as the main driver of chemo-resistance and relapse. Gene knockout of NR3C1 in diagnostic PDX material, confirmed dex-resistance as a main consequence of NR3C1 loss. Moreover, we have identified few pro-survival members of BCL2 protein family and several negative regulators of mTOR pathway as essential mediators of leukaemic propagation in this relapse PDX sample. Relapse PDX cells were also treated with ABT-199 and several mTORC1 inhibitors. We have observed these cells to be significantly more sensitive to BCL2i than its matched diagnostic pair. Moreover, both diagnostic and relapse samples showed high synergy when treated with BCL2 and mTOR inhibitors, highlighting this novel non-genotoxic drug combinations for further preclinical evaluation.

## Specific targeting of RUNX1/ETO; delivery of siRNA using Lipid Nanoparticles in vitro and in vivo

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The fusion protein RUNX1/ETO is generated by the chromosomal translocation t(8;21) and found in 15% of all pediatric acute myeloid leukemia (AML). It causes leukemogenic transformation by blocking differentiation and promoting self-renewal. Direct targeting using RNA interference promises a leukemia-specific therapeutic approach, but remains associated with poor pharmacokinetics. Here, we aimed to explore lipid nanoparticles (LNP) for safe and efficacious siRNA delivery. LNPs were prepared using microfluidic mixing techniques. Efficacies were examined in cell lines, patient-derived xenografts (PDX) and primary material. RUNX1/ETO mRNA and protein levels decreased more than twofold in all cultures. This reduction was associated with reduced proliferation, loss off clonogenicity and G1 cell cycle arrest. In vivo colocalisation studies, using fluorescence and bioluminescence, demonstrated that the LNPs reached the leukemic cells. Pharmacodynamic analyses proved RUNX1/ETO knockdown in all animals, as well as accordingly changed target gene expression levels. To conclude, LNP-mediated siRNA delivery is a promising new approach for specific targeting of fusion gene dependent cancers.

## Characterization of mechanisms of acquired Venetoclax-insensitivity in B-cell precursor acute lymphoblastic leukemia

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The FDA approved BCL-2 inhibitor Venetoclax shows high activity in relapsed/refractory CLL and promising results in preclinical and clinical studies in ALL. Still, resistance can be acquired over time. In this study, we modeled VEN resistance in a BCP-ALL cell line and investigated underlying mechanisms in order to identify strategies to overcome VEN insensitivity. After continuous exposure to increasing concentrations of VEN over time, VEN insensitive lines displayed increasing EC50 values from 4 nM to 26.2 µM. We could not identify any mutations in the BCL2 gene. No differences in the protein expression of BCL-2 and the anti-apoptotic protein BCL-XL, but a significant up-regulation of the anti-apoptotic protein MCL-1 were observed when comparing VEN insensitive to sensitive lines. Using BH3 profiling, we could identify a clearly reduced dependence on BCL-2 and an increased dependence on MCL-1 in all VEN insensitive lines. Upon VEN exposure, the pro-apoptotic protein BIM is released from BCL-2 but sequestered by MCL-1. By co-targeting BCL-2 and MCL-1 with VEN and the MCL-1 inhibitor S63845 we could synergistically induce cell death by releasing BIM from both BCL-2 and MCL-1.

## MicroRNA - 497~195 cluster suppresses cell cycle progression by targeting CCND3/CDK4 in acute lymphoblastic leukemia

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Recurrent deletions suggest an important role of proliferation in B cell precursor-acute lymphoblastic leukemia (BCP-ALL). We investigated microRNA (miRNA) function in ALL development. In two independent cohorts of BCP-ALL diagnostic and patient-derived xenograft (PDX) samples we found that low miR-497~195 expression was associated with high risk of early relapse and inferior event-free survival. MiR-497~195 overexpression in PDX samples delayed in vivo engraftment and prolonged survival. By ex vivo study and gene expression profiling we showed that the tumor suppressive role was due to inhibition of CDK4/CCND3-mediated cell cycle progression. We observed a higher proportion of early relapses in cases with low miR-497/195 expression co-occurring with CDKN2A/B deletions, suggesting that the lack of both regulatory mechanisms promotes leukemia aggressiveness. Altogether, we found that poor outcome in BCP-ALL is often associated with co-occurrence of low miR-497~195 expression and deletion of CDKN2A/B. MiR-497~195 cluster plays a tumor suppressor role, inhibiting CDK4/CCND3-dependent cell cycle progression, indicating the potential of targeting cell proliferation in ALL treatment.

