PROJECT FINAL REPORT

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4.1 Final ASSET Publishable Summary

Executive Summary

ASSET applied a systems level approach to study embryonal tumours (ET), which include neuroblastoma (NB), medulloblastoma (MB) and Ewing sarcoma family tumours (ESFT). These tumours arise in babies and children, and are devastating for the patients and their families. Combining state-of-the-art genomics, transcriptomics, proteomics and mathematical modelling, ASSET aimed to (i) deconvolute the plethora of molecular pathogenetic cancer aetiologies to the common core principles; (ii) develop better patient stratification; and (iii) devise new drug targets, drugs, and drug combinations that will open new therapy options for ETs.

ASSET was highly successful and not only delivered on its objectives but also developed new research directions, e.g. the adoption of zebrafish as an animal model, which is rapidly becoming a new gold standard animal model for neuroblastoma. Another major success was the integration between basic, translational and clinical research through computational modelling. This aspect of the project can serve as a blueprint for implementing systems medicine approaches that leverage breakthroughs in basic research to accelerate translational research and clinical applications.

ASSET scientific discovery highlights

- New molecular mechanistic insights into the pathogenesis of ETs arising from the integrated application of omics technologies and computational modelling
- New methods to stratify patients based on advanced interpretation of omics results
- New drug targets identified through systems approaches and data integration
- New drug combinations identified
- New computational resources developed
- Identifying the role of miRNAs in ETs
- Development of new computational biology resources
  - A comprehensive resource for data collection and analysis: the ASSET Data Warehouse
  - Development of data analysis pipelines for mutations
  - New tools for data integration and contextualisationIntegration of genomic and transcriptomics data for enhanced patient stratification
  - Development of statistical tools for analysing timecourse series of gene expression data
  - Developing new statistical tools for analysing drug cooperation
  - Developing reusable code for conducting Markov chain Monte Carlo (MCMC) inference

ASSET impact highlights

ASSET has generated a number of scientific innovations that will have lasting impact on science and the scientific community. The most important achievements in this respect are briefly listed below:

- Proof of concept for systems medicine approaches
- Systems guided drug design and deployment
- New software tools for the analysis and interpretation of omics data
- New tools for data integration and data comparison
- Nucleating a community of researchers and clinicians interested in systems medicine approaches
- Broad and effective dissemination of results

Summary description of project context and objectives

Project aims and objectives

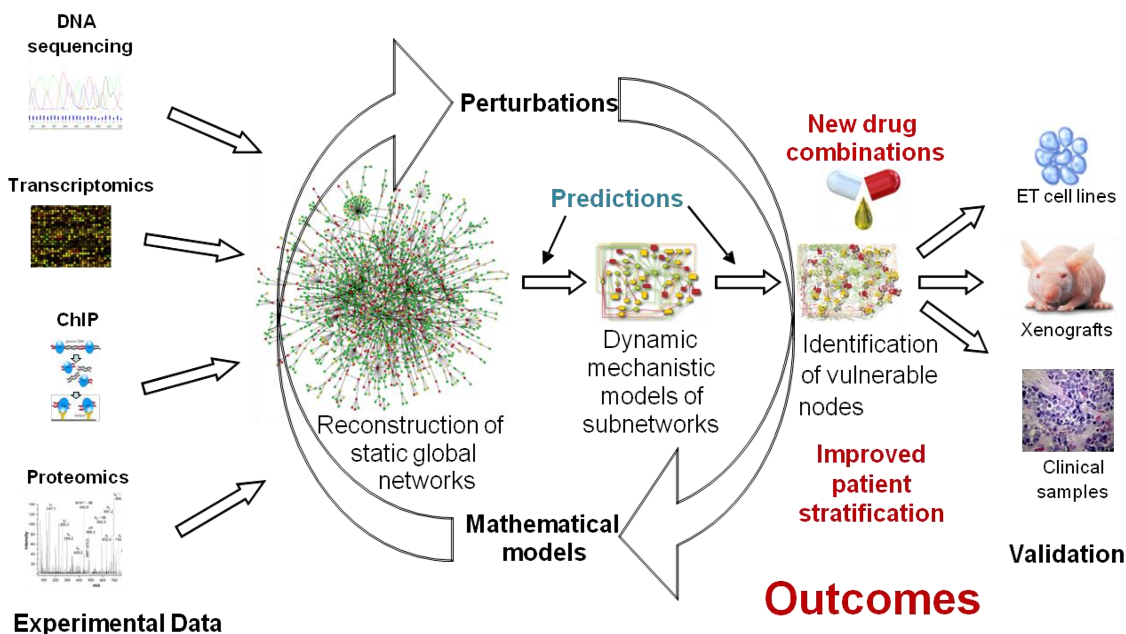
ASSET applied a systems level approach to study embryonal tumours (ET), which include the entities neuroblastoma (NB), medulloblastoma (MB) and Ewing sarcoma family tumours (ESFT). These tumours arise in babies and children, and are devastating for the patients and their families. ETs pose significant clinical challenges in terms of disease stratification for prognosis and treatment as well as in the paucity of drugs available for treatment. ETs seem to share common aberrations in core signalling networks with "modulator" pathways determining disease-specific manifestations. Combining state-of-the-art genomics, transcriptomics, proteomics and mathematical modelling, ASSET has analyses ETs with the aims (i) to deconvolute the plethora of molecular pathogenetic cancer aetiologies to the common core principles; (ii) to develop better patient stratification and to (iii) devise new drug targets, drugs, and drug combinations that will open new therapy options for ETs.

Towards these aims the ASSET objectives were

using a systems biology-driven discovery and validation engine for

- the combined analysis of genomic mutations, transcriptome, miRNA expression and dynamic proteome changes in ET model cell lines
- mathematical modelling to elucidate molecular pathogenetic networks and their emergent properties
- systematic perturbations to probe and refine these networks
- implementation of a virtuous cycle of model making and validation in relevant biological model systems (cell culture models and preclinical mouse models) and clinical samples
- using a systems medicine approach in order to identify new drug targets, drugs and drug combinations based on a molecular mechanistic understanding of pathogenic network aberrations

The ASSET concept and workflow



ASSET outcomes and achievements

ASSET was highly successful and not only delivered on its objectives but also developed new research directions, e.g. the adoption of zebrafish as an animal model, which is rapidly becoming a new gold standard animal model for neuroblastoma. Another major success was the integration between basic, translational and clinical research through computational modelling. This aspect of the project can serve as a blueprint for implementing systems medicine approaches that leverage breakthroughs in basic research to accelerate translational research and clinical applications.

ASSET research highlights

Ewing Sarcoma

- Analysis of aberrant signalling in Ewing sarcoma leading to the discovery of new targets
- New functional insights into EWS-FLI1, the main driver oncogene of Ewing Sarcoma
- A central role for the histone deacetylase SIRT1 in Ewing Sarcoma
- Post-transcriptional gene regulation exerted by EWS-FLI1 dependent microRNAs
- Role of miRNAs in Ewing sarcoma
- Small molecules and new targets for Embryonal Tumours
- New drug combinations for treating Embryonal Tumours
- Insights from mathematical models of EWS-FLI1 signalling
- Single cell transcriptomics
- Whole genome sequencing of Ewing sarcomas

Neuroblastoma

- Targeting MYCN signalling through a network based approach
- Mathematical models as informative biomarkers in neuroblastoma (NB)
- Insights from whole genome sequencing in neuroblastoma
- Identifying vulnerable nodes in neuroblastoma by synthetic siRNA screens
- Modelling and predicting chemotherapy survival of neuroblastoma cells
- New roles for MYCN in neuroblastoma (NB)
- New roles for ALK in neuroblastoma (NB)
- Proteomic analysis of TrkA signaling

Medulloblastoma (MB)

- The role of miRNAs in MB
- Synthetic lethal siRNA screens in MB
- Synthetic lethal kinome screens in MB

Crosscutting Findings

- The role of miRNAs in ETs
- Crosstalk between receptor tyrosine kinases (RTKs) and nuclear receptors for steroid hormones

Technology Advances

- Systematic and elective high throughput perturbation screens to identify vulnerabilities in Embryonal Tumour (ET) cells
- New targets arising from the screens

- Investigating the dynamic behaviour of protein signalling networks by reverse protein microarrays

Development of new computational biology resources

- A comprehensive resource for data collection and analysis: the ASSET Data Warehouse ASSETmart
- Development of data analysis pipelines for mutations
- New tools for data integration and contextualisation
  - *Cell-type specific models to simulate the different cell lines and predict the effect of drugs*
  - *Tools to perform analyses and comparison of the mutational profiles of the cell lines and primary samples within the context of large international cancer patient datasets*
  - *Tools for comparing gene expression profiles from cell lines and primary patient samples*
- Integration of genomic and transcriptomics data for enhanced patient stratification
- Development of statistical tools for analysing timecourse series of gene expression data
- Developing new statistical tools for analysing drug cooperation
- Developing reusable code for conducting Markov chain Monte Carlo (MCMC) inference

ASSET impact highlights

ASSET has generated a number of scientific innovations that will have lasting impact on science and the scientific community. The most important achievements in this respect are briefly listed below:

- Mathematical models as informative biomarkers in neuroblastoma
- New biomarkers for high risk neuroblastoma
- Systems guided drug design and deployment
- New software tools for the analysis and interpretation of omics data
  - *Atlas of Cancer Signalling Network (ACSN) database*
  - *NaviCell Web Service*
  - *A* new version of *BiNoM Cytoscape plugin*
  - *NBPrognostics*, a comprehensive catalogue of prognostic down- und up-regulated genes in NB
  - *SwitchFinder* is a novel statistical method developed for the analysis of time-series data.
  - *Lora* is a package for Markov chain Monte Carlo (MCMC) inference
- New therapeutic targets
- Circadian regulation of RTK signalling as a new type of therapeutic intervention
- Development of new technologies for analysing pathogenetic mechanisms in ETs
- Establishment of ASSETmart, a data warehouse and analysis platform for ETs for use by the scientific community
- New tools for data integration and data comparison
- Proof of concept for systems medicine approaches
- Nucleating a community of researchers and clinicians interested in systems medicine approaches
- Broad and effective dissemination of results

Description of the main S&T results/foregrounds

Advanced Insights into in the Molecular Pathology of Embryonal Tumours (ETs)

Ewing Sarcoma

The driving force of Ewing sarcoma pathogenesis is the chimeric gene regulatory protein EWS-FLI1. It combines the DNA binding domain of an ETS transcription factor with a portion of a nuclear protein, EWS, with functions in general transcription, RNA splicing, and DNA recombination. EWS-FLI1 perturbs gene expression on a genome-wide level leading to unlimited proliferation and blocked differentiation. In this project we applied a systems approach to identify hubs in the gene regulatory network of EWS-FLI1 and unravel mechanisms of expression dysregulation. To this end, a Ewing sarcoma model cell line (A673) with switchable EWS-FLI1 expression was fully characterized on the genomic, epigenomic, transcriptional and proteomic level in EWS-FLI1 on and off states. We obtained a genome-wide map of EWS-FLI1 binding [1] and described the first Ewing sarcoma specific epigenome [2] and microRNA regulome (Schwentner et al., submitted). Integrating the high throughput data sets, patterns of EWS-FLI1 driven gene activation and repression were obtained, among them modifiers of therapeutic response [3].

Analysis of aberrant signalling in Ewing sarcoma leading to the discovery of new targets

Ewing sarcoma is hallmarked by the expression of a EWS-FLI1 fusion oncogene. Careful analysis of time series data of Ewing sarcoma cell line conditionally inhibited for EWS-FLI1 expression, led to the identification of new promising targets for new therapeutic approaches in Ewing sarcoma. One of such was Protein Kinase C Beta ($PRKC_{\beta}$) as a gene strongly activated by EWS-FLI1. Comparison with other paediatric or bone cancers showed that $PRKC_{\beta}$ is highly and specifically overexpressed in Ewing sarcomas. Its transcriptional activation is directly regulated by the EWS-FLI1 oncogene. This protein is responsible for the phosphorylation of histone H3T6, allowing global maintenance of H3K4 trimethylation on a variety of gene promoters. In the long term, $PRKC_{\beta}$ RNA interference induces apoptosis *in vitro*. In xenograft mice models, complete impairment of tumour engraftment and even tumour regression were observed upon $PRKC_{\beta}$ inhibition, highlighting $PRKC_{\beta}$ as a most valuable therapeutic target for new therapeutic approaches in Ewing sarcoma.

