Systems biology analysis for Ewing sarcoma

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This chapter follows a non-standard format

Abstract

Ewing sarcoma (EwS) is a highly aggressive pediatric bone cancer that is defined by a somatic fusion between the *EWSR1* gene and an ETS family member, most frequently the FLI1 gene, leading to expression of a chimeric transcription factor EWSR1−FLI1. Otherwise, EwS is one of the most genetically stable cancers. The situation when the major cancer driver is well known looks like a unique opportunity for applying the systems biology approach in order to understand the EwS mechanisms as well as to uncover some general mechanistic principles of carcinogenesis. A number of studies have been performed revealing the direct and indirect effects of EWSR1−FLI1 on multiple aspects of cellular life. Nevertheless, the emerging picture of the oncogene action appears to be highly complex and systemic, with multiple reciprocal influences between the immediate consequences of the driver mutation and intracellular and intercellular molecular mechanisms, including regulation of transcription, epigenome and tumoral microenvironment. In this chapter, we present an overview of existing molecular profiling resources available for EwS tumors and cell lines and provide an online comprehensive catalogue of publicly available omics and other datasets. We further highlight the systems biology studies of EwS, involving mathematical modeling of networks and integration of molecular data. We conclude that despite the seeming simplicity, a lot has yet to be understood on the systems−wide mechanisms connecting the driver mutation and the major cellular phenotypes of this pediatric cancer. Overall, this chapter can serve as a guide for a systems biology researcher to start working on EwS.

Keywords: Ewing sarcoma, EWSR1-FLI1, cancer systems biology, omics data, network, mathematical modeling, data integration.

Running title: Systems biology analysis for EwS

1 Introduction

Ewing sarcoma (EwS) is a rare aggressive bone and soft tissue cancer, with the peak of 15 years in age distribution. Its world−wide incidence rate is about 2−3 cases per million children, being lower in African population compared to European population. The 5−years survival is currently 70−80% for the cases with localized tumors and drops to 30% for those with metastases ***(1)***.

From the point of view of cancer genomics, EwS represents one of the most genetically stable cancers. Together with rhabdoid tumors, EwS was ranked as the cancer having the lowest somatic mutation frequency among 27 analyzed cancer types ***(2)***. Therefore, EwS is also relatively homogeneous at the genomic level. At the same time, the major genetic cancer driver event in EwS is well established: it is a balanced chromosomal translocation leading to the fusion of a member of the FET gene family with an ETS transcription factor. In 85% of the cases, this leads to appearance of a chimeric transcription factor EWSR1−FLI1, which activity leads to the widespread changes of the cellular molecular profiles and phenotypes. In the following we will refer to the systemic properties of EWSR1−FLI1 gene and protein, unless explicitly specified, since other translocation types generally have similar characteristics and are rare.

EWSR1 gene encodes a multi−functional protein that is involved in various cellular processes, including gene expression, cell signaling, RNA processing and transport. The protein includes an N−terminal prion−like low−complexity domain PrLD and a C−terminal RNA−binding domain (the latter is commonly missing in the fusion protein). Characterizing the precise normal biological function of EWSR1 protein is difficult due to its potency to interact with many other proteins, which is usually articulated as that EWSR1 connects together several important biological functions such as transcription and splicing ***(3)***. From the network biology point of view, EWSR1 frequently plays the role of a major hub in the protein−protein interaction (PPI) networks of proteins associated to diseases ***(4)***. Genomic translocations involving EWSR1 are not exclusively attributed to EwS; they serve driver mutations in a broad variety of mesenchymal lesions which includes Ewing's sarcoma/peripheral neuroectodermal tumor, desmoplastic small round cell tumor, clear cell sarcoma, angiomatoid fibrous histiocytoma, extraskeletal myxoid chondrosarcoma, and a subset of myxoid liposarcoma ***(5)***.

FLI1 is a member of the large E26 transformation−specific (ETS) transcription factor family characterized by a specific binding DNA motif with consensus sequence CAGGAAG. It is normally implicated in the development of different animal tissues. For example, it transcriptionally regulates genes that drive normal hematopoiesis and vasculogenesis ***(6)***.

The fusion between two normal genes *EWSR1* and *FLI1* leads to the new properties of the resulting protein, EWSR1−FLI1, the most remarkable of which is its ability to bind microsatellite sequences containing exact GGAA repeats, which are rather abundant in the human genome and are not functional in healthy cells ***(7,8)***. Upon EWSR1−FLI1 binding, the microsatellites become potent enhancers of genes located sometimes at

hundreds of thousands of base pairs away. This new property has a profound effect, with activation of EWSR1−FLI1 leading to the drastic rewiring of gene expression and epigenetic re−programming. These changes appear to be detrimental to cells and lead to apoptosis for the majority of human cell types, but can be tolerated in few, including pediatric mesenchymal stem cells (MSCs) or neural crest stem cells (NCSC) ***(1)***.

Importantly, the process of EwS tumorigenesis can be recapitulated to some extent in several inducible cell line models, in which the expression of EWSR1−FLI1 can be modulated through doxycycline−controlled short hairpin RNA (shRNA) or application of siRNA−based oncogene knock−down. Reducing the expression of EWSR1−FLI1 in EwS cells to some minimal level (e.g., 20% from its initial concentration) leads to the drastic reduction of proliferation and changes in the cell morphology towards MSC−like phenotype ***(9)***. This process can be reversed by re−activating the chimeric oncogene ***(10)***. Further, reduction of the EWSR1−FLI1 expression leads to apoptosis, showing addiction of the EwS cells to the oncogene activity. Inducible systems have been extensively used to study the role of other genetic and epigenetic actors and the downstream mechanisms of the EWSR1−FLI1 action. The development of genetically engineered mouse models of EwS has not been very successful so far, probably due to the distribution of GGAA−microsatellites in the mouse genome being different from human ***(11)***.

Understanding the mechanisms of adult cancers is usually heavily complicated by genomic complexity and genetic heterogeneity of the tumors, which might be a result of long (sometimes, decades−long) history of their evolution. In adults, the tumorigenic process is usually a direct and indirect consequence of a combinatorial action of several driver genes, with importance of usually vaguely determined temporal sequence of events. EwS looks a much simpler case and hence it seems, at first glance, as a promising target for application of the systems biology approach in order to unravel this particular mechanism of carcinogenesis.

A systems biology approach is here understood as studying a biological phenomenon by combining a collection of system−wide molecular information: in particular, by applying multiple perturbations or profiling series of tumor samples, and recapitulating the available data in the form of mathematical models that reflect some aspects of the cellular life.

Indeed, EwS is a cancer type which is relatively well characterized at the molecular level (see description of available molecular data in the corresponding sections of this chapter and some examples in Table 1).

Understanding EwS and other pediatric cancers through the analysis of “big" omics data, applying mathematical modeling and, more recently, machine learning approaches stimulated several interdisciplinary consortia to launch large−scale European projects with this motivation in mind. The ambition of these projects is to unravel the mystery of pediatric (sometimes called embryonal) tumors, including EwS. The driving idea is that many embryonal tumors exploit the same type of fragilities such as the regulation of cell cycle or

apoptosis and the blockage of normal developmental and differentiation programs ***(12)***, which enable the appearance of these cancers early in human life, without accumulation of large number of mutations.

Examples of such large projects are the FP6 project “European Embryonal Tumour Pipeline” (EEPT), aimed at generating omics data for EwS, and the FP7 project “ASSET: Analysing and Striking the Sen-sitivities of Embryonal Tumours" which collected efforts of 14 partner institutions (https://cordis.europa.eu/project/id/259348/). The “European Network for Cancer research in Children and Adolescents" (ENCCA) facilitated and structured networking activities for prioritization of, access to and clinical research on innovative, biologically targeted drugs for the treatment of childhood cancers. Currently, in the Horizon−2020 program, iPaediatricCancer (iPC) consortium (21 partner institutions, including major cancer research centers in Europe and USA, IBM Research and Barcelona Supercomputer Center). works on integrating large−scale omics data on several pediatric cancers and making them available, using computational clouds, for machine learning and modeling−based analysis (https://ipc-project.eu/). The above-mentioned European consortia represent truly interdisciplinary teams, putting in close collaboration experts in cancer biology, computer science and computational biology. The ambitious long−term goal of such efforts is to provide individualized diagnostic and prognostic tools for ongoing and future clinical trials focused on pediatric tumors such as EURO EWING, MOSCATO 02, MAPPYACTS.