## Enhancer hijacking on the MYCN amplicon in neuroblastoma

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MYCN amplification drives one in six neuroblastomas. In addition to harboring the MYCN oncogene, amplicons are characterized by complex structural rearrangements and co-amplify non-coding elements of the genome. The functional relevance thereof, however, is incompletely understood. Inspecting copy number data of 240 neuroblastomas, we observed an asymmetric distribution of MYCN amplicon boundaries. We showed that this pattern can be explained by a selective pressure to co-amplify a key MYCN-driving enhancer in 90% of cases, much more recurrently than expected by chance. Subsequent epigenomic analysis indicated that this and other enhancers remain functional after amplification. Intriguingly, amplicons lacking the key local enhancer frequently incorporate distal parts of the genome. Sequencing-based amplicon reconstruction revealed that local enhancer loss can be compensated by distal enhancers juxtaposed to MYCN. As Hi-C analysis showed, these cases of enhancer hijacking arise through novel topologically associated domains. Taken together, we demonstrated how non-coding elements shape intra- and extrachromosomal MYCN amplicon structures in neuroblastoma.

## Testing ABT-263 for the treatment of pediatric acute myeloid leukemia

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Deregulation of the anti-apoptotic members of the BCL-2 family is a well-known tumor evasion strategy. Consequently, a drastic decrease of leukemic burden can be achieved by genetic knockdown or pharmacologic inhibition of the BCL-2 family members. Here, we evaluated the potential of the BCL-2/-XL inhibitor ABT-263 (Navitoclax) as a therapeutic option. Various AML cell lines as well as leukemic blasts from AML patients were subjected to increasing concentrations of ABT-263 in vitro. Human peripheral blood derived CD34+ cells served as control. A patient-derived-xenograft (PDX) model in immunocompromised MISTRG and NSG mice was subsequently used to examine the in vivo response. Mixed lineage leukemic blasts (MLL) and cell lines exhibited clear antiproliferative response. Subsequent testing in PDX models further

highlighted this effect and showed a survival benefit for MLL in vivo. We observed a strong correlation between survival benefit and BCL-2 – but not BCL-XL expression. Our findings emphasize the importance of the corresponding pathway in the maintenance and progression of MLL-rearranged AML and give an outlook towards unexplored targeted therapeutic opportunities.

## Investigating the role of activated telomerase in genetically engineered neuroblastoma mouse models

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**DOI** 10.1055/s-0040-1709777
High-risk neuroblastoma is defined by the presence of telomere maintenance mechanisms, whereas such mechanisms are lacking in spontaneously regressing tumors. To evaluate whether telomerase inactivation impairs initiation and growth of neuroblastoma in in vivo models, we crossbred Th-MYCN;Th-ALKF1174L mice with Tert-/- knockout mice. Preliminary data suggest that the incidence of neuroblastomas is comparable in Th-MYCN;Th-ALKF1174L; Tert+/+ and Th-MYCN;Th-ALKF1174L;Tert-/- mice, but that telomerase-deficient mice show substantially prolonged survival compared to telomerase-proficient mice. In a complementary approach, we are determining the relevance of telomerase activation in neuroblastoma tumorigenesis using a conditional ROSA26-Tert-transgenic mouse model, in which Tert is overexpressed in neuroectodermal cells after Cre-loxP-mediated recombination. In addition to examining the role of telomerase in neuroblastoma pathogenesis, we will use these mouse models to evaluate the growth-inhibitory activity of telomerase inhibitors, such as 6-thio-2′-deoxyguanosine and imetelstat, both of which have shown promising cytotoxic effects in human neuroblastoma cell lines in vitro and in vivo

## The function of CBP/p300 and BET-proteins in dependence of H3.3K27M mutation in diffuse intrinsic pontine glioma (DIPG)