New functional insights into EWS-FLI1, the main driver oncogene of Ewing Sarcoma

We found widespread EWS-FLI1 dependent reprogramming of the Ewing sarcoma epigenome resulting in super-activation of proliferation associated promoters, repression of widely active and differentiation associated enhancers, and de novo generation of Ewing sarcoma specific super-enhancers. The most dynamic EWS-FLI1 dependent epigenetic modification was histone H3K27 acetylation [2]. We could also demonstrate that genome-wide methylation profiling allows prediction of enhancer activity and established inter- and intra-tumour variability along two dimensions, associated with the relative strength of a mesenchymal versus stem cell signature and with the strength of the EWS-FLI1 regulatory signature (Sheffield N et al., submitted). Consistent with the importance of epigenomic reprogramming in Ewing sarcoma, epigenetic drugs, predominantly inhibitors of histone deacetylases (HDACs) and drugs targeting apoptosis inhibitors were among the agents that scored most highly when interrogating a library of >3000 compounds for EWS-FLI1 dependent toxicity (Tsafou K et al., in prep). Finally, by a high-throughput druggable siRNA screen, we identified a reader of the repressive histone H3K27 tri-methylation mark, the leucine rich repeats and WD repeat domain containing 1 (LRWD1) protein as the most sensitive target discriminating viability of EWS-FLI1 high and low Ewing sarcoma cells (Iljin K et al., submitted). Among epigenetic readers, the polycomb proteins BMI-1 and EZH2 are highly overexpressed in Ewing sarcoma. Polycomb target gene

expression in Ewing sarcoma deviates from normal tissues and stem cells and most targets are relatively repressed. However, our studies contributed to the discovery of a EWS-FLI1 dependent paradoxical up-regulation of numerous polycomb targets highly enriched for posterior homeobox group D (HOXD) genes due to a relative loss of polycomb-dependent histone H3K27 tri-methylation and gain of histone H3K4 tri-methylation at the promoters of posterior HOXD genes [4].

Central to the aberrant activation of proliferation genes by EWS-FLI1 we described a transcriptional module in which direct EWS-FLI1 binding to the promoter leads to the exchange of a repressive by an activating E2F transcription factor in a feed-forward loop. We reported an evolutionary conserved architecture of the E2F/ETS transcriptional module that we demonstrated to be aberrantly activated not only in EWS-FLI1 expressing Ewing sarcoma but also in ERG rearranged prostate cancer [1]. Time-resolved measurements of gene expression were used to mathematically model this module and confirm a direct functional interaction between EWS-FLI1 and E2F3, which we had predicted by genome-binding studies of these transcription factors and mutational analysis of their binding sites [5]. EWS-FLI1 had previously been reported to potentially associate with serum response factor (SRF) to drive the expression of growth factor induced gene sets. In healthy tissue, in response to signals delivered by RAS or RHO pathways, SRF either complexes with the ETS transcription factors ELK1 or SAP1, or with myocardin related transcription factors 1 or 2 (MRTF1/2) depending on the growth factor signal and the sequence context of SRF response elements. Integration of RNAseq and transcription factor binding studies by chromatin immune precipitation sequencing (ChIP-seq) revealed a significant overlap between EWS-FLI1 and MRTF2 binding in Ewing sarcoma cell lines. By single and combined knockdown of these transcription factors we obtained evidence that EWS-FLI1 hijacks MRTF2 target genes independent of SRF leading to growth factor independent aberrant activation (Katschnig A et al., in prep.). Motif analysis of genes bound by MRTF2 suggested that this mechanism affected predominantly target genes of the developmental JAP/TAZ signalling pathway. A further EWS-FLI1 dependent transcriptional module involves FOXO1 as a central player regulated by EWS-FLI1. We observed direct transcriptional suppression of FOXO1 expression as well as indirect regulation of FOXO1 subcellular localization via the direct EWS-FLI1/E2F regulated kinases CDK2 and AKT. We found FOXO1 to be responsible for a repressive sub-signature of the chimeric oncoprotein. We demonstrated that FOXO1 re-activation by selenic acid reduces Ewing tumour cell growth in vitro and in a xenograft mouse model [6].

A central role for the histone deacetylase SIRT1 in Ewing Sarcoma

In contrast to most adult cancers, the tumour suppressor TP53 is rarely mutated in Ewing sarcoma. Yet, basal TP53 expression and activity is indirectly modulated by EWS-FLI1 through inhibition of NOTCH signalling. We found that basal TP53 in Ewing sarcoma lacks acetylation, which is needed for functional activation of the transcriptional properties of the tumour suppressor protein. Our genome-wide studies identified the type III deacetylase SIRT1 as the missing link prohibiting TP53 acetylation, and that NOTCH activation in response to knockdown of EWS-FLI1 leads to SIRT1 suppression via activation of the NOTCH downstream effector HEY1, and consequently to TP53 activation [7]. Additionally, we found that EWS-FLI1 regulates SIRT1 via transcriptional activation of the ubiquitin specific peptidase USP1 leading to stabilization of lysine specific demethylase LSD1 which, in turn, leads to stabilization of SIRT1 by a mechanism still to be determined (Ban J et al., in prep.). In three independent series of primary Ewing sarcomas, we found SIRT1 most highly and significantly expressed in metastases. The small molecule SIRT 1/2 inhibitor Tenovine 6 (Tnv6) blocked tumour cell migration and growth in a zebrafish xenotransplantation model, identifying SIRT1 as a therapeutic target in this disease [7].

An alternative approach to SIRT1 inhibition is depletion of its co-substrate NAD. We therefore studied the influence of EWS-FLI1 on NAD metabolism and the consequences of NAD depletion on Ewing sarcoma cell growth and death. Genome-wide transcriptomic analysis identified a number of NAD

metabolic enzymes as EWS-FLI1 downstream transcriptional targets, including NAMPT, the rate limiting enzyme of the NAD salvage pathway. Blocking NAMPT led to NAD depletion in Ewing sarcoma cells, followed by a reduction of ATP levels and cell death. These effects could be rescued by nicotinic acid, a substrate of the NAD salvage pathway. Using conditional EWS-FLI1 knockdown by doxycycline-inducible shRNA revealed that the cytotoxic activity of NAMPT inhibition was significantly decreased in the absence of EWS-FLI1. These results suggest synthetic lethality of EWS-FLI1 expression with NAMPT inhibition (Mutz C et al., submitted). In contrast, de novo synthesis of NAD from the essential amino acid tryptophan did not affect Ewing sarcoma viability. Strikingly however, the knockdown of EWS-FLI1 in A673 cells led to activation of tryptophan degradation by the enzyme tryptophan 2,3-dioxygenase (TDO2) resulting in the accumulation of the primary metabolites kynurenine and kynurenic acid. Consistent with these two metabolites serving as ligands for the aryl hydrocarbon receptor (AHR), we observed nuclear AHR translocation and transcriptional activation of several downstream target genes. Thus, our studies identified a further indirect mechanism of transcriptional perturbation by EWS-FLI1 (Mutz C et al., submitted).

Post-transcriptional gene regulation exerted by EWS-FLI1 dependent microRNAs

In addition to mechanisms and consequences of epigenetic and transcriptional de-regulation by EWS-FLI1, we also investigated in great detail mechanisms of post-transcriptional gene regulation by EWS-FLI1 dependent microRNAs. Using a combination of unbiased, genome-wide assessment of physical microRNA/mRNA interactions by photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation sequencing (PAR-CLIP) and RNA sequencing upon depletion of specific microRNAs we discovered a prominent role of the EWS-FLI1 induced microRNA cluster hsa-miR-17-92 in Ewing sarcoma. We found recognition (seed) sequences for these microRNAs strongly enriched in mRNAs associated with the RNA induced silencing complex (RISC). We identified 7 known and 80 so far unknown genes as directly regulated targets for this cluster, one quarter of them annotating to the transforming growth factor beta / bone morphogenetic protein (TGFB/BMP) pathway predominantly downstream of SMAD signalling. Combining transcriptional and post-transcriptional (microRNA mediated) EWS-FLI1 signatures, our results suggest a functional EWS-FLI1-driven shift from TGFB to BMP signalling in Ewing sarcoma (Schwentner R et al., submitted).

In summary, the collaborative work performed by us with our ASSET partners resulted in a plethora of novel data that shed unprecedented insights into the gene regulatory networks driven by the EWS-FLI1 oncogene, and highlighted novel therapeutic targets for Ewing sarcoma treatment. The systems approach taken in this project combining integration of multi-level high-throughput data sets, mathematical modelling, in vitro and in vivo validation, and verification in patient materials may be taken as a paradigm to the assessment of oncogene function. As a consequence, multiple novel hubs in gene regulatory networks of Ewing sarcoma were identified that represent specific sensitivities of the disease. Therapeutic targeting of these hubs will make Ewing sarcoma treatment more specific and efficient, thus increasing life expectancy and quality of affected individuals and potentially decreasing health care costs.

Role of miRNAs in Ewing sarcoma

MicroRNAs (miRNAs) are central connections between the genome, transcriptome and proteome. Their crucial roles in cancer are increasingly recognised. The aberrations of miRNA expression in ETs and their effects on the transcriptome and proteome were characterized in order to include miRNA effects into the model of signalling describing the action of EWS-FLI1 oncogene. Global and focused approaches were combined: high-throughput cell-based miRNA transfection screens with a comprehensive library of >800 pre-mirs have generated data concerning ET cell proliferation. In addition, the profiling miRNA expression in ETs has been performed on 40 Tumours. The correlation analysis has been applied to microRNA and transcriptome patient data. Pairwise correlation coefficients have been computed for each miRNA in the dataset versus each transcript. The large-scale

analysis has permitted the identification of pair miRNA/mRNA with a negative and positive correlation. Moreover, the effect of these miRNA/mRNA pair on the phenotype of Ewing tumour (growth cell, invasion, migration) was studied. Several innovative methods for integrative analysis of mRNA and miRNA expression in tumoural samples were developed for this purpose. Thus, a new measure of connection between mRNA and miRNA expression was developed (antagonism pattern), and methods for detecting the shortening of 3'UTR in mRNAs after induction of an oncogene and methods for deconvolution of miRNA expression into independent signals were suggested. As a result, the action of several miRNAs and their genomic clusters (such as imprinted cluster) or a module of hsa-miR-145 and hsa-miR-199b. Functions of several miRNAs, including hsa-miR-30a-5p, were experimentally characterized.

Small molecules and new targets for Embryonal Tumours

CeMM, in collaboration with various ASSET partners, focused on researching therapeutics using systems-level approaches. First we developed a refined methodology to characterize the cellular target profiles of small molecules using chemical proteomics [8]. This method enhanced cognate target elution efficiency and proved to be effective and generically applicable.

Using chemical proteomics, we discovered that MTH1 (NUDT1), a nucleotide pool sanitizing enzyme, has a global role in tumourigenesis [9]. Loss-of-function of MTH1 impaired the growth of KRAS mutant tumour cells, and MTH1 inhibitors cause DNA damage in cancer cells. Moreover, we found that the (S)-enantiomer of the kinase inhibitor crizotinib is a nanomolar inhibitor of MTH1 catalytic activity while (R)-crizotinib was inactive. These results suggest that nucleotide pool homeostasis as an interesting intervention point for cancer therapy. We also developed a metabolite interaction mapping method, that relies on thermal-stability profiling in combination with mass spectrometry [10]. By detecting thermal stabilization of the protein in the intact cells, MTH1 was identified as top target of the (*S*)-enantiomer of crizotinib.