The purpose of this chapter is to 1) outline the available omics datasets suitable for application of systems biology and machine learning−based approaches and 2) highlight several applications of the systems biology approach to study EwS. Having these objectives, we catalogued the EwS−related datasets from various sources, with the intention to update this catalogue online in the future. We collected and harmonized some of the dataset collections for immediate application of various computational analyses. Therefore, this chapter serves as a guide and a resource for the future systems biology studies of EwS.

2 Omics and large-scale perturbation studies of EwS

2.1 Sources of public omics datasets

Considering its rareness, EwS is a pediatric cancer type which is relatively well studied at the molecular level. For example, International Cancer Genome Consortium (ICGC) included moderate size collection of EwS donor profiles as a multi−omics dataset available through the corresponding data portal. Otherwise, the molecular profiles of Ewing tumors or model systems are scattered across data repositories and publications. For example, querying ‘Ewing’ in ArrayExpress returns 135 datasets. EwS molecular profiling is part of data resources specialized in pediatric cancers such as Kids First initiative (https://kidsfirstdrc.org/), which also provides an associated computational cloud Cavatica (https://cavatica.squarespace.com/). Some

EwS−related datasets can be obtained through a specialized instance of cBioPortal (focused on adult cancers), named PedcBioPortal which has been developed for the Childhood Cancer Genomics program (pediatric cancer−specialized data resource, supporting the curation and pan−cancer integration of public, pediatric cancer genomics datasets, http://pedcbioportal.org).

In this section we made a systematic effort to identify EwS−related molecular data across multiple sources, having in mind their potential usefulness in the future systems biology or machine learning−based studies. We focused on molecular data generated either as a result of profiling of tumor cohorts or the results of chemical or genetic or epi−genetic perturbations applied to model systems (mainly cell lines). This effort resulted in a catalogue which is available as an online table at https://github.com/sysbio-curie/EwingSarcoma_Omics_Atlas. Some exemplary datasets from this catalogue are listed in Table 1.

Previously, an effort to summarize available large−scale EwS−related omics data has been undertaken in a review that we strongly recommend ***(13)***. However, in ***(13)*** the effort was to summarize the biological insights obtained from the sequencing studies of EwS, while here we focus on the data itself and its possible re−use in a systems biology−oriented approach. We consider all data generation efforts for EwS, not only limited to sequencing, and mention more recent (e.g., single cell) studies as well as the types of data not previously summarized (such as proteomics and metabolomics).

2.2 Genomic studies of EwS

The genome of EwS patients has been studied for decades. After the identification of the genomic fusion as initiating event, the focus of the studies has shifted towards the identification of secondary events. The initial efforts were drawn towards the identification of chromosome copy number variations (CNV) through Microarray-based Comparative Genomic Hybridization (aCGH) approaches ***(14-18)***. Later, Next-Generation Sequencing (NGS) allowed characterizing the genome of EwS in a more precise way.

2.2.1 Microarray-based Comparative Genomic Hybridization studies

Ferreira et al. ***(19)*** studied 25 patients, 23 primary tumors obtained before any treatment and relapse for two of them. The authors observed a median number of three aberrations per case, with 21 tumors (84%) showing at least one DNA copy number aberrations (CNA). The most frequent gains were entire chromosomes 8 (56%), 12 (20%), 18 (12%), 20 (12%) and the short arm of chromosome 5 (5p) (20%). The most frequent losses involved entire chromosomes 10 (16%) and 19 (16%), and partial regions of chromosome arms 16q (16%) and 7q (25%). They computed the fraction of the genome affected (FGA) by this instability. The median of FGA for all samples was 6%.

An unsupervised clustering analysis was performed, using the smallest overlapping regions of imbalance (SORI) as variable ***(19)***. Two subgroups were identified: one genomically unstable characterized by a high

number of aberrations with a median number of 8 SORI per case (range: 4-18) and one genomically stable, with a median number of one SORI per case (range: 0-3). Trisomy 8, the most common secondary aberration in EwS, was equally found between both groups.

Based on the 20 patients with tumor samples at diagnosis for whom clinical and follow−up data were available, the genomically stable group showed a higher tendency to achieve complete remission during or after treatment than the genomically unstable group (100 vs 62%). In addition, genomic unstable group was more refractory to chemotherapy and thus associated to a poor prognosis.

Savola et al. ***(20)*** studied 31 samples by aCGH: 23 were primary tumors, two recurrences, and six metastatic tumors. They confirmed most of the findings previously obtained by conventional CGH and array CGH studies ***(19)*** about the most recurrent CNVs. They also observed a significantly better prognosis for primary tumors with three or less CNVs than for tumors with higher number of CNVs both in terms of event−free and overall survival.

Finally, Mackintosh et al. ***(21)*** screened 67 tumors from untreated patients and 16 cell lines for CNAs by aCGH. They confirmed most of the previous findings: most frequent gains comprised the entire chromosome 8, and the chromosome arms 1q and 12p, while most frequent losses were located in 3p, 9p, 16q and 17p. In their cohort, twice bigger than previously discussed cohorts, 1qG, detected in 31% of tumors, was the CNA with the highest clinical impact, associated with relapse and poor overall and disease−free survival. It was also confirmed that the number of CNAs drives the clinical outcome of patients.

Altogether, several seminal studies of EwS genome involving relatively small cohort sizes (between 30 and 70 tumors) converged to similar conclusions about small but clinically relevant genomic instability in EwS.

2.2.2 Genome−wide association studies (GWAS) of EwS

Two large−scale GWAS studies of EwS have been published so far in order to find genetic determinants for predisposition to this cancer type. One of the major questions addressed by these studies is explaining the geographical differences in the occurrence frequencies of EwS, considering the fact that this cancer is not considered to be highly heritable. In one of the studies, genotypes of 401 French individuals with EwS, 684 unaffected French individuals and 3,668 unaffected individuals of European descent and living in the United States have been analyzed ***(22)***. In a more recent analysis, 733 EwS cases and 1,346 unaffected individuals of European ancestry were genotyped ***(23)***. Both studies showed consistent results pointing to six susceptibility regions with the effect sizes larger than observed in the majority of cancers. Interestingly, the exact mechanism underlying one of the loci located close to *EGR2* gene was revealed ***(24)***. EwS cell proliferation was shown to depend on the activity of EGR2. It appeared that a single SNP can increase the number of consecutive GGAA motifs in a genomic region located near *EGR2* with epigenetic characteristics of an active regulatory element, and thus increase the EWSR1-FLI1−dependent enhancer activity. Similar

mechanism explained clinically relevant upregulated activity of *MYBL2*, a potent regulator of cell proliferation and cell survival in some EwS tumors and not the others, thus illustrating a possibility of cooperation of cancer drivers with regulatory germline variants, even if some of them were not yet identified in case-control GWAS ***(25)***.

2.2.3 Sequencing EwS genome, WES & WGS approaches

With the outbreak of NGS, more precise characterization of both coding and non-coding parts of the genome became possible. Several simultaneously finalized large-scale studies have investigated the EwS tumor genomes. Tirode et al. ***(26)*** performed whole-genome sequencing (WGS) of 112 tumors, and showed that EwS tumors have rather stable genomes, with a median number of somatic SNVs of 319, of somatic coding mutations of 10 and of somatic SVs of 7 per tumor. Somatic CNAs were frequent, as previously demonstrated. The most frequently mutated genes where *STAG2* (in 19 cases, 17% of the patients), TP53 (8 cases) and EZH2 (3 cases). Interestingly, a significantly greater number of structural variants (SVs) was observed in *STAG2* mutated cases but *STAG2* mutation was not associated with the number of SNVs and indels. The authors also identified in the one hand, mutual exclusivity between *STAG2* mutation and *CDKN2A* deletion and, on the other hand, a significant co−association between *STAG2* and *TP53* mutations. Finally, linking these recurrently mutated genes to clinical features showed that patients with *STAG2* or *TP53* mutations had a significantly lower probability of survival, patients with neither *STAG2* nor *TP53* mutations had the highest probability of survival, and patients with both genes mutated had the worst outcome. A last track investigated by the authors was that subclonal *STAG2* mutations may expand at relapse.