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Diffuse intrinsic pontine gliomas (DIPG) represent the most aggressive pediatric high grade brain tumor entity with fatal prognosis. 85 % of all DIPG carry a mutation in one of the Histone 3 genes (H3K27M) leading to hypotrimethylation of H3K27 and simultaneous hyperacetylation. Accordingly, it becomes highly suggestive that the H3K27 histone acetyltransferase CREB binding protein (CBP) & p300 and the histone acetylation- "reading" Bromodomain and Extra-terminal domain (BET) proteins play a tumor-promoting role in H3K27M-mutated DIPG. To investigate their function, siRNA-mediated knockdown of these proteins is performed in isogenic DIPG cell lines carrying H3K27M or H3WT and subsequent functional and molecular biological assays have been performed to determine the differential impact of CBP/p300 and BET-proteins in dependence of the H3 mutation status. Preliminary results reveal that CBP knockdown had the strongest effects on tumor-biological features in comparison to knockdown of the BET-proteins. In conclusion, H3K27M-mutated DIPG appear to markedly stronger depend on CBP than on p300 or BET proteins.

## Investigating miR-125b target gene Arid3a in trisomy 21-associated leukemogenesis

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Myeloid leukemia associated with Down syndrom (ML-DS) is characterized by the triad of fetal origin, trisomy 21 and truncating Gata1 mutations. Chromosome 21-encoded microRNAs of the miR-99a~125b tricistron are highly upregulated in ML-DS. We identified miR-125b as the dominant microRNA within this cluster synergizing with Gata1s during leukemogenesis. Combining RNA-sequencing with an shRNA-based positive selection screening in fetal hematopoietic stem/progenitor cells, we identified Arid3a as the main target of miR-125b, responsible for the oncogenic phenotype. While downregulating Arid3a lead to increased proliferation, restored expression of ARID3A in miR-125b high expressing human cell lines as well as patient-derived-xenografts impaired proliferation. Moreover, low ARID3A expression is associated with poorer overall survival in pediatric AML patients. Mapping the Arid3a protein interaction network, chromatin occupancy and transcriptional activity revealed its downregulation as a crucial event in pathogenesis of ML-DS. Hence, we established a novel role of transcription factor ARID3A as miR-125b target involved in myeloid malignancies.

## Comparison of Curcumin and Curcuminoids in terms of mechanisms of action and treatment efficiency in DIPG

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Diffuse intrinsic pontine glioma (DIPG) is a pediatric high-grade glioma of the brainstem, characterized by a very poor prognosis. 85 % of DIPG carry a mutation in lysine 27 of histone 3 (H3K27M), leading to an epigenetic imbalance. Due to lack of efficient conventional treatment options parents of affected children often use therapeutic adjuvants such as Curcumin (Cur). To investigate the molecular and biological effect of Cur on DIPG cells in dependence of the H3K27M mutation isogenic cell lines were subjected to cell viability assays, immunoblotting, and RNA sequencing. In addition, to overcome the limited therapeutic use due to poor bioavailibility, we further compared Cur with synthesized Curcuminoids (CurO) in terms of their mechanisms of action. Our preliminary results showed that Cur and CurO similarly reduce cell viability in DIPG via induction of autophagy, independently of the underlying H3K27-status. However, both Cur and CurO modulate the H3K27M-induced epigenetic imbalance. In summary, Cur and CurO induce anti-tumor effects probably throughout similar processes. Thus, CurO with its improved bioavailability might serve as potent therapeutic adjuvant for DIPG in future.

## Multi-drug chemotherapy dose optimisation in preclinical leukaemia models using HPLC-MS/MS

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Translation of novel drug combinations to clinical trials requires testing of drugs in carefully designed in vivo experiments. In the literature, the doses of chemotherapeutic drugs vary drastically across preclinical studies and selection of the clinically relevant and non-toxic doses is challenging. Moreover, in childhood leukaemia where repurposing of existing drug therapies and novel drug combinations are generating interests, it has become difficult to identify the appropriate dosages to undertake preclinical studies. To address this, we have developed analytical methods to quantify plasma drug levels in mice of multiple-drugs for a novel quadruple drug combination developed for the treatment of ALL. After drug administration to mice, small volumes of plasma were collected at appropriate time points and drug concentrations in plasma were calculated using an HPLC-MS/MS system. This strategy allowed us to identify and adjust the dosages of drugs whilst assessing toxicity without excessive use of animals. Quantification of drug plasma levels in mice guided the selection of clinically applicable doses.