We closely worked together with several groups within the ASSET consortium, thus we were involved in a broad Ewing sarcoma-focused drug screen. To investigate single drug activities in Ewing sarcoma interfering with the EWS-FLI1 regulatory machinery, a broad range of ~3000 compounds were screened for viability inhibitory activity against in both presence and absence of EWS-FLI1, revealing interactions between compounds and signalling pathways driven by EWS-FLI1. We identified differential sensitivity of Ewing sarcoma cells to inhibitors of anti-apoptotic BCL2 family members based on EWS-FLI1 dependent expression of MCL1 *(manuscript in preparation).*

New drug combinations for treating Embryonal Tumours

Combinations of inhibitors are used to overcome the resistance caused by compensatory pathways and to lessen the toxic side effects through reduced dosing, which is especially appealing in pediatric tumours. Using a parallel phenotypic combinatorial screening approach, we identified disease specific interactions of targeted agents. We observed a highly potent synergy in neuroblastoma, between the tyrosine kinase inhibitor lapatinib and anticancer compound YM155. We found that the inhibition of ABCB1 efflux transporter by lapatinib led to considerable increase in intracellular concentration of YM155. This allowed the prolonged and elevated cytotoxicity specific for resistant neuroblastoma cells expressing high levels of ABCB1 *(manuscript in preparation)*. Although YM155 was developed as a survivin inhibitor, the precise molecular mechanism is unknown. We used a haploid genetic screen to reveal an absolute interdependency between YM155 action and SLC35F2, a member of the solute carrier protein family that is overexpressed in a number of malignancies [11]. We further showed that YM155 conferred its cytotoxicity via DNA intercalation in cells expressing SLC35F2, leading to a DNA damage response and apoptotic cell death. This gene-drug interaction might offer a specific targeting strategy of DNA damage to tumour cells with elevated levels of SLC35F2 expression.

We also found drug combinations effective Ewing sarcoma; e.g. the clinically evaluated multikinase inhibitor PKC412 and IGF1R/INSR inhibitors were strongly synergistic. We profiled PKC412 by chemical proteomics and found that it inhibits crucial Ewing sarcoma signaling routes. We showed that a particular drug combination-induced alteration of phosphorylation events was responsible for the synergistic effect since a large portion of signaling events were unique for the combinatorial treatment *(manuscript submitted)*.

Insights from mathematical models of EWS-FLI1 signalling
In order to systematically characterize the function of a number of genes in Ewing sarcoma genesis resulting from the analysis of expression data as well as mathematical modeling, a high-throughput screening of several hundreds of gene using siRNA-based strategy coupled with automated microscopy has been performed. The result of knocking down of a particular gene function was systematically quantified using several phenotypic read-outs such as cleavage of CASP3 (apoptosis), staining of cyclinB1, EdU and DAPI, total number of cells. Due to the multi-dimensional character of the measured cell phenotype, new mathematical methods of data treatment have been developed and applied to obtained experimental screening results. A mathematical modelling of the downstream effect of EWS-FLI1 has been undertaken which depicted a complex network governing the cell fate decisions between proliferation and apoptosis affected by the chimeric oncogene at various levels (https://navicell.curie.fr/navicell/maps/ewing/master/). A part of this biological network was validated using systematically collected siRNA/qPCR data in order to test and refine the network. Mathematical modelling allowed identifying a number of putative direct targets of EWS-FLI1 including *cul1* gene [12].

Single cell transcriptomics
Taking advantage of single cell RNA sequencing, which was newly established at CURIE, we further investigated the oncogenic EWS-FLI1 mechanisms of action. We used the ASSET reference cell line: the doxycycline-regulated, sh-based system that enables to control for EWS-FLI1 expression in Ewing cells. And we sequenced the transcriptomes of single cells at different time points during EWS-FLI1 re-expression. We have used a matrix factorization technique, Independent Component Analysis (ICA) to identify independent biological pathways activated in single cells. Our study demonstrates the power of combining single cell transcriptomics and ICA to distinguish the direct and indirect effect of an oncogene, and to gain insights into the cell cycle machinery and how it is coupled to the direct action of an oncogene. In addition it provided us with a shortlist of putative direct targets of EWS-FLI1, which are candidate transcriptional entry points for the action of EWS-FLI1.

Whole genome sequencing of Ewing sarcomas
We have also explored in more details the genome of 100 Ewing sarcomas by whole genome sequencing and identified STAG2, CDKN2A and TP53 as the most frequent secondary alterations in Ewing sarcoma [13]. This study also highlighted that subclones with STAG2 mutation may expand at relapse. We have further investigated the susceptibility to Ewing sarcoma among Caucasians and discovered that the EGR2 gene is an essential gene for Ewing sarcoma development [14], and that EGR2 is regulated by EWS-FLI1 via a polymorphic GGAA repeat that presents genetic variants that are more frequently observed in Ewing sarcoma patients than in the general population [15].

Neuroblastoma
Neuroectodermal tumours, including Neuroblastoma, are the most common among solid tumours in paediatric oncology, but are rare disease compared with adult cancer entities, such as lung and breast cancer or colorectal carcinoma. Nevertheless, the burden placed on public health is high. Overtreatment of less aggressive neuroblastoma is a huge clinical problem with current treatment strategies, as it can result in secondary, chemotherapy induced diseases later in life. On the other

hand, current treatment options largely fail to cure highly aggressive tumours. There is a lack of biomarkers to stratify patients accurately, and also a lack of treatment options that are tailored to individual patients and the severity of their disease. Accordingly, neuroblastoma research in ASSET focused on (i) better biomarkers for patient stratification; (ii) concepts reducing treatment intensity at the time of diagnosis using molecularly informed risk prediction tools, and on (iii) the development of novel molecularly based treatment options that more specifically target drivers of tumour development, malignant progression and drug resistance.

Targeting MYCN signalling through a network based approach

Neuroblastoma (NB) is characterised by MYCN gene amplification occurring in 20% of cases, as well as, with very low levels of recurrent somatic mutations [16]. The paucity of recurrent somatic mutations in NB hampered classical genetic approaches, which rely on frequently altered oncogenic drivers, results in the need to identify novel targets for the treatment of high-risk NB. Moreover, the MYCN oncoprotein is often regarded as undruggable, since it is a basic helix loop helix transcription factor with no domains that could be targeted by small molecules. To overcome this obstacle we have taken an integrated and network-based approach to elucidate novel therapeutic targets for NB, which focuses on the functional status of downstream biological networks rather than genetic mutations alone. In order to map these networks we combined transcriptomic analysis of MYCN target genes, MYCN DNA-binding analysis and proteomic identification of MYCN-binding proteins under a number of conditions, as well as, with genome wide RNAi screens. A panel of core ASSET consortium NB cell lines were analysed ranging from MYCN non-amplified cells (SH-SY5Y) to MYCN amplified cells (MNA), such as IMR-32, Kelly and the patient matched cell lines KCN and KCNR. We also used Tet-ON inducible system for ectopic MYCN overexpression in SH-SY5Y-MYCN cell line.

Different types of omics analyses have enabled us to reconstruct highly connected transcriptional and protein-protein interaction networks regulated by MYCN. RNA-seq revealed that MYCN overexpression predominantly repressed target gene expression at the levels of mRNA, miRNA and non-protein coding RNA [17-19]. In contrast to overexpression, MYCN amplification predominantly upregulated targets. Thus, MYCN's effect on target gene expression is fundamentally different depending on whether it is overexpressed (repressor) or amplified (activator). We also found that MYCN controls its own protein interaction network by transcriptionally regulating its binding partners [19]. Our network-based approach identified vulnerable therapeutically targetable nodes that function as critical regulators or effectors of MYCN in NB. These nodes were also validated by siRNA knockdown screens and further corroborated by functional studies and the analysis of patient data [19, 20].

Our studies indicated the potential utility of the GSK3 inhibitors LiCl and BIO (bioacetoxime) as therapeutic agents for NB treatment [20]. LiCl is in clinical use for the treatment of bipolar disorders, and hence may be quickly translatable for the therapy of NB. LiCl also showed the most specificity for cancerous cells while not affecting the viability of differentiated neurons. LiCl also altered many of the genes and upstream regulators of the gene signature for stratification of poor prognosis NB patients. In addition, we identified β-estradiol and MAPK/ERK as being novel targetable vulnerabilities of MYCN-amplified NB. Interestingly, we demonstrated that Wnt/β-catenin signalling is a bi-directional vulnerability of NB, malignant melanoma and colorectal cancer, with hyper-activation or repression of the pathway both representing a promising therapeutic strategy, even within the same cancer type[19]. Hyper-activation directs cancer cells to undergo apoptosis in cells driven by β-catenin, while Wnt inhibition blocks proliferation of cancer cells and promotes NB differentiation. We also showed that Wnt and retinoic acid (RA) co-treatments synergise, representing a promising combination treatment for MYCN-amplified NB. RA induced neuronal differentiation of NB is employed in NB treatment as both primary and maintenance therapies. Despite the effectiveness of retinoid treatment for some patients, it is ineffective for many high-risk patients and this is largely due to MYCN-induced resistance. We employed RNA-seq and network-based analysis to globally profile the

functional status of regulatory networks affected by RA. Besides reconstructing MYCN-regulated transcriptional networks upon RA induction, we have also identified MYCN-RA-TGFβ cross talk at a number of molecular levels, including MYCN's genomic binding, transcriptional activities and protein-protein interactions[21]. Our findings prompted us to further investigate the therapeutic potential of pharmaceutically activating TGFβ signalling in NB. We showed that the small molecule TGFβ activator Kartogenin (KGN) has differentiating potential in NB cell lines, and that its effectiveness in promoting differentiation should be investigated further, particularly its effects in normal non-oncogenic MYCN single-copy neuroblast cells.

Mathematical models as informative biomarkers in neuroblastoma (NB)

In our analyses we also postulated that the dynamics of pathway activity may contain prognostically relevant information different from that contained in the static nature of other types of biomarkers. To investigate this hypothesis, we characterized the network that regulated stress signaling by the c-Jun N-terminal kinase (JNK) pathway in NB cells. We generated an experimentally calibrated and validated computational model of this network and used the model to extract prognostic information from NB patient–specific simulations of JNK activation. An inability to initiate switch-like JNK activation in the simulations was significantly associated with poor overall survival for patients with NB with or without MYCN amplification. This analysis identified a group of high risk NB patients without MNA, for which currently no biomarkers exist [22]. More generally, this analysis showed that dynamic mathematical models can be very powerful biomarkers as they capture pathogenetic mechanisms and are have a high information content that supersedes single biomarkers [23].

Dynamic modelling was also employed for analysing the MYCN amplification and apoptosis sensitisation, with a specific focus on DNA-damage induced apoptosis. This model potentially can explain the poorly understood chemoresistance in MYCN-amplified NB. Since TrkA expression is a favourable prognostic parameter in NB, we also have systematically assessed downstream signalling pathways and constructed a mathematical model of TrkA signalling that could predict which network components are critical for mediating cell fate decisions. As NB cells are devoid of TrkA expression we engineered cell lines with inducible TrkA expression. Mathematical modelling has indicated that TrkA signalling can induce apoptosis which is mediated by activation of the JNK pathway and simultaneous inhibition of the protective PI3K pathway (in preparation). In summary, this work demonstrates that mathematical modelling can help to clarify molecular decision mechanisms that arise from nonlinear network structures and thereby make predictions how these decisions can be manipulated.