Two other studies reported similar findings ***(27,28)***. A limitation of these studies in the use of their data is the variability of the sequencing strategy used on their cohorts and the limited number of normal−tumor available pairs. In Crompton et al., the authors performed WES of tumor−normal pairs from 26 patients, tumors from 66 patients, and 11 cell lines. They also performed WGS on 7 paired samples, genotyping array (SNP array) on 29 samples, and transcriptome sequencing (RNA-Seq) on 30 EwS samples. In Brohl et al., 101 samples were investigated: 65 tumors (including 13 normal−tumor pairs) and 36 cell lines. Only 6 patients were investigated using paired WGS and 80 samples (including cell lines) were subjected to targeted sequencing, with a panel based on the mutations detected by WGS. In addition, RNA-Seq was performed on 30 samples.

In the end, the three independent studies reported similar conclusions, using independent cohorts and different sequencing strategies, which reinforced their findings.

Another major dataset of EwS WGS data should be released during this year by the Kids First project, with the sequencing of around 1,000 families with an EwS case (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs001228.v1.p1).

2.3 Studies of EwS transcriptome

2.3.1 Expression of coding genes in EwS

EwS is relatively well characterized at the transcriptomic level. By browsing public repositories, we catalogued more than 1,300 distinct transcriptomic profiles obtained at bulk level, among which approximately 600 were profiles of tumoral samples and 600 were profiles of cell lines under different conditions and perturbations. The absolute majority of transcriptomic profiles have been generated using various microarray platforms, with Affymetrix HG−U133Plus2 chip being the most popular (40% of profiles). Among experimental systems used for gene expression profiling, cell lines A−673 (Cellosaurus id: CVCL_0080) and SK−N−MC (Cellosaurus id: CVCL_0530) appear to be the most popular. Ironically, both these cell lines are marked as problematic because of confusion with their cancer of origin: A−673 was initially thought to be a rhabdomyosarcoma, SK−N−MC a neuroblastoma cell line. Both of them are included in the Cancer Cell Line Encyclopedia (CCLE) and are well characterized at genomic and epigenomic levels (including gene expression, DNA methylation and chromatin mark profiles). For example, A−673 cell line was transformed to inducible cell line system, where the expression of EWSR1−FLI1 transcript can be modulated, and time series of transcriptomic changes measured upon inhibition or re−activation have been produced ***(10)***. To this collection, one can add a rich dataset of single cell RNA-Seq profiles (9,000 profiles) recently published and comprising time−resolved measurements of the inducible A−673−derived cell line upon activation of the oncogene, cells in several patient−derived xenografts (PDXs) and in xenograft−inducible cellular systems ***(29)***. Another recently published large single cell dataset contains 9783 scRNA-Seq profiles for three EwS cell lines CHLA9, CHLA10 and TC71 ***(30)***.

Even if few hundreds of EwS tumors have been profiled at gene expression level, their simultaneous comparative study is complicated by the fact that different transcriptomic platforms have been used in these studies: therefore, one has to deal with laboratory−specific and platform−specific effects. The largest cohort of 117 EwS tumors has been profiled in the frame of the French Cartes d'Identité des Tumeurs (CIT) from the Ligue Nationale Contre le Cancer (http://cit.ligue-cancer.net) and made publicly available ***(22)***. Two sets of samples for 44 EwS tumors have been profiled at the level of gene expression in order to characterize the inflammatory response of the tumoral tissues compared to cell lines ***(31)***. More recently, 85 EwS tumors from two sample sources were profiled in order to construct and validate transcriptomic signatures of survival for EwS in ***(32)***. International Cancer Genome Consortium (ICGC) openly provides 57 processed bulk RNA−Seq profiles of EwS (as a part of BOCA−FR dataset), released as a part of a large−scale EwS genomic study ***(26)***. Few smaller datasets with the profiles of EwS tumors have been released in the public domain ***(9,33-35)***.

We collected most of the identified transcriptomic datasets in a form suitable for further computational analysis in a unified collection of data matrices available at https://github.com/sysbio-curie/EwingSarcoma_Omics_Atlas.

2.3.2 Expression of non-coding parts of the genome: microRNA & lncRNA

Deregulations in gene expression have also been studied in non-coding elements of EwS cells, like miRNA and lncRNA. Some of these datasets are listed in Table 1. Sand et al. in ***(13)*** reviewed, among others, expression studies on non-coding elements performed. We briefly recapitulate here the main studies and discuss more recent works.

One focus is to identify miRNAs deregulated in EwS cells compared with control cells. Mostly, limited number of cell lines were used to highlight the function of one specific miRNA, as in ***(36-38)*** and a series of papers published by the group of Tsumura ***(39-43)***. For example, in the mentioned studies, they took advantage of one microarray experiment in 5 human EwS cell lines and human mesenchymal stem cells to show the role of let−7a, miR−16 and miR−29b in the cell cycle ***(39)***, miR−138 on EwS cell proliferation, invasion, and migration ***(43)***, miR−301a on cell proliferation ***(40)***, miR−20b in EwS cell proliferation ***(41)*** and miR−181c in apoptosis ***(42)***. A recent study by Parafioriti et al. ***(44)*** used larger sample size of tumor samples, 20 patients affected by primary untreated tumors and normal MSCs from 4 normal donors. The miRNAs microarray analysis of 954 miRNAs showed 58 significantly differentially expressed in EwS samples compared to MSCs, with 36 being up- and 22 being down-regulated. They suggest to consider BCL−2 as a novel biomarker for EwS. This dataset was then used in the paper by Liu Y, ***(45)***, together with a gene expression dataset, to demonstrate the potential use of miR−21/CD166 as diagnostic markers and therapeutic targets for this disease.

Another focus is to identify miRNAs deregulated between different stages/characteristics of the disease. The aim is to either identify prognostic miRNAs or to compare the effects of the founding translocation type on the mirnome landscape. In such studies, sample sizes are usually bigger and tumor samples are preferred to cell lines. In the work published by Nakatani et al. ***(46)***, the authors studied 34 primary tumors to identify prognostic miRNAs, related to treatment response and outcome, comparing 21 tumors from patients who had an early tumor relapse with 13 tumors from patients who never recurred. They concluded that miR−34a expression was a strong predictor of outcome in EwS. In the work by Karnuth et al. ***(47)***, the cohort was composed of 40 EwS biopsies with different translocation types, six EwS cell lines and mesenchymal stem cells from 6 healthy donors. Of the 35 differentially expressed microRNAs between tumors and controls (over 377 investigated), 19 were higher and 16 lower expressed in EwS. miR−31 was the most differentially expressed microRNA, with lowest expression in mesenchymal stem cells. It was described as a potential tumor suppressor in EwS with influence on proliferation and invasiveness. In addition, no significantly

differentially expressed microRNAs were detected between EwS samples with EWSR1−FLI or EWSR1−ERG translocations.

Finally, we mention two studies that differ from the main topics previously described. Teicher et al. ***(48)*** screened 63 human adult and pediatric sarcoma cell lines including 23 ES with 100 FDA approved and 345 investigational agents. Both microRNA expression and gene expression were measured. The drug and compound response, gene expression and microRNA expression data are publicly available at http://sarcoma.cancer.gov. De Feo et al. ***(49)*** applied an innovative approach, looking for the first time at the exosome of CD99, one of the hallmark surface molecules of EwS ***(50)***. The cells are prone to differentiate toward neural lineage if deprived of CD99 ***(50)***. Using relatively small sample size, three CD99-positive exosomes and four CD99-negative exosomes, the authors could decipher the repertoire in these 2 types of exosomes and identify miR−199a−3p as contributing to EwS aggressiveness.