## CRISPR/Cas9-based screens identify an essential long noncoding RNA locus in pediatric acute myeloid leukemia cells

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Long noncoding RNAs (lncRNAs) have emerged as important players in numerous biological processes, yet the vast majority lack functional characterization. To address this in the context of pediatric AML, we screened 619 lncRNA genes from 9 stem cell-AML and subgroup-specific signatures via a CRISPRi-based dropout approach. One candidate was essential for 6 of the 8 tested cell lines – LNC666, a low-abundance nuclear transcript. On chr15, it is flanked by 2 coding genes, which both appear dispensable for AML cells. In contrast, LNC666 repression and excision both reduce proliferation. Ectopic expression and shRNA-mediated knockdown did not yield notable growth effects, implying a cis mode of action. Indeed, CRISPRi tiling of the locus distinguished 3 key regions, including the LNC666 promoter, all of which enhance transcription from a minimal promoter. Saturating mutagenesis further refined these regions to short stretches where intact sequence is vital. Our data directly implicate the deregulation of lncRNA loci in pediatric AML, and suggest LNC666 as a new player in its pathophysiology. We also highlight the power of CRISPR/Cas9 approaches for interrogating complex genomic loci.

## Mapping chromatin occupancy of GATA1 and RUNX1 isoforms in Down syndrome myeloid leukemia

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Down syndrome myeloid leukemia (ML-DS) is characterized by exclusive expression of an N-terminus truncated GATA1 (GATA1s) and disequilibrium of RUNX1 isoform expression. ML-DS blasts are dependent on intact RUNX1, while GATA1s and RUNX1A synergize to induce leukemia in mice. Here, we studied differential chromatin occupancy of GATA1 and GATA1s as well as RUNX1 isoforms at target gene promoters and their effect on gene transcription.

To this end, GATA1 and GATA1s-mutant as well as RUNX1A and RUNX1C isoforms were lentivirally introduced into the ML-DS cell line CMK and in gene-edited primary murine fetal liver cells. After doxycycline induction of transgene expression, transcription factor binding to target genes was analyzed by CUT&RUN. RNA sequencing revealed differential target gene activation by the different transcription factor isoforms. This was accompanied by differential promoter occupancy of GATA1s compared to GATA1, which is mainly characterized by loss of DNA-binding. Furthermore, we found a dominant negative effect of RUNX1A over RUNX1B/C, but also RUNX1A specific functions. These data explain the synergistic effect of GATA1s and RUNX1A during the pathogenesis of ML-DS.

## Combined infection with oncolytic viruses (OVs) induces synergistic and additive cell death in glioblastoma multiforme (GBM) cell lines

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Experiments documented the use of OVs as a promising therapy of targeting only GBM tumor cells. Studies revealed that a combination of pathways is necessary to profit the most of immunogenic reactions. We analyzed the form of cell death after single and simultaneously administered OVs infection performing MTT, flow cytometry, Immunofluorescence, and Caspase-Glo 3/7-Assay in U87 and U373. We could prove that a combined infection of Reovirus (RV) with Parvovirus H-1 (PV) has a synergistic cell-killing effect with higher rates in apoptosis, necroptosis, and necrosis than an infection with one of these OVs alone, always applied in equivalent MOI. RV plus Newcastle Disease Virus (NDV) coinfection shows additive tumor cell killing effects only in necrosis and necroptosis. Apoptosis remains equivalent to a single NDV infection. PV plus NDV coinfection is less apoptotic despite more cell death, thus resulting in a shift towards necroptosis and necrosis related cell death. Our data indicate that a synergistic anti-tumor effect can be achieved by targeting different cell death mechanisms through combined infection with OVs in two different GBM tumor cell lines.

## High-throughput imaging analysis for drug combination efficacy in childhood acute lymphoblastic leukaemia

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Childhood acute lymphoblastic leukaemia responds to standard treatment, but more targeted drugs are needed. Patient-derived xenografts (PDX) more closely resemble patient cancers than cell lines. PDXs do not proliferate well ex vivo without mesenchymal stromal cells (MSC). Separation of the cell types may allow greater accuracy and insight into patient drug responses observed in the clinic. Drugged PDX-MSC cells were stained with a fluorescent DNA dye and imaged. After QC images were analysed by object-based (OB) or pixel-based (PB) classification pipelines, using supervised machine learning. Ground truth images determined the accuracy and precision of each approach. Combination treatments were assessed using SynToxProfiler. OB classification resulted in an excellent correlation with ground truth PDX counts, but not MSCs ($R2 = 0.93$, 0.36 respectively). Overlapping pixels between ground truth and called objects gave a false positive rate of 0.4 % for PDX and MSC, but the false negative rate was 23 % and 47 % respectively. PB improved on cell number correlation for both cell types (0.98, 0.83), and false positive/negative scores were reduced (PDX < 0.1 % & 15 %, MSC 0.2 % & 32 %).