Insights from whole genome sequencing in neuroblastoma

Roughly half of Neuroblastomas regress spontaneously or are cured by limited therapy. In contrast, high-risk Neuroblastomas have an unfavourable clinical course despite intensive multimodal therapies, and their molecular basis has remained largely elusive. We performed whole genome sequencing of 56 representative cases (high-risk, n=39; low-risk, n=17), complemented by transcript, DNA methylation and histone modification profiling of the same cases. We discovered recurrent genomic rearrangements affecting a chromosomal region (5p15.22) proximal of the telomerase reverse transcriptase gene (*TERT*) [24]. These rearrangements occurred only in high-risk neuroblastomas (12/39, 31%) in mutually exclusive fashion with amplified *MYCN* oncogene and *ATRX* mutations, which are known genetic events in this tumour type. In an extended case series (n=217), *TERT* rearrangements defined a subgroup of high-risk tumours with particularly poor outcome. Despite the large diversity of these rearrangements, they all induced massive transcriptional upregulation of the *TERT* gene. The median *TERT* expression was 92-fold higher in *TERT*-rearranged tumours than in low-risk tumours. In the remaining high-risk tumours, *TERT* expression was also elevated in *MYCN*-amplified tumours as it is a transcriptional target gene of the MYCN oncoprotein. Alternative lengthening of telomeres was present in Neuroblastomas without *TERT* or *MYCN* alterations, suggesting that telomere lengthening/activation represents a central mechanism defining this

subtype. The 5p15.22 rearrangements juxtapose the *TERT* coding sequence to strong enhancer elements, resulting in massive chromatin remodeling and DNA methylation of the affected region. Accordingly, the region upstream of *TERT* that usually consists of condensed chromatin in most somatic cells keeping *TERT* in a silenced state is targeted by genomic repositioning rather than by gain of *TERT* copy numbers. We could further demonstrate that this mode of oncogene activation ("enhancer hijacking") in high-risk NBs is not restricted to the *TERT* oncogene. Similar rearrangements can activate other Neuroblastoma-relevant oncogenes such MYCN, MYC or ALK. In support of a functional role of TERT, neuroblastoma cells bearing rearrangements or amplified *MYCN* exhibited both upregulated *TERT* expression and enzymatic telomerase activity. *TERT* rearrangements occurred in approximately one quarter of high-risk Neuroblastomas. Our results indicate that most high-risk tumours are affected by either *TERT* rearrangements, *MYCN* amplification, or *ATRX* mutations, all of which funnel into telomere stability, thus providing a molecular, mechanistic definition of this neuroblastoma subtype. The most aggressive subtype of neuroblastoma was defined by telomerase activation as a result of either *TERT* rearrangement or *MYCN* amplification. With the further development of telomerase inhibitors, our finding might point to a novel therapeutic option for the most aggressive subgroup. By contrast, low-risk tumours are characterized by the absence of such alterations and low *TERT* expression levels, presumably precluding immortal proliferation of indefinite replicative capacity.

Identifying vulnerable nodes in neuroblastoma by synthetic siRNA screens

Based on a MYCN synthetic lethal screen using a druggable genome siRNA library DKFZ identified vulnerable nodes in neuroblastoma cells with high MYCN expression. We found methionine cycle and methyltransferase (e.g. EZH2, MLL2, DNMT1) inhibition as synthetic lethal interactions with amplified MYCN. MYCN induces a metabolic adaptation in the methionine cycle via activation of several methionine cycle enzymes, in turn favoring an epigenetic state of excessive methylation reactions. We provided mechanistic evidence that this metabolic switch is involved in aberrant histone and DNA methylation (CpG island methylator phenotype, CIMP), as well as large-scale transcriptional reprogramming in MYCN-amplified NB cells. In an integrative approach, we further analyzed methylomes, transcriptomes and copy number variations in 105 neuroblastomas, complemented by primary tumour- and cell line-derived global histone modification analyses and epigenetic drug treatment *in vitro*. DNA methylation patterns defined strongly divergent patient subgroups with respect to survival and clinicobiological variables, including amplified *MYCN* [25] (Henrich et al. *in press*). Transcriptome integration and histone modification-based definition of enhancer elements revealed intragenic enhancer methylation as a mechanism for high risk disease associated transcriptional deregulation.

Further, we provide evidence for PRC2 activity (primarily EZH2) and DNA methylation (e.g. DNMT1) collaborate in ongoing repression of pro-differentiation programs in high-risk neuroblastomas. Intriguingly, these programs can be effectively re-induced via combination treatment targeting the repressive effect of both PRC2 and DNA methylation. Our data considerably extend the understanding of how epigenetic deregulation contributes to neuroblastoma pathogenesis and can inform novel diagnostic and therapeutic development for children with unfavorable neuroblastoma.

Modelling and predicting chemotherapy survival of neuroblastoma cells

A particularly challenging problem is how the knowledge of genetic lesions and epigenetic dysregulation coming from molecular studies described can be converted into improved therapy. We addressed this problem using a combination of mathematical modeling and model-driven, quantitative experiments. We focused on how MYCN amplification deregulates systems-level checkpoints for cell proliferation and DNA repair after chemotherapy. Using a combination of transcriptomics, proteome analyses and live-cell imaging of individual cancer cells and their pedigrees, we established that high MYCN levels in the cell abolish a key checkpoint for cell proliferation, the so-

called restriction point, resulting in fast growth of the tumour. Conventional chemotherapy successfully eliminates a large fraction of these fast-growing cells. However, an appreciable fraction survives treatment. Here we have shown that the same MYCN-driven molecular mechanisms that abolish the restriction point in dividing neuroblastoma cells push cells back into proliferation after chemotherapy. Single-cell analyses show that while the majority of these re-proliferating cells die due to DNA damage sustained by chemotherapy, a small fraction of "resister cells" regain full viability and give rise to growing clones of tumour cells. This phenomenon of near eradication of the tumour upon chemotherapy and re-growth of a few surviving cells mimics a relapse scenario often observed with neuroblastoma patients. Therefore, we further characterized the properties of these resister cells, leading to suggestions for new combinations of conventional chemotherapy and molecularly targeted drugs to treat aggressive, MYCN-amplified neuroblastoma.

New roles for MYCN in neuroblastoma (NB)

We used in depth data mining analyses on large data sets from human neuroblastoma samples in combination with data obtained from MYCN driven tumours in mouse models in order to identify new MYCN regulated processes that can be exploited as therapeutic targets. As such, we discovered replicative stress resistance (RSR) as a novel cancer hallmark implicated in NB. RSR protects NB cells against harmful events during DNA replication and cell division. Extensive experiments in cellular model systems and in a novel zebrafish model demonstrated the crucial role of BRIP1 in allowing cancer cells with high MYCN activity to cope with replicative stress. We also were able to show that RSR is druggable, using a molecule that drastically increases replicative stress. Moreover, in combination with other drugs we could show that synergistic effects can be generated.

We also identified the transcription factor SOX11 as novel contributor to MYCN induced cancer formation. High SOX11 activity levels make NB cells more aggressive. Mechanistically, MYCN and SOX11 protein interact in the cell and bind to overlapping target genes. Both a mouse and zebrafish model have been generated for SOX11 overexpression in order to generate pre-clinical models for drug testing and to study the biology and role of SOX11 in neuroblastoma in depth.

Using a MYCN driven mouse NB model we established a unique data set representing the dynamic up and down regulation of coding and noncoding genes. This resources has proven instrumental for the analysis of candidate genes from other studies. It allowed to identify a series of putative synergistically acting transcription factors in this tumour opening new venues for treatment.

New roles for ALK in neuroblastoma (NB)

The second most frequently altered gene in NB is ALK, a TGFβ receptor that normally responds to growth signals assuring an appropriate growth response. In cancer cells ALK1 is switched on constitutively due to gene amplification, mutation or gene fusion thus constantly firing pro-growth signals in the cell. Here, we have identified two important genes in the ALK1 signalling cascade that offer new approaches for drugging ALK1 activated tumour cells. First, we showed that HBP1, a negative regulator of MYCN is suppressed by ALK1, explaining why ALK activation in MYCN overexpressing cells further increases the aggressiveness of these cells. We fully dissected the signalling pathway controlling HBP1 levels [26] and also discovered new therapeutic drug combinations [27] based on this observation which are currently being tested in pre-clinical mouse and zebrafish models. Secondly, we identified ETV5 as a novel neuroblastoma oncogene controlled by ALK1 signalling showing that ETV5 activation may contribute to a process termed "epithelial-mesenchymal-transition" or EMT which is well known to accelerate invasion and metastasis.

Proteomic analysis of TrkA signaling

Many neuroblastoma tumours are prone to spontaneous regression or they differentiate into a more benign state rendering patients off therapy. These favorable biological characteristics are associated

with nerve growth factor (NGF) signaling via its high-affinity receptor, the TrkA RTK. To identify the underlying molecular mechanisms responsible for this phenomenon we performed a comprehensive proteomics analysis of NGF signaling dynamics in TrkA-expressing neuroblastoma cells using quantitative mass spectrometry. Our dataset encompassing time-resolved interactome, proteome and phosphoproteome provided a unique view into NGF-TrkA signaling as well as the possibility to discover new protein candidates involved in regulating this process and thus, with the potential to function as new biomarkers or drug targets in neuroblastoma.

The work was published in Science Signaling and made the cover page with the title: "Temporal proteomics of NGF-TrkA signaling identifies an inhibitory role for the E3 ligase Cbl-b in neuroblastoma cell differentiation". The manuscript describes the global and proteome-wide changes in NGF-TrkA signaling dynamics underlying the differentiated favorable patient phenotype using a neuroblastoma cell culture model system. Signaling activation elicits neurite outgrowth in neuroblastoma cells, a hallmark of neuronal differentiation. Dynamics of NGF-TrkA signaling was explored by a quantitative mass spectrometry-based approach. Besides characterizing three unique layers of signaling information (the interactome, the phosphoproteome and the proteome) spanning 5 minutes – 48 hours of duration, the study led to the identification of an inhibitory E3 ubiquitin ligase.

In more details, we performed a temporal analysis by mass spectrometry of changes in the proteome in response to NGF in neuroblastoma cells. We found that NGF not only activated TrkA, but also initiated its degradation by promoting the interaction of TrkA with the E3 ubiquitin ligase Cbl-b, which resulted in the ubiquitylation and degradation of both proteins. Neuroblastoma cells with reduced Cbl-b levels had increased TrkA signaling and produced longer neurites. In addition to identifying this inhibitory role for Cbl-b, the proteomics data are a resource for further investigation of TrkA signaling dynamics.

In a second project we focused our attention on a different receptor tyrosine kinase in neuroblastoma, which expression correlates with poor prognosis. Aberrant signaling by ALK is found in a subset of neuroblastoma patients and increased ALK activity contributes to a more malignant disease phenotype. To delineate signaling pathways underlying oncogenic ALK-mediated growth of neuroblastoma cells we applied a quantitative mass spectrometry-based proteomics approach.
We treated an ALK-amplified neuroblastoma cell line, displaying constitutive ALK activity, with three clinically relevant ALK tyrosine kinase inhibitors (crizotinib, TAE684 and LDK378). By combining information on different layers of signaling (gathering interactome, phosphoproteome, proteome and peptide pull-down data) our analysis provides unique insights to how perturbations of ALK activity affects signaling and growth of neuroblastoma cells.
Our multi-layered proteomics analysis revealed a number of new ALK effector molecules in neuroblastoma. This comprehensive global analysis of the ALK signaling network using quantitative proteomics has revealed novel aspects of ALK function in neuroblastoma and can function as a resource within the field of ALK signaling and neuroblastoma.

Medulloblastoma (MB)

MB is the most common childhood tumour of the central neural system. Histological and molecular stratification have revealed that the transcription factor c-Myc is frequently deregulated in this disease and is often associated with poor patient outcome. Since strategies to target c-Myc directly remain elusive, the signaling networks controlled by the oncogene are considered to offer a promising alternative approach for less toxic and patient-tailored therapies.