As miRNA, lncRNAs are attractive as tissue−specific biomarkers. Marques Howarth et al. ***(51)*** performed RNA-Seq to look for novel transcripts regulated by EWSR1−FLI1 (six tumor samples). They compared pediatric human mesenchymal progenitor cells (pMPCs) expressing EWSR1−FLI1 with control pMPCs and identified 157 genes with higher expression in cells expressing EWSR1−FLI1, while only 13 genes had reduced expression. They focused primarily on genes not previously established as EWSR1−FLI1 targets, identifying 16 candidate genes, of which 15 were protein coding genes and a single lncRNA of unknown function, *EWSAT1*. They showed that EWSAT1 is a lncRNA specifically upregulated as a consequence of the oncogenic fusion.

2.4 Characterizing the EwS's epigenome

As EwS, apart from EWSR1−FLI1 fusion, rarely shows recurrent mutations, improvements in the knowledge of its epigenomic landscape can provide novel breakthrough. In this sense, DNA methylation profiling provides a valuable approach to study the states of EwS cells. Some efforts have been drawn in this direction to boost the understanding of the interplay between DNA methylation and the pathogenesis of EwS and, in general, the carcinogenesis of human malignancies.

Indeed, methylation profiling has been carried out on a relatively large set of cohorts such as on 52 EwS tumors, three cell lines and eight MSC using bead chip methylation ***(52)***; on 69 EwS tumors by Illumina GoldenGate Methylation Cancer Panel I microarray ***(53)***; on 15 EwS tumors, seven cell lines, ten healthy tissues and four human MSC by Infinium Human Methylation 450K ***(54)*** and on 140 EwS tumors, 16 EwS cell lines, 32 MSCs by reduced representation bisulfite sequencing (RRBS) ***(55)***. Sheffield et al. ***(55)*** defined a DNA methylation signature of EwS samples that resulted in an accuracy close to 100% for distinguishing EwS samples from various other tumor types. Differences between tumors were further assessed by comparing aggregate DNA methylation profiles and the Methylation−based Inference of Regulatory Activity score. A continuous disease spectrum underlying EwS and between mesenchymal and stem cell signatures

was identified. This is another way to say that Ewing tumors are not characterized by well-defined molecular subtypes that can be defined at the level of DNA methylation.

Since EWSR1-FLI1 is a transcription factor, an important part of the EwS omics atlas constitutes a collection of ChIP-Seq and ATAC-Seq profiles. Historically, characterizing the binding affinity of EWSR1-FLI1 to GGAA microsatellites was a result of introducing genome-wide ChiP-on-chip technology which was later replaced by ChIP-Seq ***(7,8)***. EwS model systems such as A-673 cell line, its inducible modifications and related constructs based on MSC cells are relatively well-characterized in terms of genome-wide ChIP-Seq profiles for EWSR1-FLI1 itself (sometimes using antibodies against FLI1) and some other important co-factors and transcription factors (such as *MYBL2* or *E2F3*) ***(25, 56-58)***. ChIP-Seq profiles are generated in various conditions (e.g., with activated or knocked-down oncogene), and include recently published time-resolved profiles ***(29)***.

ChIP-Seq profiling was used to characterize the state of chromatin modifications and their dependence on the activity of EWSR1-FLI1, in inducible model systems ***(57,58)***. In ***(58)***, an impressive effort was undertaken to chart the “epigenome map" (collection of RNA-Seq, ATAC-Seq, DNA methylation, ChiP-Seq profiles including histone mark modifications) of the A-673-derived inducible model system. The complete dataset was made public, easily available online (http://www.medical-epigenomics.org/papers/tomazou2015/) and was reused in a number of EwS studies.

These datasets present a unique and unprecedented opportunity to use computational and machine learning methods for the quantification of EwS heterogeneity between and within tumors at epigenetic level.

2.5 Proteomic studies

The EWSR1−FLI1 fusion is a key driver in EwS oncogenesis. As such, downstream effectors and target proteins of EWSR1−FLI1 are likely implicated in disease pathogenesis and are thus of interest to the discovery of new biomarkers and therapeutic targets, but also to the identification of protein interactions and signaling pathway partners playing a key role in the onset and progression of cancer hallmarks.

Proteomic profiling has been performed mostly on EwS cell lines: in 293, and A-673 cell lines ***(59)*** and in TC32J and CHLA10 under serum−starved conditions ***(60)***.

Few publications investigate the consequences of EWSR1−FLI1 modulation on EwS proteomic profiles. Madoz-Gurpide et al. profiled and compared the proteomic expression of TC−71 EwS cell lines relative to an EWSR1−FLI1 knockdown TC−71 cell line variant, using 2D−DIGE ***(61)***. Franzetti et al. measured the proteomic expression of A-673 and SK−N−MC cell lines prior and after EWSR1−FLI1 knockdown ***(62)***.

Proteomic profiling has also been used to compare EwS profiles of patients from different prognosis groups, to identify synergistic drug combinations that improve clinical efficacy and to elucidate the mechanism of acquired drug resistance. For instance, Kikuta et al. examined the proteomic profile of 8 biopsy samples from

EwS patients (with good and bad retrospective prognosis) using two−dimensional difference gel electrophoresis ***(63)***.

Lamhamedi−Cherradiff et al. generated 37 EwS cell lines resistant to IGF−1R− or mTOR−targeted therapy ***(64)***. In this study, reverse-phase protein lysate arrays (RPPAs) revealed proteomic changes linked to IGF-1R/mTOR resistance, and selected proteins were validated in cell-based assays, xenografts, and within human clinical samples.

Puerto−Camacho et al. ***(65)*** investigated the therapeutic value of ENG targeting, a core receptors of the TGF− family, through characterization of ENG, sENG and MMP14 expression by flow cytometry analysis in a panel of ten EwS cell lines and by IHC analysis in a set of three EwS xenografts, nine PDX models and 43 FFPE patient samples, assaying them for the efficacy of targeted antibody therapy.

In a systems biology perspective, a new method for target discovery which can be used as surrogate tool for the analysis of the proteome has been proposed in ***(66)***. The approach consisted in the analysis of whole cellular transcriptomes by RNA-Seq to identify candidate cell proteins. As a proof of concept, the method was applied on three EwS cell lines (A-673, TC-32 and TTC-466) and two MSC lines, and revealed a set of candidate target proteins differentially expressed in tumor cells.

2.6 Metabolomic studies

EwS is a unique model system to study metabolic alterations caused by the oncogene and to increase the understanding of metabolic reprogramming in general, in particular the metabolic switch from oxidative to glycolytic metabolism (Warburg effect).

To our knowledge, only two studies have characterized EwS cell lines metabolome. Jonker characterized the metabolome of A-673, SK−N−MC and A-673−C1 doxycline inducible cell lines under different conditions ***(67)***, in EWSR1-FLI1^{HIGH} and EWSR1-FLI1^{LOW} conditions, and time−resolved metabolomics time series after inhibition of the oncogene. Metabolic analysis identified twenty−four commonly changed metabolites in different pathways, implicated in such processes as energy metabolism, the tryptophan pathway, N−glycosylation, fatty acid synthesis and glutathione metabolism. Consistently with the Warburg effect, a partial reversion from glycolysis to ATP generation through oxidative phosphorylation was observed upon EWSR1−FLI1 inhibition.

Tanner et al. characterized the metabolome of A-673 cell lines after EWSR1−FLI1 silencing by shRNA and on a control knockdown ***(68)***. In agreement with the previous study, this metabolomic analysis revealed distinct separation of metabolic profiles in EwS−knockdown versus control−knockdown cells. Metabolites in several metabolic pathways were altered and phosphoglycerate dehydrogenase was found to be highly expressed in EwS and correlated with worse patient survival.

