LETTER TO THE EDITOR

Integrative gene network and functional analyses identify a prognostically relevant key regulator of metastasis in Ewing sarcoma

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The identification of cancer stemness genes is crucial to understand the underlying biology of therapy resistance, relapse, and metastasis. In pediatric tumors – despite being largely composed of undifferentiated stem cell-like cells – mutations generally do not involve canonical stemness/metastasis-associated genes [1]. Ewing sarcoma (EwS), the second most common bone cancer in children and adolescents [2], is a highly aggressive malignancy associated with dismal outcome in metastatic disease [3], wherefore deciphering mechanisms of metastasis is imperative. EwS is characterized by a remarkably 'silent' genome with a single driver mutation generating an oncogenic fusion transcription factor (EWSR1-ETS) that promotes stem cell features [4, 5]. Thus, EwS constitutes an ideal model to study how perturbation of a transcriptional network by a dominant oncogene can mediate metastasis.

To identify highly relevant EWSR1-ETS target genes involved in the stemness/metastasis axis we implemented a systems biology approach to analyze published transcriptome profiles of 5 Ews cell lines with/without shRNA-mediated knockdown of EWSR1-FLI1 or -ERG (< 20%) for 96 h [6] as described in Fig. 1a. This analysis yielded 348 differentially expressed genes (DEGs) being up- or downregulated (|log2 FC| ≥ 1.5) after knockdown of EWSR1-FLI1 or -ERG across all cell lines (Supplementary Table 1). We next filtered these DEGs for genes annotated with the significantly enriched gene ontology term ‘Regulation of Cell Differentiation’ using PantherDB (P = 4.93 × 10^{-12}, false-discovery rate = 1.97 × 10^{-9}). The resulting 76 DEGs (Supplementary Table 2), were subjected to network analysis for pathway, physical, and genetic interactions using Cytoscape and GeneMania (Fig. 1b, c). To identify clinically relevant key hubs within this network we focused on TFs (n = 11) that were highly interconnected (> 10 interactions) with a significant (P < 0.05, Mantel-Haenszel test) association with overall survival in a cohort of 166 EwS patients with matched gene expression and clinical data [7]. After adjustment for multiple testing (Supplementary Table 3), transcription factor 7 like 1 (TCF7L1, alias TCF3) emerged as the most promising candidate for functional follow-up, whose low expression was associated with poor patients’ overall survival (nominal P = 0.0057; P = 0.0228 Bonferroni-adjusted).
Next, we explored TCF7L1 protein levels in association with clinical outcome in tissue micro-arrays (TMAs) comprising 114 EwS tumors and found that low TCF7L1 protein expression was significantly (P = 0.035) associated with worse overall survival (Fig. 1e), supporting that low TCF7L1 upregulation of TCF7L1 (Supplementary Table 4), which was confirmed in 5 EwS cell lines by qRT-PCR (average ~6-fold upregulation) (Supplementary Fig. 1b) and validated in A673 cells xenografts with/without conditional knockdown of EWSR1-FLI1 (A673/TR/shEF1 cells) at mRNA and protein levels (Supplementary Fig. 1c).

Prior reports suggested that TCF7L1 may have either oncogenic or tumor suppressor properties depending on the cellular context. Indeed, TCF7L1 expression has been linked to promotion of cell proliferation, tumor growth and sphere formation in breast cancer [8], colorectal cancer [9, 10], acute lymphoblastic leukemia [11] and skin squamous cell carcinoma [12], while ectopic expression of TCF7L1 inhibits self-renewal of liver cancer stem cells [13]. To explore its role in EwS, we first analyzed the TCF7L1 expression pattern across 18 cancer entities using microarray data from the Cancer Cell Line Encyclopedia (CCLE) and sphere formation in breast cancer [8], colorectal cancer [9, 10], acute lymphoblastic leukemia [11] and skin squamous cell carcinoma [12], while ectopic expression of TCF7L1 inhibits self-renewal of liver cancer stem cells [13]. To explore its role in EwS, we first analyzed the TCF7L1 expression pattern across 18 cancer entities using microarray data from the Cancer Cell Line Encyclopedia (CCLE) and an own study that compiled well-curated microarray data from the same cancer entities [14]. Surprisingly, TCF7L1 was very highly, but variably, expressed in EwS cell lines and primary tumors (Fig. 1f).