The role of miRNAs in MB

Despite their recent discovery as powerful diagnostic markers, very little is known about the role of miRNAs in brain neoplasia and in particular in MB. We have performed work on miR-125b in MB [28]. This was based on our previous work showing that the phosphoinositide 3-kinase (PI3K) pathway plays an important role in the regulation of medulloblastoma cell survival and proliferation, although the molecular mechanisms and downstream effectors underlying PI3K signaling still remain elusive. The impact of RNA interference (RNAi)-mediated silencing of PI3K isoforms p110α and p110δ on global gene expression was investigated by cDNA microarray analysis in MB cell lines [28]. A subset of genes with selectively altered expression upon p110α silencing in comparison to silencing of the closely related p110δ isoform was revealed. Among these genes, the leukemia inhibitory factor receptor α (LIFRα) was validated as a novel p110α target in MB. A network involving c-Myc and miR-125b was shown to be involved in the control of LIFRα expression downstream of p110α [28]. Targeting the LIFRα by RNAi, or by using neutralizing reagents impaired medulloblastoma cell proliferation *in vitro* and induced a tumour volume reduction *in vivo*. An analysis of primary tumours revealed that LIFRα and p110α expression were elevated in the sonic hedgehog (SHH) subgroup of medulloblastoma, indicating its clinical relevance. Together, these data reveal a novel molecular signaling network, in which PI3K isoform p110α controls the expression of LIFRα via c-Myc and miR-125b to promote MB cell proliferation [28].

Synthetic lethal siRNA screens in MB

We performed a high-throughput siRNA screen in c-Myc-transfected and empty vector-transfected medulloblastoma cells. Statistical analysis revealed genes whose silencing decreased cell viability more in c-Myc than in vector-transfected cells. The candidates identified in the screen were EPHA7, PCTK1, AKAP12 and CDKL1. PCTK1 is a member of CDK family implicated in cell cycle, proliferation, vesicle trafficking, neurite growth and spermatogenesis. We then synthesized a small molecule PCTK1 inhibitor in order to perform validation experiments using pharmacological inhibition of PCTK1. The results indicated that PCTK1 depletion reduced cell proliferation in a dose-dependent manner in c-Myc-overexpressing cells. More detailed analysis of cell cycle indicated that inhibition of PCTK1 results in G2/M arrest. Considering that the knowledge about the PCTK protein family still remains obscure, we employed a bioinformatics analysis. The results indicated that GSK3β, a protein kinase implicated in a variety of different pathways and deregulated in many cancers, was the top candidate linked to PCTK1 in MB. Indeed, experimental results have confirmed that GKS3β phosphorylation (Ser9 site inactivating GKS3β) decreased upon PCTK1 inhibition. This observation indicates that GSK3β is inactivated by PCTK1. Accordingly, PCTK1 inhibition induced a decrease in c-Myc expression levels, providing a molecular basis for the increased sensitivity of c-Myc-over-expressing MB cells to PCTK1 inhibition. The PCTK1 inhibitor also induced a decreased in tumour formation by c-Myc-over-expressing MB cells in an *in vivo* assay. Taken together, PCTK1 was uncovered as a novel druggable molecular target in MYC over-expressing medulloblastoma [29].

We have also performed a kinome-wide siRNA screen in established MB cell lines to identify genes involved in cell survival and resistance to the chemotherapeutic agent cisplatin. A set of 6 genes comprising *ATR, LYK5, MPP2, PIK3CG, PIK4CA, and WNK4* were identified as contributing to both cell proliferation and resistance to cisplatin treatment in MB cells. An analysis of the expression of the 6 target genes in primary MB tumour samples and cell lines revealed overexpression of *LYK5* and *PIK3CG*. The results of the siRNA screen were validated by target inhibition with specific pharmacological inhibitors. A pharmacological inhibitor of p110γ (encoded by *PIK3CG*) impaired cell proliferation in MB cell lines and sensitized the cells to cisplatin treatment. Together, our data show that the p110γ phosphoinositide 3-kinase isoform is a novel target for combinatorial therapies in medulloblastoma [30]

Synthetic lethal kinome screens in MB

In parallel, we have performed a kinome-wide siRNA screen in established neuroblastoma (NB) cell lines to identify genes involved in cell survival and resistance to Cisplatin. As a result we identified the fibroblast growth factor receptor 2 (FGFR2) as an important determinant of cisplatin resistance. Pharmacological inhibition of FGFR2 confirmed the importance of this kinase in NB chemo-resistance. Silencing of FGFR2 sensitized neuroblastoma cells to cisplatin-induced apoptosis, which was regulated by the downregulation of the anti-apoptotic protein BCL2. Mechanistically, FGFR2 was shown to activate protein kinase C delta (PKC-δ) to induce BCL2 expression. FGFR2, as well as the ligand FGF-2, were consistently expressed in primary NB and NB cell lines, indicating the presence of an autocrine loop. Gene expression profiling data revealed that FGFR2 expression correlates with *MYCN* amplification and with advanced stage disease, demonstrating the clinical relevance of FGFR2 in neuroblastoma. These findings suggest a novel role for FGFR2 in chemo-resistance and provide a rationale to combine pharmacological inhibitors against FGFR2 with chemotherapeutic agents for the treatment of neuroblastoma [31].

Crosscutting Findings

The project was designed as an integrated effort of deploying systems approaches to gain new insights into molecular pathogenicity of embryonal tumors, and design new diagnostics and therapeutic strategies. Therefore, many activities ranged across different ET entities.

The role of miRNAs in ETs

Concentrating on several growth factors and their receptors, we studied downstream signaling and the initiation of transcriptional events in response to specific growth factors. Specifically, we portrayed a wave-like program, which initiates by rapid disappearance of two-dozen microRNAs, followed by an abrupt rise of immediate early genes (IEGs), relatively short transcripts encoding transcriptional regulators. Concurrent with the fall of IEGs, some 30-60 min after stimulation, a larger group, the delayed early genes, is up-regulated and its own fall overlaps the rise of the final wave of late response genes. This late wave persists and determines long-term phenotype acquisition, such as invasiveness. Key regulatory steps in the orderly response to growth factors provide a trove of potential oncogenes and tumour suppressors.

Crosstalk between receptor tyrosine kinases (RTKs) and nuclear receptors for steroid hormones

Importantly, our work contrasted receptor tyrosine kinases (RTKs) and nuclear receptors for steroid hormones, because both groups are essential for body homeostasis, but the cross-talk between these receptor families is poorly understood. We observed that glucocorticoids inhibit signalling downstream of the EGFR RTK. The underlying mechanism entails suppression of the EGFR's positive feedback loops and simultaneous triggering of negative feedback loops that normally restrain EGFR signalling. Our studies in mice revealed that the regulation of EGFR's feedback loops by glucocorticoids translates to circadian control of EGFR signalling: EGFR signals are suppressed by high glucocorticoids during the active phase (night-time in rodents), while EGFR signals are enhanced during the resting phase. Consistent with this pattern, treatment of animals bearing EGFR-driven tumours with a specific kinase inhibitor was more effective if administered during the resting phase of the day, when glucocorticoids are low. These findings support a circadian clock-based paradigm in cancer therapy.

Technology Advances

The project used a range of modern technologies, and their application also produced advances in methodology as well as useful scientific results.

Systematic and elective high throughput perturbation screens to identify vulnerabilities in Embryonal Tumour (ET) cells

A main goal of ASSET was to identify mechanistically understood network vulnerabilities that can be exploited for new approaches to the diagnosis and treatment of major paediatric tumours. Therefore, VTT performed high-throughput perturbation screens targeting key driver oncogenes, (MYCN, c-Myc and EWS-FLI1) and their signalling networks to identify druggable pathways, drug targets and growth inhibitory compounds. The ASSET ET cell models used in these perturbation screens were (i) SY5Y/6TR (EU)/pTrex-Dest-30/MYCN cells with a tetracycline inducible MYCN construct as neuroblastoma mode; (ii) UW-228 cells with 4-OTH inducible c-Myc expression as a medulloblastoma model; and (iii) A673/TR/shEF cells with doxycycline inducible small hairpin RNA downregulating EWS-FLI1 expression as Ewing sarcoma model. To identify MYCN, c-Myc and EWS-FLI1 dependent vulnerabilities in these ET cell models, HTS using commercial miRNA, siRNA and compound libraries were made in both in presence and absence of induction. Cell viability was measured as the endpoint 3 days after miRNA, siRNA or compound exposure using CellTiter-Glo® Luminescent Cell Viability Assay. The miRNA screens with Dharmacon miRIDIAN® mimics (gain-of-function, N = 810) and Dharmacon miRIDIAN® inhibitors (loss-of-function, N = 896) were performed at least twice in each condition and siRNA screen targeting druggable genome (Qiagen Druggable genome v3, targeting ~7000 genes, screened as pools of 4 siRNA molecules per target gene) was performed once per each condition. Compounds in high-throughput perturbation screens were studied with at least two different concentrations and included Multisource Spectrum (2000 compounds, known drugs, other bioavailable compounds and natural products), Biomol International (84 kinase and phosphatase inhibitors), Sigma LOPAC (1280 compounds, FDA approved drugs and other compounds pharmacologically relevant structures) and Selleck (N = 522, a selection of FDA approved drugs and additional experimental compounds) libraries. The raw HTS results were normalized and deposited to ASSET data warehouse to facilitate their use by the ASSET consortium. Selected results were validated as collaborative efforts among ASSET partners. The overview of the main findings from each ET model is summarized briefly below.

Neuroblastoma (NB) screens

Genome wide miRNA screen identified several novel putative tumour suppressive and oncogenic miRNAs. 180 miRNA candidates were selected based on high-throughput screening results and mapped to the human genome. This analysis retrieved five miRNAs from 1p, showing striking growth inhibitory potential. These miRNAs included well-known mir-34a as well as four candidates that had not been functionally characterized in NB [32]. Moreover, dozens of growth inhibitory and growth promoting miRNAs were mapped to two large miRNA clusters in the human genome, C19MC at chromosome 19 and the 14q32 miRNA cluster. Out of 180 candidates, four of the strongest anti-proliferative miRNA candidates were selected for further validation in other NB cell models and all four miRNA candidates were shown to activate p53 pathway. These results were integrated with miRNA expression results from NB tumours and are being prepared for publication.

High-throughput siRNA screen identified 257 anti-proliferative hits (hit rate 4%) and out of these, 79 reduced cell viability only in induced MYCN overexpressing NB cells. The siRNA screen included perturbation data for 1684 “prognostic genes” defined by U. Bonn using a novel bioinformatics approach based on analyses of genome-wide transcriptomic data derived from NB tumour samples [33]. Out of the prognostic genes targeted by siRNA molecules, 66 were identified as anti-proliferative hits. Gene ontology analysis indicated that these genes are involved in cell cycle, protein ubiquitination, translational elongation, neuronal development and apoptosis – all potential druggable processes for the inhibition of NB cell proliferation. In addition, high-throughput compound screening identified several compounds with more potent anti-proliferative effect in MYCN over-expressing NB cells.

Comparison of results from HT siRNA and compound screens identified multiple connections and some of these were selected for further studies to understand mechanism behind MYCN driven vulnerabilities in more detail [17]. High-throughput RNAi screening method as well as the network-based interpretation leading to potential diagnostic and therapeutic utilisation of some of siRNA targets have identified beta-estradiol, MAPK/ERK, and the WNT pathway as promising targets [19, 21].

Ewing sarcoma family tumour (ESFT) screens

To identify EWS-FLI1 driven vulnerabilities high-throughput perturbation screens were initiated 72 hours after shRNA induced EWS-FLI1 silencing (knock-down was ~90%). Genome wide miRNA screening identified nine pre-mir candidates only expressed in the presence of EWS-FLI1. The anti-proliferative effect for two of these (miR-552 and miR-631) was validated showing that miR-552 induced a G1 arrest while miR-631 elicited a G2 cell cycle arrest. We further undertook a bioinformatics analysis of the results by assessing whether experimentally validated mRNA targets for each miRNA are enriched in certain pathways or processes. The only experimentally validated mRNA target enriched among EWS-FLI1 selective miRNA hits was GAPDH, a key component of glycolysis. These results support previous suggestion that targeting glycolytic activity is a way to treat Ewing sarcoma. The high-throughput siRNA screen highlighted the role of Leucine rich repeats and WD repeat Domain containing 1 (LRWD1) as a potent regulator of EWS-FLI1 driven cell proliferation [34]. A further exploration of HT drug screening results was done by VTT together with UCPH, CEMM, and CCRI, and a manuscript describing these findings together with exploration of molecular mechanism behind EWS-FLI1 driven differential response to BCL2 family inhibitors is in preparation.