Characterization of the metabolome remains costly. Other strategies have been proposed in the literature to study EwS cell metabolism through exploration of targeting metabolic dependencies. Using such approach,

Dasgupta et al. studied the metabolic dependencies in A-673, TC−71, MHC EwS cell lines and 2 nonmalignant cells ***(69)***; Sen et al. in SK−N−MC, TC−32, HCT116 and HEK-293T ***(70)***, and Issaq et al. in TC71, EW8, and 5838 cell lines ***(71)***.

2.7 Systematic perturbation studies, drug and gene invalidation screenings

EwS cell lines were extensively used in screenings in which either gene functions were systematically invalidated or when the cells were exposed to a set of drugs. In particular, a set of EwS cells participated in the large-scale drug sensitivity profiling projects such as screening the Cancer Cell Line Encyclopedia (CCLE) or Genomics of Drug Sensitivity in Cancer (GDSC) ***(72,73)***. Both screenings resulted in predicting potential drugs for EwS among the main reported results. Olaparib (PARP1 inhibitor) was suggested in the GDSC paper, and irinotecan (topoisomerase 1 inhibitor) appeared to be efficient for killing EwS cell lines with elevated SLFN11 expression, in the CCLE study. Both drugs were tested in clinical trials of EwS although with limited success. The sensitivity of EwS cell lines to olaparib was particularly surprising because it was thought to be efficient in cancers with increased BRCA1-dependent genomic instability while EwS cell lines usually have robust expression and no mutations in BRCA1. One of the possible explanations was that PARP1 appears to be a direct target of EWSR1-FLI1 and at the same time acts as its transcriptional co-factor. This creates a positive feedback loop, on which EwS cells depend, and its disruption leads to inactivation of the driver oncogene ***(74)***.

Since 2012, several more focused efforts have been made to screen vulnerabilities of EwS cells. To give some examples, siRNA-based screening in ***(75)*** targeted around 7,000 genes in the low and high EWSR1-FLI1 activity conditions, highlighting the particular and clinically relevant sensitivity to *LRWD1* gene. The largest, to our knowledge, drug screening tested more than 300,000 compounds and highlighted the proteasome addiction of EwS cells ***(76)***. The druggable interactome of EWS-FLI1 was nicely charted in ***(77)*** based on a screening by 3,325 compounds in two EWSR1-FLI1 activity conditions in the form of a hallmark-like image. ’Apoptosis’, ’Translation’, ’Histone deacetylation’, ’Transcription regulation’, ’Topoisomerase activity’ and ’Microtubule organization’ appeared to be the ’hallmarks’ of EwS druggability.

Combinatorial screening identified some synergistic drug effects in EwS context, in particular, the synergy between PKC412 and IGF1R inhibitors ***(78)***. This result is of particular interest in the light of that most of IGF1R inhibitors used alone lead to drug resistance in treating EwS. Finally, the novel genome-wide CRISPR-Cas9-based screening identified druggable dependencies in EwS cells having wild-type TP53, which is the representative genetic background for the majority of EwS tumors ***(79)***.

Quite interestingly, some of the EwS screenings were preceded by *in silico* predictions. Thus, the Connectivity Map database was used in order to identify those drugs whose transcriptomic signature would have a potential to “reverse" the signature of EWSR1-FLI1 ***(80)***. Two drugs, auranofin, a thioredoxin

reductase inhibitor, and ganetespib, an HSP90 inhibitor, were predicted to have anti-cancer activities *in silico* and were confirmed active across a panel of genetically diverse EWS cells. Moreover, their combined effect appeared to be synergistic.

Besides simple viability screens, EwS cells were subject to siRNA-based High-Content Screening. 672 EwS-relevant genes were invalidated followed by microscopy imaging which allowed quantifying not only the number of cells, but also distinguishing mitotic and apoptotic cells, as well as distribution of cells in different cell cycle phases, in a fully automated fashion ***(81)***.

[Table 1 about here.]

3 Computational systems biology studies of EwS

Characterizing EwS at multiple levels of molecular description allows combining different types of data in order to either validate the conclusions made in one particular dataset or apply joint integrative data analysis, finding biological signals emergent across several levels of molecular description. This is the purpose of multi−omics and integrative data analysis in cancer systems biology which becomes a major tool in deciphering the complexity of cancer disease ***(82)***.

3.1 Multi−omics EwS datasets

Some large−scale efforts such as The Cancer Genome Atlas (TCGA) systematically collect multi−level molecular description of adult cancers, and were already subject to multi−omics data analysis ***(82)***. Nevertheless, multi−omics datasets of pediatric cancers are still rare and contain only few samples with matched molecular profiles, e.g. the one generated for medulloblastoma ***(83)***. In the case of EwS, several datasets exist where multiple−level of omics profiling were combined for a sufficient number of samples (e.g. more than 20). First of all, such a dataset is publicly available as a part of International Cancer Genome Consortium database (ICGC), under the reference BOCA−FR. This dataset contains molecular characterization of 98 tumor samples, of which 43 samples combine three levels of molecular description: genome (CNVs, somatic mutations, including structural somatic mutations), transcriptome (profiled by RNA−Seq) and DNA methylation (profiled by reduced representation bisulfite sequencing).

Other examples of multi−omics tumor description in EwS include joint profiling of mRNA and miRNA expression such as in the study ***(84)*** (39 EwS tumors) or systematic characterization of cell lines available

in the standard collections such as NCI Sarcoma Cell Line panel (23 EwS cell lines) or Cancer Cell Line Encyclopedia (12 EwS cell lines) ***(72)***.

Processed versions of some of the datasets for the multi−omics analyses in EwS are available as a part of the data repository associated with this chapter, https://github.com/sysbio-curie/EwingSarcoma_Omics_Atlas, and will be updated in the future.

3.2 Integrative biology studies combining several data types and sources

Several studies have been performed combining several sources of data in order to address various biological questions, such as clarifying the origin of EwS cells. For example, in the study ***(9)*** integrative analysis of transcriptomic data from EwS inducible cell lines, EwS tumors, transcriptomes of MSC and other cell types, provided arguments in favor of mesenchymal stem cells as potential precursors of EwS cells. Indeed, knocking−down the EWSR1−FLI1 expression in EwS cell lines resulted in convergence of the tumor gene expression profiles to that of mesenchymal stem cells. At the same time, another integration of transcriptomic data of various origins showed that the expression profile of EwS is more similar to that of neural crest stem cells than other cell types such as mesenchymal stem cells ***(85)***. Ten years later, the origin of EwS cells is still disputed between mesenchymal stem cells and neural crest stem cells ***(1)***. In a recent study, more than 40000 publicly available RNA-Seq profiles (including 260 EwS) have been re-analyzed and defined a set of 'EwS-like' transcriptomes corresponding to the neural crest cells, induced pluripotent stem cells, human embryonic stem cells, MSCs. The manifold learning-based analysis of EwS transcriptomes in the context of EWS-like profiles showed that the EwS transcriptome can be placed at an intermediate position on the developmental trajectory connecting pluripotent, neuroectodermal, and mesodermal cell states ***(30)***. We believe that multi−level data integration (rather than comparing only gene expression profiles) might provide the right metric to resolve the several decade−old question on the origin of EwS cells, or suggest a new concept.

Another important question is characterizing the transcriptional response for the induction of EWSR1− FLI1, including up- and down-regulated genes. In 2008, a meta−analysis of 13 different model systems and transcriptomic profiles has been performed in order to define the consensus or core 'transcriptomic signature' of EWSR1−FLI1 (503 up- and 293 down-regulated genes) ***(86)***. In another study, a molecular function map of EwS was built based on the joint analysis of EwS cell lines and tumors together with MSC transcriptomes. This analysis highlighted distinct clusters of activities for EWSR1−FLI1 regulated genes in EwS and revealed another definition of the transcriptional EWSR1−FLI1 signature (367 up- and 252 down-regulated genes) ***(87)***. However, both analyses were not able to distinguish direct and indirect downstream effects of EWSR1−FLI1 activity, that requires using more molecular description layers, specific mathematical modeling or single cell approaches as described below.