Weighted Gene Correlation Network Analysis (WGCNA) based on enriched gene-sets in TCF7L1-correlated genes in 166 EwS tumors showed that EwS tumors with low TCF7L1 expression were enriched in embryonic pathways (Fig. 1g). Although TCF7L1 is generally highly expressed in EwS, these data suggested that suppression of its transcription by EWSR1-FLI1 is associated with embryonic processes that may be linked with poor patient outcome. Thus, we generated two EwS cell lines (SK-N-MC and TC-71, with lowest TCF7L1 expression across the 5 EwS cell lines tested (Supplementary Fig. 1a) with a doxycycline- (DOX)-inducible re-expression of TCF7L1 (Fig. 1h). Consistent with the hypothesis that TCF7L1 may be a EWSR1-ETS-repressed downstream transcription factor (with a potentially indirect regulation, possibly partially mediated by TCF7L1 promoter hypermethylation as depicted in Supplementary Fig. 1d–g), transcriptome profiling of two EwS cell lines after either knockdown of EWSR1-ETS or re-expression of TCF7L1 showed a highly significant (P = 2.57 × 10^{-165} or P = 5.34 × 10^{-272}) overlap of concordant DEGs (Supplementary Fig. 1e). Congruently, conditional re-expression of TCF7L1 significantly reduced cell proliferation (Fig. 1i), clonogenic...
Fig. 1 (See legend on previous page.)
growth in two-dimensional (2D) cultures (Supplementary Fig. 2b), anchoragel-independent and three-dimensional (3D) growth (Fig. 1), and local tumor growth in a preclinical subcutaneous xenotransplantation mouse model (Fig. 1k). The xenografts expressing specific re-expression of TCF7L1 (DOX(+)-group) (Supplementary Fig. 2e,f) showed a reduced mitotic index (Fig. 1l), and a significant decrease of the proliferation marker Ki67 (Fig. 1m). Similarly in vitro and in vivo experiments performed with both cell lines ‘empty’ controls exhibited no significant differences (Supplementary Fig. 2a, c-d, g-i).

Since stemness features, such as elevated clonogenic capacity and anchorage-independent growth, are essential for circulating tumor cells to colonize and develop into clinically apparent metastases in distant organs, we reasoned that TCF7L1 may be linked to the metastatic process in EwS. In support of this hypothesis, transcriptome profiling, subsequent gene-set enrichment and WGCNA of TCF7L1-reexpressing SK-N-MC and TC-71 EwS cells uncovered that low TCF7L1 levels led to overrepresentation of gene-sets involved in cellular migration (Fig. 2a). Accordingly, re-expression of TCF7L1 resulted in significantly reduced transwell migration (Fig. 2b), invasion, and single-cell 3D migration in fibrin gel using advanced microfluidic chambers (Fig. 2c).

Next, we employed an orthotopic spontaneous in vivo metastasis model by injecting TCF7L1 re-expressing cells into the proximal tibiae of immunocompromised mice. Remarkably, while there was no significant difference in local tumor growth in the limited space of the tibial plateau (Supplementary Fig. 3a,b), we observed a strong inhibition of macrometastatic spread to liver, lungs, and kidneys upon re-expression of TCF7L1, and significantly reduced micrometastatic burden in the same organs (Fig. 2d,e, Supplementary Fig. 3c-e). In agreement with these in vivo findings, we observed that TCF7L1 was significantly ($P = 0.016$) lower expressed in EwS metastases ($n = 7$) compared to primary tumors ($n = 118$) in situ as defined by RNA-sequencing of tumor tissue (Fig. 2f). Strikingly, this result became even more significant when focusing on gene expression data of 4 matched pairs of metastases and primaries ($P = 0.0078$) (Fig. 2g).