Medulloblastoma MB screens

High-throughput miRNA screening in MB model identified five precursor miRNAs and two miR inhibitors reducing cell viability only in c-Myc over-expressing cells. Results from validation experiments indicated that the strongest decrease in cell proliferation in c-Myc-overexpressing MB cells was identified after inhibition of hsa-miR-106b. Several bioinformatics analyses were performed to identify potential targets of miRNA candidates and serine/threonine kinase PCTAIRE-2 was validated as a downstream target of mir-106b in Medulloblastoma [35]. High-throughput siRNA screen identified 13 putative targets whose silencing reduced cell viability especially in MYC over-expressing MB cells. Out of the candidates chosen for validation, the strongest c-Myc dependent anti-proliferative and pro-apoptotic effect was seen in response to RNA-binding protein 8A (RBM8A) silencing. RBM8A is a core exon junction complex factor, which binds to mRNA molecules during splicing. Results from a recent study indicate that RBM8A is involved in proliferation and differentiation of embryonic neural progenitors supporting the HTS results on vital function of RBM8A in MB. High-throughput compound screen identified many compounds sensitizing c-Myc over-expressing MB cells to anti-proliferative effect. Twenty of these compounds were selected for validation in other MB cell lines. These experiments indicated that some of the selected hit compounds reduced c-Myc and MYCN expression in cultured MB and NB cells as well as tumour growth in transgenic NB zebrafish models. Corresponding manuscripts are under preparation.

New targets arising from the screens

We have also investigated the potential of targeting the axis of the insulin-like growth factor-1 receptor (IGF-1R) and PI3K signalling in the two embryonal tumour entities neuroblastoma and medulloblastoma. By treating neuroblastoma and medulloblastoma cells with R1507, a specific humanized monoclonal antibody against the IGF-1R (ROCHE), we could observe cell line-specific responses and in some cases a strong decrease in cell proliferation. In contrast, targeting the class I PI3K p110α with the selective inhibitor PIK75 resulted in broad anti-proliferative effects in a panel of neuroblastoma and medulloblastoma cell lines. Additionally, sensitization to commonly used chemotherapeutic agents (cisplatin and doxorubicin) occurred in medulloblastoma and neuroblastoma cells upon treatment with R1507. Furthermore, by studying the expression and

phosphorylation state of IGF-1R/PI3K downstream signaling targets we confirmed down-regulated signaling pathway activation by R1507 and PIK75. In addition, apoptosis occurred in embryonal tumour cells after treatment with PIK75 or R1507. Together, our studies demonstrate the potential of targeting the IGF-1R/PI3K signaling axis in embryonal tumours [36].

Investigating the dynamic behaviour of protein signalling networks by reverse protein microarrays

Reverse Protein Arrays allow the antibody based simultaneous quantification of proteins of interest using very small amounts of cell lysates. This assay is complemenatry to the MS based discovery approaches as it allows the generation of targeted measurements comparing different conditions or timecourse treatments. Protocols to prepare protein lysates for Zeptosens Phosphorylation Array Analysis were optimized for SH-SY5Y cell lines engineered to express either TrkA or TrkB receptors. There receptors drive opposite cell fates in neuroblastoma cells, i.e. differentiation and apoptosis versus proliferation. Among 122 analyzed proteins/phosphorylated proteins 16 showed regulation. Among the regulated proteins are pAkt, CDK1, CyclinD1, pGSK3β, pMEK1/2, pStat3, pPKCα, pPLCγ, pTyk2, S6 ribosomal protein and SHP2. The overlap of similar regulated proteins between NGF (TrkA) and BDNF (TrkB) signalling was seen in 15 out of the 16 regulated proteins. A promising target was Phospholipase C gamma (PLCγ) which was selectively upregulated in SY5Y-TR-TrkA cells after NGF stimulation and not in SY5Y-TR-TrkB cells after BDNF stimulation. This indicates a role of PLCγ in cell death and differentiation. These results were validated by Western blotting.

Development of new computational biology resources

The systems medicine approach required the development of new computational tool for data analysis, integration and storage.

A comprehensive resource for data collection and analysis: the ASSET Data Warehouse ASSETmart

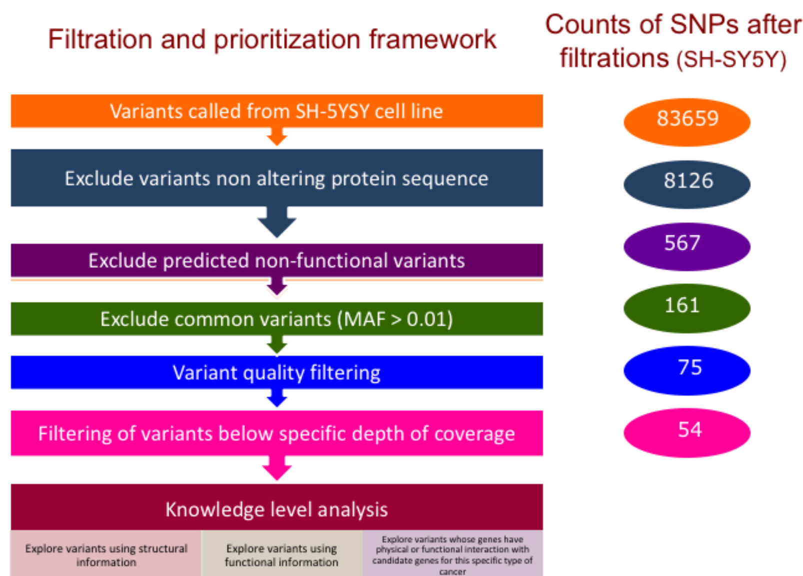
To harvest, manage and preserve data generated in ASSET an interrogable project database for holding shared experimental data was developed. This allowed the high-throughput data submitted by the partners to be integrated at a gene as well as cell line level as well as the possibility to include public data sets. This is a powerful queryable repository for extracting data and building pipelines on. At its heart is a data warehouse to support and enable the combination of project proprietary data with publicly available data sources. Core data types included in the repository are gene expression microarrays, CHiP sequencing, RNA sequencing, Exome sequencing, proteomics, compound screening, siRNA screening and miRNA screening. Data is represented to that the user can directly find the experiment type or platform used, information on the samples studied including the cancer type or the cell line name, specific conditions describing each sample, number of replicates used or additional important information, not described above (e.g. type of measurement). Public data sources that are included in the database include Ensembl, PubChem, ChEMBL, COSMIC and InWeb.

ASSETmart has a subversion repository holding the source code and the data produced by the ASSET partners hosted at the DTU subversion archive. However, to facilitate the usability of the warehouse we have developed a user-friendly web interface, enabling thus the ASSET partners to browse, explore and combine in-house and publicly available data. ASSETmart is available at: https://asset-mart.cbs.dtu.dk. The interface allows users to either query for one or multiple biological entities (e.g. genes, proteins, transcripts, compounds or microRNAs) or experiments and experimental conditions of interest. Furthermore, the database is directly linked with the visualisation tool Plotly (https://plot.ly) which provides a web-based user-friendly interface and platform for data analysis, graphing and representation.

This database will be made accessible to the scientific community at the end of the project, and represents the lasting legacy of ASSET to the scientific community.

Development of data analysis pipelines for mutations

Various data analysis pipelines were constructed on ASSET. In particular, variant prioritisation was a key theme in processing exome sequencing data representing the tumourigenic burden of mutations. A key element is data reduction in order to select mutations that are likely pathogenic or germane to the tumour development and potentially contributing to the perturbed cell signalling necessary for pathogenesis. Understanding these key mutations is important in mechanistic insights as well as in understanding potential treatment strategies. The figure illustrates the use of a variant prioritisation and annotation pipeline on the neuroblastoma cell line SY5Y, where 83,659 original variants were triaged into selecting 54 mutations of high interest for further validation and studies.



New tools for data integration and contextualisation

A main goal of ASSET was to develop new tools to integrate and contextualize the data collected by the consortium. These activities can be broadly subdivided into the development of:

Cell-type specific models to simulate the different cell lines and predict the effect of drugs

We used simulations based on networks to create computational models of the cell lines. Networks of pathways were defined, where each node is a pathway (a set of proteins that are functionally related to each other and define a biological process) and regulatory interactions between pathways were inferred from the literature using information collected from online databases. The approach was based on the Boolean framework, which represents the cell as an assembly of logic circuits that describe how the various nodes (biological processes) turn on and off in response to different inputs. This model allows the simulation of activation and repression of the different pathways, with each node of the network only able to take binary values (ON/OFF). Such simplified models were used to simulate processes inside a single cell, for example whether it was growing, dividing or killing itself (apoptosis). The simulation of populations of cells was implemented using multiple instances of this model running inside each cell. This procedure is needed to reproduce *in silico* the experiments in which gene expression datasets were generated, which involve pooling together millions of cells and measuring the total amount of expression in the population. The final ingredient to make the simulations more closely represent the biology of a specific cell line is using binarised expression data

(ON/OFF for each gene) to establish the fractions of cells in the simulated population in which each pathway and connection are present. For each node and edge in the network we assigned a probability of presence proportional to the number of genes that were present within each node or edge, as measured by expression in the specific cell line of interest. We can thus make cell-type specific models that can be run in the computer and used to simulate experiments, which can be useful to predict which real experiments in the laboratory would be most informative.

In one application, the estimation of activation of different pathways in specific cell lines was used to predict drug synergies. We used publicly available databases with information about which proteins are affected by specific drugs to infer interactions between each drug and the different pathways. The interactions were stronger when the pathway contained more of targets of the drug. The association between drugs and pathways targeted is made cell-type specific by considering only proteins inside the pathway that are present, as evidenced by expression in the cell-type of interest. One can thus obtain a cell-type specific profile of drug-pathway interactions that can be used to compare drugs to each other. We defined the similarity of two drugs based on how similar their interactions with all the different pathways were (that is measuring the correlation of the drug-pathway interaction profiles). We could then use these values of drug-drug similarity to predict the effect of drugs alone or in combination. For example, if two drugs were shown to synergise, we could predict that substituting one of the two drugs with another similar drug would also lead to a synergistic effect. We also developed a framework that can integrate different networks describing different types of biological regulation mechanisms. This way we can potentially follow how the action of a drug can be propagated as a signal to different components in the cell, which will facilitate the interpretation and prediction of why two drugs act synergistically.

Tools to perform analyses and comparison of the mutational profiles of the cell lines and primary samples within the context of large international cancer patient datasets

An important part of ASSET involved looking at DNA sequence mutations in cell lines and patient derived cells that affect protein coding regions (exome sequencing). These experiments were performed on the three core cell lines (SH-SY5Y, IMR575 and ASP14) and a cohort of Ewing Sarcoma patients. Knowing in what proteins the mutations are found is just the first step towards understanding how these mutations might be related to the cancer studied. We, therefore, developed and applied different bioinformatic tools to predict whether these mutations were likely to be causally associated with tumorigenesis or simply the result of random mutations and without consequences. To this end we used ASSETScout, a suite of computational bioinformatic tools that was developed to perform the analysis of mutations found by exome sequencing, assess their pathogenetic relevance, and associate them with biological processes. The platform takes as an input a list of variants (specific changes of DNA sequence compared to the human reference genome sequence), and outputs the predicted damage potential for each variant, a description of the biological processes that might be affected, and the frequency of these variants across different sets of publicly available datasets for mutations in cancer patients , e.g. International Cancer Genome Consortium IGC), The Cancer Genome Atlas (TGCA).