Few other examples of integration of several data types are represented in the genomic landscape of EwS papers described above, as well as several joint studies of coding and non−coding gene expression ***(45,84)***.

3.3 Assessing EwS tumor composition via mathematical modeling

Mathematical modeling of the bulk tumor transcriptome as a complex mixture of various cell types can shed light on the composition of tumor microenvironment via applying so−called computational deconvolution tools ***(88)***. To our knowledge, there exists only one study where the state−of−the−art deconvolution method, CIBERSORT, has been applied in order to determine the relative fraction of 22 immune cell types using 197 microarray expression profiles for EwS tumors ***(89)***. From this analysis, it followed that the most abundant type of immune cells present in the microenvironment of EwS tumors are immunosuppressive M2 type macrophages, and that increased number of neutrophils, albeit a low number, was associated to poor survival (although with a border−line statistical significance). A minority of EwS tumors appeared to be in the “hot" state, with dominating T−cells populations. Interestingly, this study pointed to a link between hypoxia and the immunogenic status of the EwS tumor, with high hypoxia been associated with the “cold" state, characterized by decreased in infiltration of T−cells.

Deconvolution of DNA methylation profiles can also serve the purpose of quantifying immune tumoral composition (e.g., via MeDeCom tool ***(90)***) but has not been applied to EwS so far. Nevertheless, some deconvolution−related approaches have been used to estimate the levels of the within−sample heterogeneity (WSH) of Ewing tumors ***(55)***. In a recent study, the RRBS profiles of EwS were used to benchmark six different WSH measures, concluding that different WSH measures may be more suitable to quantify different aspects of WSH measured through DNA methylation (cell type heterogeneity, DNA methylation erosion, cellular contamination or allele−specific methylation) ***(91)***.

3.4 Network modeling approaches

The wide−spread action of EWSR1−FLI1 is caused by its properties as a potent transcription factor and as a protein able to interact with many other proteins. This oncogene is frequently referred to as a “network hub" regulating various biological mechanisms such as splicing ***(92)***. The pleiotropic effect of EWSR1−FLI1 appeared a difficult case from the point of view of mechanistical modeling, since the perturbation caused by its activity is distributed across major cellular functions: therefore, it is difficult to define the potential model borders. Some of the published studies aimed at understanding the networks transducing the immediate action of EWSR1−FLI1 downstream to the major cellular phenotypes.

For example, transcriptome dynamics upon inhibition and re−activation of EWSR1−FLI1 in the A-673 cell line transformed into inducible system was used to define a list of candidate genes connecting the oncogene with apoptosis and cell cycle phenotypes ***(10)***. The selection of the genes was achieved using a model−based approach, assuming sigmoid−like response in the gene expression, which appeared to be more sensitive than the standard fold change−based approaches and more adapted to the temporal nature of the data.

As a result, a complex influence network of downstream action of EWSR1−FLI1 has been inferred, using literature knowledge about biological interactions (see Figure 1A and the interactive version at https://navicell.curie.fr/navicell/maps/ewing/master/ created using the online network visualization platform NaviCell ***(93)***). A limited number of genes was further selected for data−based network inference in order to validate part of the mechanistic connections between the network members. A complete perturbation−response matrix has been experimentally constructed for 11 selected genes (FOXO1A, IER3, CFLAR and their known regulators), by systematically knocking down them one by one using specific siRNAs. The response was quantified by qRT−PCR for each gene in the set. The perturbation−response matrix was binarized and analyzed together with the influence network in order to distinguish direct and indirect effects of each perturbation.

The network analysis validated a number of direct interactions between EWSR1−FLI1 and its targets and discovered few new ones, in particular, a member of E3 ubiquitin−protein ligase complex CUL1, indicating a link between the oncogene and the protein turnover regulation in the context of EwS. The hypothesis that *CUL1* is a direct target of the oncogene was further supported by ChIP−Seq data analysis. More generally, the reconstructed network can serve as a basis for the mechanistic modeling of the EWSR1−FLI1 action, for example, using probabilistic Boolean modeling approach ***(94)***.

Another study applied mathematical modeling to better understand the functional synergy between the action of EWSR1−FLI1 and the cellular E2F dependent gene regulatory network which is the central part of the cell cycle progression mechanism ***(95)***. The model focused on explaining the observation that knocking down EWSR1−FLI1 is accompanied by loss of *E2F3*/pRB (activator complex) and gain of *E2F4*/p130 (inhibitory complex) occupancy at E2F target promoters. The originality of the approach was in that four alternative mathematical models based on the standard formalism of chemical kinetics suggested to explain this phenomenon (see Figure 1B). The parameters of all four models were fit to the expression dynamics of four genes (*EWSR1−FLI1*, *E2F3*, and two E2F target genes *ATAD2* and *RAD51*) measured by qRT−PCR in 14 time points following the knock−down of EWSR1−FLI1. The Bayesian model selection approach was used to rank the models accordingly to their ability to explain the data. One of the four models was way more probable accordingly to this analysis (Figure 1B). It predicted the synergy either through physical and/or functional interaction between EWSR1−FLI1 and an E2F3 complex as a necessary prerequisite for combinatorial promoter binding and activation. This study provided an excellent example for the power of systems biology in the study of complex gene regulatory mechanisms that are otherwise difficult to assess experimentally.

[Figure 1 about here.]

4 Single cell studies of EwS

From the point of view of systems biology, emergence of technologies allowing to study biological systems at single cell level dramatically improve our understanding of the mechanisms of tumorigenesis, genetic and epigenetic tumoral heterogeneity connected to resistance to treatment. Each cell represents a possible state of a biological system under specific (even though not completely characterized) conditions. Therefore, with an advent of the single cell technologies, the amount of data available for reverse−engineering the biological mechanisms dramatically increased in the last 5 years.

The first recently published single−cell study of EwS provided a valuable resource comprising several single cell datasets ***(29)***. First, the doxycycline−inducible system based on the A−673 cell line was profiled in 7 time points at single cell level using C1 single−cell system (Fluidigm) (383 cells at all time points), tracing the induction of the EWSR1−FLI1 from the meta−stable state where its expression was the lowest to the meta−stable state where the expression of EWSR1−FLI1 was high. RNA velocity−based analysis combined with pseudo−time quantification showed a picture of relatively rapid transition of Ewing cells between twometa−stable states EWSR1−FLI1^{LOW} and EWSR1−FLI1^{HIGH} (see Figure 2A). For each individual cell, the duration of the transition appeared to be much shorter than the total duration of the experiment (15 days) which allowed quantification of the RNA velocity vectors. In each metastable state, EwS cells can proliferate: in the EWSR1−FLI1^{LOW} state, the proliferation appears to be possible as soon as 2 days after the doxycycline was removed from the system. In the EWSR1−FLI1^{HIGH} state, non−proliferating cells were rare. Two cellular trajectory types described the heterogeneity of the transition between two states. In the first scenario, followed by the majority of EwS cells, the activation of proliferation approximately coincided with the full activation of the oncogene or even preceded it, in the second, the activation of proliferation was delayed after the oncogene activation (Figure 2A).

The cell line dataset was jointly analyzed with several other scRNA−Seq datasets, including EwS PDXs (142 cells) and a xenograft implanted with the inducible cell line system (215 cells). Independent Component Analysis (ICA) was applied in order to identify distinct sources of gene expression heterogeneity ***(96)***, which allowed to distinguish the proper activating transcriptional program of EWSR1−FLI1, called IC−EwS (220 genes), from seemingly indirect and non−specific to EwS effects such as the transcriptional program of G1/S or G2/M phases or response to hypoxia. The results of the functional enrichment analysis of the identified independent components can be browsed online at http://bioinfo-out.curie.fr/projects/sitcon/mosaic/toppgene_analysis/.

The IC−EwS signature was validated by time−resolved bulk FLI1 ChIP−Seq measurements in the same time points as used for the transcriptomic profiling, and H3K27ac histone mark modification profiling in EWSR1−FLI1^{HIGH} and EWSR1−FLI1^{LOW} conditions. The conclusion was that IC−EwS is strongly enriched with direct targets of EWSR1-FLI1. The downregulation program of EWSR1−FLI1 was recapitulated in a

component called IC−ECM (for extracellular matrix) but appeared to be non−specific to EwS tumors and less directly associated with the direct action of the oncogene.