## Identification of novel therapeutic approaches in a xenograft model of juvenile myelomonocytic leukemia (JMML)

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Juvenile myelomonocytic leukemia (JMML) is a highly aggressive myeloproliferative disorder of early childhood and the only curative treatment is hematopoietic stem cell transplantation (HSCT). However, the main cause of treatment failure is relapse in up to 30 % of patients urging the strong need for novel therapies. In JMML, malignant transformation is driven by constitutive activation of the RAS signaling pathway suggesting attractive candidates for therapy. By using our xenograft model that closely mimics human disease, we are testing different novel therapies including BH3-mimetics, RAS pathway inhibitors and HSP90 inhibitors to identify treatment strategies able to cure JMML. It is our primary goal to deplete leukemia-initiating cells to prevent relapse after HSCT. In addition, our studies will contribute to a better understanding of pathogenetic mechanisms of JMML. Eventually, it is our goal to transfer our preclinical observations to phase I/II clinical trials and in that way improve care of JMML patients.

## High-throughput sequencing of Ig/TCR genes for MRD monitoring in B-ALL patients receiving immunotherapy

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**Introduction** While immunotherapy is highly effective in leukemia, one of the main obstacles to MRD monitoring in ALL patients are CD19- relapses that impede FCM. Detection of clonal rearrangements of Ig/TCR genes is used as a routine method for MRD monitoring along with FCM, and unlike latter is applicable in all immunotherapy patients. Materials and methods: We tested 210 bone marrow samples from 35 patients diagnosed with refractory or relapsed B-ALL aged 1-21 years, receiving immunotherapy. Initial detection of clonal rearrangements was carried out by 8 multiplex PCRs of Ig and TCR loci followed by NGS. MRD detection included NGS of previously detected rearrangements in post-treatment samples and quantitative analysis. Results: In our cohort CD19- relapses were observed twice as often as CD19+ relapses. Both CD19- and CD19+ relapses were detected timely or at least 1 month earlier by NGS than by any other method, including FCM. Conclusions: High-throughput sequencing in patients undergoing immunotherapy is necessary for an adequate assessment of MRD level. The study is supported by RFBR grants 20-015-00462, 18-315-20038 and 18-29-09132 and Charity foundation Podari Zhizn

## Overcoming differentiation blockage by inhibition of LSD1 as novel therapeutic strategy in pediatric AML

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Recently the lysin-specific histone demethylase 1 (LSD1) has been identified as novel therapeutic target in acute myeloid leukemia (AML), where it is overexpressed in 60 % of patients thereby contributing to the disease defining differentiation blockage. Here, we investigated pharmacological LSD1 inhibition as potential treatment strategy in pediatric AML. LSD1 inhibiton induced cellular differentiation in MLL-rearranged AML, acute megakaryoblastic leukemia with (ML-DS) and without Down syndrome (AMKL) accompanied by impaired proliferation in cell lines and patient-derived cells in vitro. Intriguingly, LSD1 inhibition triggered the JAK-STAT-signaling pathway. Consequently, combination with ruxolitinib, a JAK1/2 inhibitor already used for the treatment of myeloproliferative neoplasms, demonstrated synergistic effects on ML-DS samples, which were shown to frequently harbor mutations within the JAK-STAT pathway. Future in vivo studies accompanied by molecular characterization of the consequences of LSD1 and JAK-STAT inhibition will further help shaping the role of LSD1 as therapeutic target in pediatric AML.

## A cost-effective quasi single-cell assay for deciphering of clonal architecture of leukemic cells

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**Introduction** Clonality assessment using IG/TCR rearrangements is an essential tool for clonal evolution analysis and minimal residual disease monitoring in acute lymphoblastic leukemia (ALL). Here we present a new multiplex method for ALL clonal structures analysis at the single-cell level. Material and Methods: DNA from bone marrow (BM) samples of 20 T-ALL and 60 B-ALL patients was used for initial rearrangements detection by targeted high-throughput sequencing. The nuclei from all 80 BM samples were extracted, measured, pooled together evenly and sorted by FACS in 96-tube plate 75 nuclei per tube. The detection of initially identified IG/TCR was performed in all nuclei aliquots. Results: The presence of all patient-specific IG/TCR was detected and analyzed for each sample. The clonal structure was resolved by pairing of clonal and subclonal rearrangements in aliquots. Discussion: The developed method is a useful tool for single-cell level clonality analysis and can be easily implemented in routine ALL diagnostics. The work is supported by RSF grant 18-14-00244, RFBR grants 20-015-00462 and 18-29-09132 and Charity foundation Podari Zhizn.