The prediction of whether the mutation observed could be damaging the protein's function is performed using a set of methods including two main applications that we developed: StructurePPI and KinMut. StructurePPI estimates whether a specific variant is likely to affect protein function by overlapping the genomic location of the variant with the location of protein features from 7 different public databases. This allows us to see whether the variants affects parts of the protein that bind ligands or DNA, usually important for the protein function, or whether mutations in this region of the protein were seen in other cancer datasets. KinMut looks for whether the mutations affects kinases at the level of the whole protein and its physico-chemical proteins, or disrupting specific protein domains or even single amino acids that are known to be disrupted in cancer. This suite of tools

allowed us to identify which of the mutations in the cell lines might be related to the cancers in the patients against a background of ransom mutations. The comparison of mutations seen in our cell lines and mutations seen in large cancer genomics projects such as ICGC, showed a strong overlap with genes mutated in a Neuroblastoma cohort.

We also looked closely at mutations in a large group of Ewing Sarcoma patients and saw that one of the most commonly mutated proteins was STAG2. This protein has a function in ensuring correct cell division and in some cancers patients with STAG2 mutations have worse prognosis. However, in other cancers, like bladder cancer and an aggressive type of leukaemia, mutations in this protein are actually found in patients that are more likely to survive. We hypothesised that STAG2 may have roles in other biological processes than cell division, since it is also involved in maintaining the three-dimensional structure of chromatin inside the nucleus. This suggested a possible epigenetic effect. We tested our hypothesis by looking at which genes change together with STAG2 when the ASP14 cell line is treated with different drugs. These genes were found to be related to splicing. Therefore, STAG2 might have an additional function, which adds new mechanistic insights into the molecular pathogenesis of Ewing Sarcoma, and potentially may reveal new targets.

Tools for comparing gene expression profiles from cell lines and primary patient samples
One of the main objectives of ASSET was to study cell lines derived from embryonal tumours to gain a better understanding about the tumours themselves and provide new therapeutic options for the patients. We, therefore, developed tools to enable the comparison of data generated from cell lines with that from patient samples. This is not trivial, due to the transformation that cells undergo in the process of cell line derivation, and because of the presence of non-tumour cells in primary samples (including healthy cells from tissues surrounding the tumour and cells from the immune system). These differences make it difficult to directly establish similarities of gene expression profiles between cell lines and primary samples that would allow us to use a cell line or group of cell lines as a model for specific patients. We constructed a mathematical model which identified the genes that are mostly responsible for the separation between gene expression of all cell line samples and patient ones. We then removed these genes from the expression profiles and again established similarities of all the samples. After this correction we could separate the cell lines by type and see how some patients resemble more to some of them than other. This is an important result as it suggests that our understanding of the different embryonal tumour derived cell lines can be used for designing personalised targeted treatments for the patients.

Integration of genomic and transcriptomics data for enhanced patient stratification

Here, the goal was to increase our knowledge about the dysregulation of the gene regulatory networks in NB in order to propose novel targets for interventions, and develop an equitable, risk-related stratification of neuroblastoma patients. To this end we undertook a systemic approach by integrating genomic, transcriptomic and clinical data with functional perturbation data. As result, we established a comprehensive catalogue of genes over- and under-expressed in neuroblastoma implicating poor prognosis, called NBPrognostics [17]. The functional annotation of the genes showed that the downregulated prognostic genes are involved in the development and maturation of the peripheral nervous system, in the differentiation of neurons, neuritogenesis, axonogenesis etc. The dysregulated pathways included NGF receptor signalling, SLIT/Robo signalling, Ephrin, plexin receptor signalling, Rho/RAC pathway, retinoic acid receptor signalling, WNT, beta-catenin, calcium and calmodulin signalling. The upregulated prognostic genes are involved in the cell proliferation, cell cycle progression, DNA damage and DNA repair, ribosomal biogenesis. These results are similar to the results obtained by integration of transcriptomics and proteomics data [19, 37] demonstrating the robustness of our data integration approaches. Furthermore, NBPrognostics identified genes whose expression is affected by 11q and 3p deletions and 17q gain, which are common genomic alterations in NB that are independent of MYCN amplifications. The NBPrognostics catalogue was consolidated

with the results from the high-throughput siRNA screening and compound screening. The hit compounds, found to reduce proliferation and viability of neuroblastoma cells, were linked to those prognostic genes, which are described as targets of these compounds in chemical databases. The drug-target connectivity represents a useful platform for the exploration of neuroblastoma vulnerabilities and generation of hypotheses towards better therapeutic interventions. In particular, the Bcl-2 antagonist obatoclax was focused on in further validations since it was indicated as a potent anti-proliferative hit and its target MCL1 (myeloid cell leukemia sequence 1) was indicated as prognostic overexpressed gene and as hit in siRNA screens. Validation in zebrafish model showed that obatoclax decreased tumour growth in our zebrafish model of MYCN-driven neuroblastoma. Moreover, validation results indicated that the anti-proliferative response to obatoclax was potentiated by MYCN over-expression. Taken together, the BCL2-family signalling is proposed as an interesting vulnerability to be targeted in neuroblastoma.

Development of statistical tools for analysing timecourse series of gene expression data

Inference of gene regulatory networks is impossible without time-resolved gene expression data, which give insight into the dynamics of gene expression programs. For this purpose we developed SwitchFinder, a statistical method for the analysis of time-series data, and implemented it as Web application. The approach fits a change-point model to each individual gene expression time-course, thus indicating the switch-points between increasing and decreasing activities of the gene. We introduced features of the switch-points: growth, decay, spike and cleft, reflecting important dynamic aspects. With this, the gene expression profiles are represented in a qualitative manner, enabling to deduce meaningful groups of genes with common dynamic patterns. Our Web application enables users to upload their data, run the algorithm and download the results. The query interface facilitates interactive exploration of the gene dataset. SwitchFinder was applied to the gene expression time-series measured at seven time-points in neuroblastoma cell line upon treatment with the differentiation agent ATRA (all-trans retinoic acid). The analysis revealed eight types of dynamic transcriptional responses to ATRA. The results indicated that the transcriptional response of NB cells to ATRA treatment is the time-resolved result of BMP, Wnt, Notch, FGF, G-protein coupled receptor and neurotrophin TRK receptor signalling. This coincides with the gene regulatory programs during differentiation of the neural crest cells in course of the development of the sympathetic nervous system. The immediate and early responses were rich in transcription factors involved in the determination of cell fates and regulation of embryonic development. For example, immediate early genes were responsible for axon guidance and axonogenesis. The results indicated a contribution of ATRA to the migratory phenotype of neuroblastoma cells. Repressed were the genes involved in cell cycle regulation and cell proliferation, DNA metabolic process, DNA damage response, DNA repair signalling etc. Many of the prognostic down-regulated genes (as determined by NBPrognostics) were induced by ATRA, while many of the up-regulated were repressed. This supports the hypothesis that neuroblastoma pathogenesis results from a block of cell differentiation, the promotion of cell proliferation, and maintenance of cell stemness.

Developing new statistical tools for analysing drug cooperation

Determining whether two drugs cooperate and in which way (synergistic, additive, antagonistic) is an experimentally very laborious task, that requires careful planning and data interpretation. Often it is logistically impossible to generate all the data points desirable for fully characterised dose response curves. To overcome this problem we have developed new statistical analysis methods that can deal with incomplete and noisy data in drug combination screens. This method was applied to a screen for identifying drug combination that block the proliferation of Ewing Sarcoma cells. In these experiments we measured cell viability across a range of dose levels for each drug pair without maintaining the ratio of dose levels constant, in order to obtain a broad screening across drug combinations and dose levels. The trade-off for covering a broader concentration range is the increasing difficulty to determine smooth dose-response curves that allow analysis by the established tools. Our approach

has enabled to perform statistical analysis on the available data to meet the needs of the screen. In this way we could calculate the combination index (CI) for each combination data point under the non-constant ratio design. The CI has then been mainly employed for classifying drug pairs as synergistic or antagonistic using a set of empirical classification rules.

Developing reusable code for conducting Markov chain Monte Carlo (MCMC) inference

MCMC methods are a widely used in computational biology for the inference of parameter distributions that are often the basis for constructing biochemical pathway models described by systems of ordinary differential equations (ODEs). This coding endeavor is a long-term and ongoing contribution to the broader systems biology and scientific computing community. More specifically, we have refactored the Lora package, which is a package for MCMC inference written using the Julia programming language. Such refactoring has been essential in order to accommodate an MPI implementation for the thermodynamic integration scheme of Nial Friel [38]. Partner Warwick will continue this work beyond ASSET to work towards adding the thermodynamic integration implementation to the amended version of Lora, along with an implementation of adaptive Metropolis-within-Gibbs (AMWG) sampling [39]. Variations of AMWG provide the building block of popular species of software (such as BUGS and Stan) for MCMC inference for hierarchical models. Once the development cycle for i) thermodynamic integration and ii) AMWG sampling is completed, Lora will be in a unique position internationally, due to its broad scope covering functionality that is partially met by existing software dedicated to sub-goals of Lora.

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The potential impact of ASSET

Scientific impact

ASSET has generated a number of scientific innovations that will have lasting impact on science and the scientific community. The most important achievements in this respect are briefly listed below:

Mathematical models as informative biomarkers in neuroblastoma

A conceptual innovation developed in ASSET was the use of mathematical models as highly accurate and personalisable biomarkers for patient stratification and therapy choice. We generated an experimentally calibrated and validated computational model of the JNK stress response network [1]. We used the model to extract prognostic information from NB patient–specific simulations of JNK activation. An inability to initiate switch-like JNK activation in the simulations was significantly associated with poor overall survival for patients with NB with or without MYCN amplification. This analysis identified a group of high risk NB patients without MNA, for which currently no biomarkers exist. More generally, this analysis showed that dynamic mathematical models can be very powerful biomarkers as they capture pathogenetic mechanisms and are have a high information content that supersedes single biomarkers.

New biomarkers for high risk neuroblastoma

Using whole genome sequencing we identified Telomerase aberration as marker for high risk neuroblastoma [2]. This finding will substantially improve neuroblastoma classification and risk prediction at the time of diagnosis. We also have defined mechanisms of telomere activation that will be integrated into the new diagnostic classification systems and will be further evaluated in nation-wide clinical trials in Germany.

Systems guided drug design and deployment

We used different techniques to profile drugs and drug combinations and characterize cancer vulnerabilities. The results demonstrate that a holistic, integrated systems pharmacology approach can contribute towards better understanding of cancer biology and identify new alternative therapeutic regimens. The current situation in medical care is that for a vast majority of diseases a "proxy" of a patient is being treated: all individuals with the same diagnosis are considered an average patient and receive a very similar therapy. Precision medicine is a medical model that proposes an individualized approach to treating patients. Advanced omics technologies and computational techniques enable better understanding of pathway and network aberrations in paediatric tumours that in turn can aid selection of drugs and drug combinations that could benefit specific patients. The aim is to provide for highly specific, effective and minimally toxic treatment for each patient, and thanks to the design of our studies and focus on translational aspects our results can enable clinicians to test the new drugs and drug combination in due course.

New software tools for the analysis and interpretation of omics data

The ASSET project contributed to the development of several innovative bioinformatics tools for integrative data analysis in cancer systems biology. These tools comprise the following:

The ***Atlas of Cancer Signalling Network (ACSN) database***, representing the most detailed representation of biochemical processes deregulated in cancer, was partially supported by ASSET. The ***NaviCell Web Service*** allows the network-based data visualization which allows mapping high-throughput data (mRNA, microRNA and protein expressions, gene copy number and mutation profiles, simple gene lists and clinical data) onto biological network maps. It was developed in part with support of ASSET project. NaviCell and ACSN have been used to study how activation of expression of EWS-FLI1 oncogene affects diverse set of biological functions.

A new version of ***BiNoM Cytoscape plugin*** was developed that included the implementation of several methods which we have been exploited for constructing the model of signalling affected by EWS-FLI1, such as the ***Pathway Activity Quantification (PiQant)*** method. In addition, a new method for quantifying pathway activity, ***ROMA***, was developed and applied in order to better quantify the activity of EWS-FLI1 and other transcription factors in Ewing sarcoma. The ***MaBoSS tool*** has been developed to predict probabilities of cell fates in relatively large (containing hundreds of nodes) models of regulatory networks.