Moreover, the study provided data on the intratumoral heterogeneity (ITH) at single cell level in 5 EwS PDXs profiled with 10X Genomics (8,431 cells in total). Some of the transcriptional programs identified from the inducible cell line analysis, were shown to significantly contribute to EwS ITH, including those connected to proliferation, oxidative phosphorylation, splicing, hypoxia and IC−EwS (representing a surrogate measure of EWSR1−FLI1) itself. It appeared that a well−defined intermediate level of EWSR1−FLI1 activity was associated with cells in the proliferative state. Below and, more intriguingly, above this range, almost no proliferative cells were observed. Among the cells with low estimated activity of EWSR1−FLI1, there existed a sub−population characterized by increased hypoxia signaling and increased expression of genes involved in glycolysis. Those cells having the maximum estimated activity of EWSR1−FLI1 were also characterized by increased hypoxia, after regressing out the dominant signal connected with expression of EWSR1−FLI1 direct targets.

Three EwS cell lines (CHLA9, CHLA10, TC71) have been recently sequenced at single cell level using 10X Genomics (9783 cells in total), and these data were analyzed together with the above described PDX profiles with the purpose to demonstrate the existence of mesodermal-like cell subpopulations ***(30)***. In another, yet unpublished study, the authors performed single cell profiling of three EwS cell lines in the conditions with and without knocking down EWSR1−FLI1 using siRNA ***(97)***. The dataset contained close to one thousand viable cells profiled using C1 single−cell system (Fluidigm). The authors reported that they identified existence of two rare subpopulations in EwS cells: dormant−like and neural stem−like in the EWSR1−FLI1^{HIGH} state, with distinct population dynamics after knocking−down the driver oncogene. It was suggested that the existence of these rare states can provide a survival mechanism upon the stress caused by the inhibition of EWSR1−FLI1.

Overall, it seems that single cell studies (not only of transcriptome but also other modalities such as scATAC−Seq) can provide insights on the structure of intratumoral heterogeneity and shed light on the mechanisms connecting EWSR1−FLI1 activity and major cellular phenotypes such as proliferation. Ongoing single cell profiling of EwS tumors as a part of EU Horizon−2020 iPaediatricCure project should provide new insights in the mechanisms of interaction of EwS cells with the major actors of tumoral microenvironment, including immune cells.

[Figure 2 about here.]

5 Conclusion

Independently on its clinical significance, EwS is an outstanding cancer type in the light of cancer systems biology for several reasons. It is one of the most genetically stable and homogeneous cancer, lacking clear relation to normal tissues and characterized by a single known cancer driver event. There exist multiple evidences that EWSR1-FLI1 blocks some normal developmental cellular trajectories. EwS was relatively well characterized at the molecular level in the last 15 years, partially thanks to the European-level collaborative efforts, bringing together computational and cancer biologists. Despite this, researchers from both fields still seem to be far from having a mechanistically complete picture of the connection between the fact of appearance of the particular genomic fusion causing EwS and the downstream shifts in the functioning of the major cellular mechanisms at multiple levels.

The reason for this appears to be the systemic action of the chimeric oncogene, such that its complexity evades simple intuition and the usual reductionist approach. It seems that having so many molecular clues in hand, we must have already reverse−engineered the “EWSR1-FLI1 pathway”, if the action of EWSR1-FLI1 could be reduced to dysregulation of a small number of key regulators of cellular life. The biological reality, however, seems to be more complex than this simplistic representation. This complexity is reflected in that the molecular studies of EwS, besides genomic ones, do not seem to converge to a limited set of “principal molecular players" involved in this disease.

A recent study, based on single-cell analysis of the well-studied inducible cellular EwS system, resulted in a definition of the proper transcriptional signature of EWSR1-FLI1, designated as IC-EwS, statistically independent from its indirect downstream effects on cell cycle, organization of extracellular matrix, regulation of RNA splicing, etc ***(29)***. This signature appeared to be more specific to the EwS tumors than any other previously suggested signature. EWSR1-FLI1 as a transcriptional activator binds to the repetitive sequences more or less randomly distributed across the genome. As one would expect from this, the IC-EwS signature is not enriched with any particular biological process or function. Despite this, the perturbation caused by EWSR1-FLI1 fusion and expression seems to have profound and consistent changes, collectively pushing the EwS cell towards the cancerous phenotype. This apparent discrepancy between a disorganized nature of the perturbation and the well-defined malignant outcome can be called the EwS enigma, both from the biological and the systems theory points of view.

In order to resolve this enigma, we might need to understand some yet unknown principles of cellular adaptation and selection, at epigenetic level, to the drastic changes in the topology of the connections between the modules of the global molecular network. These principles should be properly formalized in the language of mathematics, because standard descriptions, such as chemical kinetics or logical formalisms, appear to be poorly suited to this situation. In order to advance in understanding cancer in general and

rationalizing its treatment, the genesis of EwS might serve us as an important prototypical real-life scenario for application of the systems biology approach.