## Characterization of RNA-Protein Interaction Networks in Acute Leukemias

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Some subtypes of pediatric acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) remain very difficult to cure. Thus, despite the effectiveness of conventional chemotherapies, there is still an urgent need to identify new targetable structures and/or pathways. While much is known about transcriptional regulation in leukemias, the post-transcriptional layer remains underexplored. Still, mutations and aberrant expression patterns of some RNA-binding proteins (RBPs) are implicated in leukemogenesis. To reveal further oncogenic RBPs, we performed CRISPR-Cas9 dropout screens targeting 490 RBPs, followed by next generation sequencing. These screens were performed in two AML and ALL cell lines each (NOMO1, 697, M07e, REH), representing either common leukemic subtypes or those associated with poor prognosis. Analysis is currently ongoing, however, we have already identified two promising RBP candidates, namely YBX1 and ZFP36L2, whose pro-proliferative potential was verified experimentally. Next, we will perform

functional assays and PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation) experiments to elucidate RBP function.

## Therapy assessment of pediatric chronic myeloid leukemia (CML) using combined analyses of RNA/DNA response dynamics

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**Background** Optimal treatment of pediatric CML requires a patient specific risk stratification and the comparability of individual therapy responses. A deeper understanding of the CML cells eradication dynamic is needed to make earlier therapy decisions. Methods: Besides transcript-based monitoring, DNA-based quantification was performed using the droplet digital PCR (ddPCR). A total of 1348 specimens from 65 pediatric CML patients were included. Therapy response models were designed separately for RNA-/DNA-monitoring. Data was evaluated using moving quantiles analyses. Results: Combined analyses show a stronger reduction of BCR-ABL1 transcripts compared to BCR-ABL1 copy numbers in the first three months of therapy. These data suggest that the initial reduction of CML cells is lower than the transcript-monitoring indicates. By applying moving quantiles analyses, patient specific therapy responses can be assigned to risk groups. Conclusion: RNA/DNA response models and moving quantiles analyses represent advanced diagnostic techniques to detect poor responders earlier. Prospectively, combined RNA-/DNA quantification may be used to assess the practicability of therapy stop trials.

## Ezh2 loss cooperates with loss of Runx1 during leukemogenesis and reactivates a fetal gene signature

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Loss of PRC2 frequently occurs in adult AML, MDS, and pediatric ML-DS, JMML. Yet, leukemogenesis is orchestrated by the cooperation of multiple genetic lesions. Therefore, we aimed to decipher the mutational and molecular context in EZH2 loss guided transformation. Using an in vitro CRISPR-Cas9 cooperation screening, we identified two highly transforming combinations of 5 gRNAs in concert with the Ezh2 gRNA. Corresponding murine transplantation experiments resulted in high penetrance and short latency leukemia that was attenuated by removal of the Ezh2 gRNA. NGS based mutation analysis and reduced complexity screenings revealed a potent cooperation between Ezh2 and Runx1 mutations. Global gene expression analysis revealed the upregulation of fetal gene signatures including Ryk, Plag1, Hif3a and Igf2bp3 to guide Ezh2 loss mediated effects. Moreover, the interplay of Runx1 and Ezh2 mutations was found to activate myeloid and stemness genes. CRISPR-Cas9 cooperation screenings were powerful to unravel cooperating effects between the losses of Ezh2 and Runx1. Uncovered oncogenic dependencies on onco-fetal and myeloid genes may be exploited for therapeutic approaches in the future.