NBPrognostics resulted from ASSET partners participating in a large-scale meta-analysis of multi-level molecular data characterizing large cohorts of tumour samples from Ewing sarcoma, neuroblastoma, medulloblastoma and several related paediatric tumours. The aim of the meta-analysis was to detect a set of biological factors which have reproducible effect in several independent datasets. The conducted analysis will help to understand common mechanisms driving tumorigenesis in paediatric cancers. We developed a comprehensive catalogue of prognostic down- und up-regulated genes in neuroblastoma, called NBPrognostics, which represents a valuable pool of prognostic markers and putative targets for therapeutic interventions. The usefulness of NBPrognostics was validated for the gene MCL1, a target of obatoclax, which was indicated as the compound reducing proliferation in neuroblastoma cells in the high-throughput drug screens and in the zebrafish model.

SwitchFinder is a novel statistical method developed for the analysis of time-series data. We also implemented it as publically accessible Web Application (https://aif.bit.uni-bonn.de/switchfinder/welcome.html).

Lora is a package for Markov chain Monte Carlo (MCMC) inference written using the Julia programming language. We have refactored this important package in order to accommodate an MPI implementation for the thermodynamic integration scheme of Nial Friel [3]. Partner Warwick will continue this work beyond ASSET to implement thermodynamic integration and adaptive Metropolis-within-Gibbs (AMWG) sampling [4] to Lora. Variations of AMWG are the basis of popular software, e.g. BUGS and Stan, for MCMC inference for hierarchical models. This work puts Lora in a unique position internationally, due to its broad scope and compatibility with many existing software packages dedicated to sub-goals of Lora.

New therapeutic targets

Better understanding of the disease mechanisms may provide more efficient and targeted treatment options with fewer side effects and therefore, increasing knowledge on oncogene driven vulnerabilities is of key importance. Our approach was based on using smart high-throughput screening (HTS) where the screens are guided by omics profiling and modelling in order to zoom in on

the most promising screening strategies early on. This targeted HTS approach identified several candidate molecules for miRNA-based therapeutics, which either compensate missing tumour suppressive miRNAs or neutralize oncogenic miRNAs in ETs. In addition, drug target candidates, druggable pathways and growth inhibitory compounds were identified in all ET cell models. These results will help bridging the gap between fundamental understanding of ET biology and possible pharmacological interventions. Moreover, HTS results provide a useful platform for hypotheses generation with a goal to strike MYCN, c-Myc and EWS-FLI1 driven vulnerabilities in ETs.

MYCN overexpression marks a clinically very challenging NB patient group for which novel treatment strategies are urgently needed. In the past years the molecular dissection of these tumours has also provided the first insights for targeted therapies as illustrated by mutant ALK small molecule therapies which are now in clinical trials. However, single compound therapies almost invariably lead to treatment resistance and therefore novel additional targets are warranted. Our work has provided important novel data sets for further investigation of neuroblastoma biology and drugging opportunities as already illustrated by several follow up studies that emerged during this ongoing program. Most importantly, we identified a novel vulnerable cancer hallmark which we discovered in MYCN driven tumours and which we named "replicative stress resistance" and identified several compound combinations for both in vitro and in vivo testing using validated model systems and propose a pipeline to accelerate testing of successful drug combinations in the clinic. In addition, based on data integration of omics approaches we have identified targets for NB in the MAPK and Wnt pathways [5-7]. We also have identified new synergistic drug combinations with new modes of action that are selectively effective against NB cells[8].

We also have identified novel molecular targets for the future development of therapies against medulloblastoma, in particular the aggressive subgroup characterized by c-Myc over-expression. In addition, we have described novel combinations of protein kinase inhibitors and chemotherapeutic agent cisplatin that can be further validated in medulloblastoma and neuroblastoma. Taken together, we have gained novel and essential insights into the gene regulatory rewiring of neuroblastoma cells and based on these data sets identified novel targets for therapy.

Circadian regulation of RTK signalling as a new type of therapeutic intervention

Very interestingly, we discovered that drugs may have very different efficacy depending on what time of the day they are administered. For instance, glucocorticoids antagonize growth factor signalling, but this might occur during daytime only. We are testing the possibility that cancer therapy might be more effective if delivered at the resting time, when GR is inactive and glucocorticoid levels are low. If correct, our studies might leverage a change in the way cancer patients are treated using kinase inhibitors and other drugs.

Development of new technologies for analysing pathogenetic mechanisms in ETs

The use of multi-layered mass spectrometry-based proteomics to unravel complex signaling networks can provide unique insights into the functions of tumours, such as neuroblastoma. This opens the possibility to discover new protein candidates involved in regulating neuroblastoma cell survival, growth or differentiation and thus, with the potential to serve as new biomarkers or drug targets in the disease.

Establishment of ASSETmart, a data warehouse and analysis platform for ETs for use by the scientific community

This ASSETmart database built on this project incorporates rich high-throughput data that was generated by the ASSET partners. The data that was submitted by the partners to ASSETmart will be made accessible to the wider scientific community at the end of the project, and represents the lasting legacy of ASSET to the scientific community. The database provides an additional benefit in linking across the data, so queries across samples can be seamlessly made.

New tools for data integration and data comparison

The integration of different types of data within ASSET and the development of tools for comparison of cell line derived data to patient data is what will bridge the gap between the scientific results of a research project and the important translational applications that we all aimed for. New personalized treatment approaches in cancer therapy have the potential to reduce the costs to society in two ways: by better stratifying patients so that only the ones that need aggressive treatment receive it and by suggesting targeted therapies for specific cases and avoid general treatments with high cost and low rates of success.

Socio-economic impact and the wider societal implications of the project main dissemination activities and exploitation of results.

Proof of concept for systems medicine approaches

ASSET has fully delivered on its aims to obtain new insights into embryonal tumours through systems based approaches that can be used for new diagnostic and therapeutic approaches. From a strategic point of view the most important finding with long term impact on society and patients was that systems medicine based approaches work when deployed in a focussed way supported by interdisciplinary expertise. Focus is of utmost importance as the complexity increases dramatically when moving from any model system to human disease, and only can kept at bay if tightly focussing on a specific clinical question. This experience is in stark contrast to the unlimited generalisation that often is portrayed as an advantage of systems approaches. The underlying error is that the insight required to formulate general rules is falsely equated to the generalisation of the implementation of these rules. In other words, understanding the rules of chess is a generalised type of insight, which does not automatically enable the user to win a chess game.

Another important lesson is that systems medicine approaches pose new challenges that are neither solved nor well developed with our current (high state of the art) in systems biology. The main obstacles are (i) the transition of discovery research into applied research; (ii) the need for data integration to make data interpretable for different disease phenotypes; and (ii) the actual clinical implementation, as there currently is a lack of guidelines that would cover a computational model based approach for clinical implementation. Thus, harvesting the full impact of systems medicine based approaches may be delayed longer than we would have anticipated.

Nevertheless, ASSET has made great progress in the undertaking of applying systems biology approaches to medical problems, in this case ETs. The main achievements include

- New insights into mechanisms of ET pathogenesis and progression through the Integration of clinical data with different types of molecular data
- Better molecular methods for patient stratification
- Identification of new drug targets
- Identification of new drug combinations

Nucleating a community of researchers and clinicians interested in systems medicine approaches

ASSET comprised both basic, translational and clinical research groups working closely with statisticians, computational and mathematical modellers. This successful experience has promoted the new way of interdisciplinary thinking that is able to drive systems medicine approaches in the future. These small but promising efforts were amplified by

- various dissemination activities, for instance video interviews with ASSET researchers, which are posted on You Tube
- training activities especially for younger researchers and clinicians which included workshops and staff exchanges

Main dissemination activities and exploitation of results

ASSET had a variety of measures in place to ensure that our project results and progress were effectively disseminated to all stakeholder groups:

Website: (asset-fp7.eu; also www.ucd.ie/sbi/asset). The ASSET project website was set up to provide information relating to the main aspects of the project and to promote its activities. It contains a public internet site to disseminate the goals, activities and results of the project to target groups and also a private, password protected extranet site for the exchange of project information and data and the links to the ASSET training videos for use within the consortium.

Social media: ASSET Twitter (@ASSETFP7) The ASSET Twitter social media site was set up to create further awareness of the ASSET project and to highlight events at which ASSET are involved in. ASSET information was also disseminated through the Systems Biology Ireland Twitter account.

Information Fliers: Fliers were prepared reflecting the project's scope, approach and objectives, together with useful contact information. The purpose of these fliers was to provide non-electronic material for partners to distribute at meetings, workshops and conferences in order to publicise the project and to make all interested parties and the general public aware of the project.

Logos: The website and all promotional material including fliers, slide decks and poster templates included the specially designed ASSET logo. The logos were used at various conferences including for example at the "International Conference on Systems Medicine – SYSMED" held in Dublin from the 9th to 13th September 2012 ASSET had a one day workshop and the ASSET logos were included on all promotional material including the SYSMED abstract book, programme and website. The ASSET flier was also included in the delegate packs. The coordinating group UCD moved into a new state of the art facility on the UCD campus in October 2013 and held a building launch in December 2013. ASSET information was disseminated during the event, in the form of promotional material such as an

ASSET pop-up stand, and the ASSET logo & web-site address was included on the event brochure. These are some of a number of examples where ASSET promotional material was displayed.

Peer reviewed publications: ASSET produced over 80 publications including high visibility papers in top journals such as Nature, Nature Genetics, Science Signalling, Cell Reports and PNAS.

Conference contributions: ASSET researchers have participated in conferences and scientific meetings relevant to the field, both to disseminate information on the progress of the project and to seek the opinions of other experts. The consortium has taken every opportunity to present the findings through oral presentations and poster presentations. Amongst the most important international conferences that member of the ASSET consortium have attended include Advances in Neuroblastoma Research Conference, American Association for Cancer Research (AACR) Annual Meetings including the Special Conference on Advances in Paediatric Cancer Research, EMBO Conference "Cellular signalling and cancer therapy"

Conference organisation: Several ASSET partners were involved in organising and hosting international conferences/meetings, these included the "Paediatric Cancer at the Interface Symposium" (2013), "14th International Conference on Systems Biology", ICSB (2013), 'Functional Genomics and Systems Biology 2013', "Advances in Neuroblastoma Research ANR Congress" (2014 and 2015) and "The International Conference on Systems Medicine SYSMED" (2012)

Press releases and dissemination via public media:

The ASSET Project was included as a success story in the EU FP7 Research Infrastructure "ISBE Infrastructure for Systems Biology Europe" promotional brochure for the public "Success Stories in Systems Biology". It featured a 2 page article entitled "Taking a Systems-Eye View of Cancers in Children" (https://issuu.com/systemsbiologyireland/docs/isbe_case_studies_full_final_july15).

The ASSET Project was featured in a news article by the science editor of the The Irish Times, Dick Ahlstrom, on June 18, 2011. Circulation of the paper is >100,000 subscribers plus online viewers daily. UCD also highlighted the ASSET award and the EU FP7 Research on its front page news article http://www.ucd.ie/news/2010/06JUN10/170610_cancer_research.html. This article also had a link to the Irish Times article above.

European Commission's DG Research and Innovation also interviewed Prof. Walter Kolch to feature the ASSET project on its websites as a success story that appeals to a wider audience, including the media, general public and other researchers and non-experts and stakeholders. This was made available on the European Commission website in early 2014. As a result of this Prof. Walter Kolch also gave a short radio interview to highlight the ASSET project, this was aired in 2014.

A number of partners also issued press releases including VTT who issued a press release to inform the public about their participation on the ASSET project (released December 12, 2011) http://www.vtt.fi/news/2011/20111207lastensyopaprojekti.jsp?lang=en.

Socio-economic impact

The impact on society will mainly materialise from new diagnostic methods and therapies that improve patient care and outcomes for ETs. These cancers are rather rare, but the young age of the patients causes severe additional emotional stress for families. Thus, any measures that can assist the fight against these devastating cancers will help to ease the burden on affected families.

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Further information

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