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List of Figures

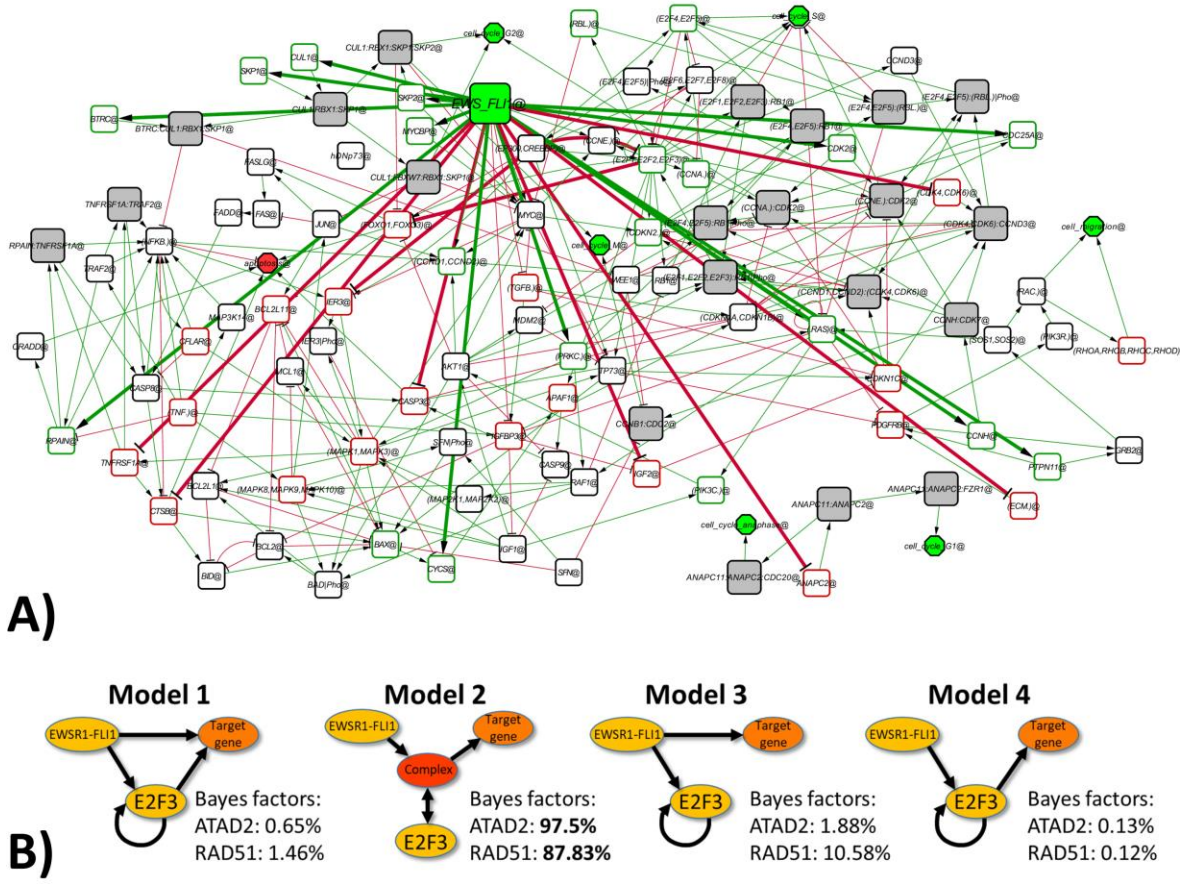

Figure 1: Examples of mathematical modeling of EwS networks. A) Reverse-engineered network of the downstream effect of EWSR1-FLI1 leading to proliferation and cell cycle phenotypes. Thick edges designate regulations inferred from transcriptome time series and siRNA/RT-QPCR data, green for activation and red for inhibition effect. White nodes are simple proteins or related groups, grey nodes are protein complexes, green pentagons are cellular phenotypes. The network image is adapted from ***(10)***. B) Use of mathematical modeling and model selection in order to test a biological hypothesis on the interplay between EWSR1-FLI1, the E2F3 transcription factor and target genes. Model 1 assumes that the EWSR1-FLI1 and E2F3 proteins independently target genes including E2F3 itself. Model 2 postulates that target gene transcription depends on the co-binding of EWSR1-FLI1 and E2F3 proteins as a complex or separately but in interdependence. Model 3 presumes EWSR1-FLI1 protein activates transcription of target genes alone without a contribution of E2F3. Finally, model 4 supposes that the EWSR1-FLI1 protein first activates the transcription of E2F3, and E2F3 protein subsequently activates transcription of target genes. The images are adapted from ***(95)***.

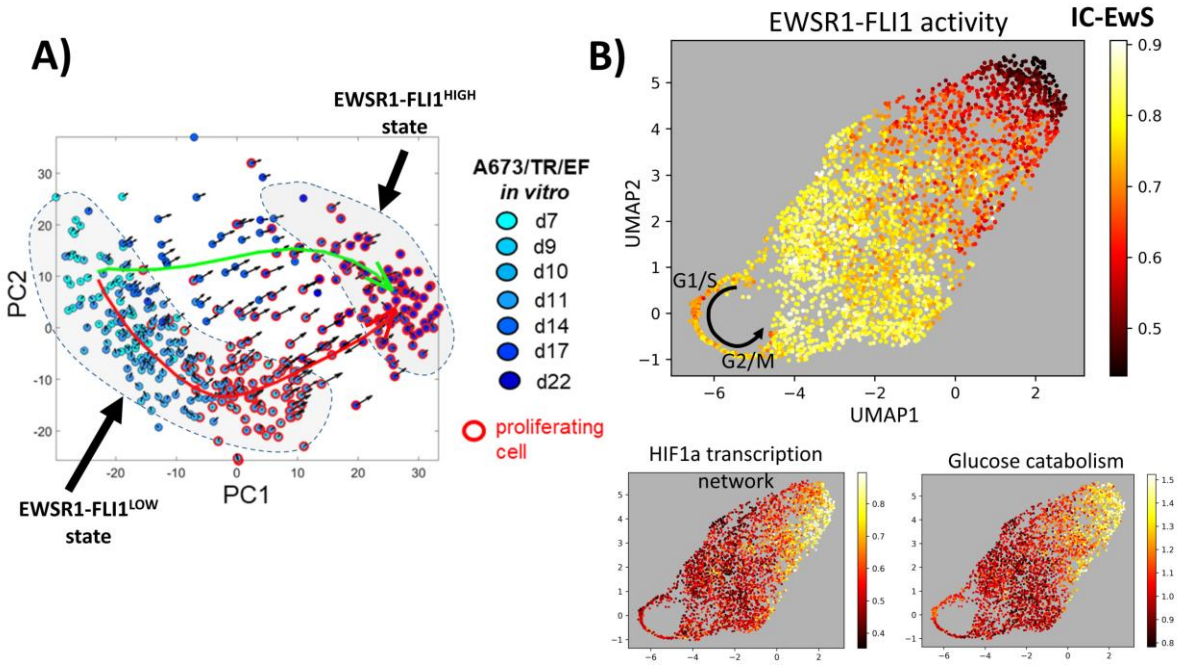

Figure 2: Single cell study of EwS. A) RNA velocity plot produced for the inducible cell line system. Each arrow shows a potential direction of the change of the transcriptome for a given cell. The image is adapted from ***(29)***. Two branches of pseudo-time (shown by green and red curves) recapitulate two types of the transcriptional dynamics after induction of the oncogene. B) Visualization of intratumoral heterogeneity in an EwS patient-derived xenograft (data from ***(29)***). Each point represents a single-cell tumoral transcriptome. The large panel shows cell heterogeneity caused by variability of the estimated EWSR1-FLI1 activity. The ring-like structure is formed by cells in the proliferative state. Small panels show two transcriptomic scores connected to hypoxia and metabolic heterogeneity.

Table 1: Exemplary omics datasets generated in EwS studies. The complete catalogue is available at https://github.com/sysbio-curie/EwingSarcoma_Omics_Atlas

Year	Experimental system and data	Dataset characteristics	Publication, ID
		Genomics	
2012	aCGH profiling of EwS	67 EwS tumors + 16 EwS cell lines	(51), GSE20355
2014	WGS + targeted sequencing	112 (WGS) and 199 (TS) tumors	(91), ICGC
2014	WES, WGS, SNP array for EwS tumors and cell lines	WES 96 tumors and 11 cell lines, WGS of 7 pairs, SNP array of 28 pairs	(15), PedcBio-Portal
2014	Sequencing of EwS tumors and cell lines	65 tumors and 36 cell lines, 6 paired WGS, 79 TS, 6 SNP arrays	(13), dbGaP phs000768.v1.p1
		Epigenomics	
2014	Epigenome of primary EwS and cell lines, MSC cells	ChIP−seq for 4 histone modifications, 5 transcription factors	(65), GSE61944
2015	“Epigenome map" of A-673 cell line	RNA-Seq, DNA methylation, ATAC-Seq, ChIP-Seq profiles with active and inhib-ited EWSR1-FLI1	(92), https: //tinyurl. com/r6ddvpb
2017	DNA methylation sequencing of EwS tumor and cell lines	140 EwS tumor samples, 16 EwS cell lines, and 32 primary MSCs	(78), GSE88826
2020	ChIP−Seq time series of A-673/TR/shEF	ChIP−Seq of FLI1 (6 time points) and H7K27ac (2 conditions)	(4), GSE129155
		Transcriptomics	
2012	French CIT program EwS tumor cohort	117 samples, Affymetrix HG−U133Plus2	(64), GSE34620
2013	EWSR1−FLI1 silencing	Time series, 13 time points	(82)
2014	ICGC transcriptomic dataset	57 EwS tumors profiled with RNA−Seq	(91)
		Proteomics	
2016	RPPA of 18 EwS cell lines resis-tant to targeted therapy	218 proteins in RPPA panels	(44), GSE78124
2018	Proteomic profiling of 2 EwS cell lines	2336 and 847 proteins of which 543 and 259 secreted proteins	(29), PXD007909
		Drug and gene screens	
2012	GDSC project	Drug sensitivity for 21 EwS cell lines	(24)
2017	siRNA-based screening of EwS	7000 genes, with low/high EWSR1-FLI1	(30)
2018	Determining druggable EWSR1-FLI1 interactome	3,325 experimental compounds in the in-ducible cell line	(94)
		miRNAome	
2012	mRNA/miRNA EwS profiling	39 EwS tumors	(54), GSE37371
2016	miRNA profiling of EwS tumors	20 tumors + 4 MSCs	(59), GSE80201
		Single cell	
2020	Single cell RNA−Seq of EwS inducible cell line and PDXs	7 time points (383 cells), 5 PDX (8431 cells)	(4), GSE130019, GSE130023, GSE130024