## Rewiring the interplay between GATA1s and secondary mutations in the leukaemogenic transformation

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Acquired mutations in exon 2 of GATA1 -in the background of trisomy 21- produce an N-terminus truncated protein, named GATA1s driving transient abnormal myelopoiesis (TAM) in newborns with Down syndrome. Additional mutations however are required to transform the pre-leukaemic TAM clones into a full-blown acute leukaemia (ML-DS). Prior work revealed common secondary mutations in ML-DS patients belonging to epigenetic modifiers, signalling pathways and cohesin complex, which were shown causative for leukaemic transformation in a murine model. However, with the observed mutational repertoire open questions remained. Here, we are utilising virus-free CRISPR techniques to introduce GATA1s and additional mutations in primary human foetal haematopoietic stem and progenitor cells. Xenotransplantation assays of edited cells showed a marked production of immature CD117 + CD41+ megakaryocytic progenitors. Comprehensive in vitro and in vivo functional assays will provide insight into the genetic players of TAM and ML-DS oncogenesis.

## Identification of cellular markers for the IKZF1plus subgroup of B-cell acute lymphoblastic leukemia

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**Background** B-ALL can be classified by the occurrence of several genetic alterations. Recently, a new subgroup – named IKZF1plus – was associated with a worse prognosis in B-ALL. IKZF1plus is defined by the co-occurrence of IKZF1 deletion and CDKN2A, CDKN2B (homozygous), PAX5 or PAR1 deletion, in the absence of ERG alteration. Therefore, we aim to identify cellular markers for the prediction of the IKZF1plus group. Methods and results: We used the TARGET database (WGS, RNA-seq and clinical data) for the analyses. A total of 125 pediatric patients were included and grouped as IKZF1plus (13 %), IKZF1 deletion only (9 %) and IKZF1 wild-type (78 %). There was an enrichment of IKZF1plus cases in the B-other subgroup, which was selected for further analyses. The differential expression analyses performed with DESeq2 was used to compare the three groups. Results showed that four genes had increased expression, while thirteen were downregulated in the IKZF1plus group. CRLF2 had the highest expression when IKZF1plus was compared with the wild-type. Conclusion: We identified potential markers for IKZF1plus. Indeed, CRLF2 expression could be a good candidate marker to identify this subtype.

## Enhancing the effect of class I HDAC inhibition in MYC amplified group 3 medulloblastoma with novel drug combinations

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Medulloblastoma (MB) is a highly aggressive pediatric brain tumor. Patients with Group 3 MB tumors harboring an amplification of the oncogene MYC exhibit a worse prognosis compared to patients with non-MYC amplified Group 3 tumors. We and others have previously demonstrated that MYC amplified Group 3 MB cells show high sensitivity towards class I histone deacetylase (HDAC) inhibition with entinostat (MS-275) in vitro. To identify drugs suitable for combination treatment (entinostat + X) of MYC amplified Group 3 MB we performed a drug screen with a library of n = 75 compounds as single agents and in combination with entinostat in n = 4 MB cell lines (MYC amplified vs. MYC-non amplified). To gain further inside in pathways altered upon class I HDAC inhibition we determined changes in protein quantity, phosphorylation and acetylation in an array based proteomic profiling of entinostat treated MYC amplified HD-MB03 cells. The drug screen revealed n = 20/75 drugs that were particularly effective in combination with entinostat treatment in the three MYC amplified cell lines. We selected three top hit drugs for further assessment. Experiments to determine drug synergy are currently ongoing.

## Genome-wide CRISPRa screen identifies determinants of Actinomycin-D resistance in alveolar rhabdomyosarcoma

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Rhabdomyosarcomas (RMS) are the most common soft tissue sarcomas in children and adolescents. Even with current multi-modal treatment strategies combining surgery, radiotherapy and poly-chemotherapy such as Actimomycin D (ActD), Vincristine and Ifosfamide, outcomes of patients with relapsed or high-risk metastatic alveolar RMS remain dismal. Even though ActD is administered to the majority of patients with RMS, the determinants of sensitivity and resistance towards ActD are largely unknown. Here, we used a CRISPR-based genome-wide gene activation screen to identify genes contributing to resistance against ActD. In addition to ABCC1, a drug pump already described to confer resistance to ActD, we identified several genes encoding for transcriptional regulators of cell differentiation to be sufficient to induce resistance to ActD in vitro. CRISPR guide RNA target genes inducing resistance to ActD were validated using cell viability assays. Comparing genes differentially expressed between RMS patient-derived xenografts that were either sensitive or resistant to ActD with the results of our CRISPRa screen further refined our list of potential regulators of ActD resistance in RMS.

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