To further investigate the mechanism of action of TCF7L1, we generated two EwS cell lines with conditional re-expression of different TCF7L1 deletion mutants for its major domains (ΔHMGB: DNA binding domain; ΔCTNNB: β-catenin binding domain) and observed that only the ΔHMGB mutants exhibited a normal clonogenic growth and migration capacity in vitro (Fig. 2h,i), and tumor growth in vivo (Fig. 2j). Moreover, further analysis of our WGCNA on TCF7L1-regulated signatures displayed in Fig. 2a highlighted several downregulated ‘driver target genes’ (aka leading-edge genes), including ANXA1, LMO7, SLC9A9, and TMEM71 as potential key mediators of TCF7L1 inhibition of the EwS migratory phenotype. These genes were selected for validation as they ranged among the top 10 downregulated genes by TCF7L1 and as their upregulation has been previously implicated in cancer progression [15–18]. As shown in Supplementary Fig. 3f, these genes are strongly downregulated upon

(See figure on next page.)

**Fig. 2** | High TCF7L1 expression inhibits metastasis in EwS through its DNA binding domain. **a** Weighted Gene Correlation Network Analysis (WGCNA) depicting functional gene enrichment of down- or up-regulated genes in TCF7L1 re-expressing EwS cells. Network depicts signatures presenting $P < 0.05$, NES > 2. NES, normalized enrichment score. Arrows depict direction of gene regulation. **b** Relative percentage of migrated cells in 6h. TC-71 and SK-N-MC EwS cells containing a DOX-inducible re-expression construct for TCF7L1 where pre-treated with or without DOX for 72h. $n \geq 7$ biologically independent experiments. Two-sided Mann-Whitney test. **c** Invasion and single cell migration distance in 15h. TC-71 and SK-N-MC EwS cells containing a DOX-inducible re-expression construct for TCF7L1 where pre-treated with or without DOX for 72h and added to a microfluidic chamber containing a fibrin compartment. $n \geq 3$ biologically independent experiments. Two-sided unpaired t-test. **d** Schematic representation of the experimental design: TC-71 or SK-N-MC EwS cell lines containing a DOX-inducible re-expression construct for TCF7L1 were injected in the right tibia plateau. Animals were subsequently randomized and treated with or without DOX. At the end of the experiment, mice were evaluated ex vivo for presence of spontaneous metastases in inner organs. Pie charts depict percentage of metastasis-free organs (blue) in each condition. **e** Graph depicts relative area of histological metastatic spread of orthotopically injected SK-N-MC EwS cells containing a DOX-inducible re-expression construct for TCF7L1. $n = 21$ slides/group. Two-sided Mann Whitney test. Pictures show representative histological images of HE stainings from the evaluated organs. Scale bar is 50 μm. M, metastasis; N, normal tissue. **f** Comparison of relative TCF7L1 expression of EwS primary tumors versus metastasis ($n = 125$), normalized using RPLP0 expression of the same samples. **g** Analysis of relative TCF7L1 expression (normalized to RPLP0 expression of the same samples) in paired metastasis/primary samples from EwS patients 1–4. Independent one sample t-test. **h** Relative colony number of colony-forming assays (CFAs) of TC-71 (left) and SK-N-MC (right) cells containing a DOX-inducible re-expression construct for TCF7L1, empty control, or one of the two deletion mutants for TCF7L1 (deletion mutant for the β-catenin binding domain, ΔCTNNB; deletion mutant for the DNA binding domain, ΔHMGB). Cells were grown either with or without DOX. $n = 4$ biologically independent experiments. **i** Relative percentage of migrated cells in 6h. TC-71 and SK-N-MC EwS cells containing a DOX-inducible re-expression construct for TCF7L1 were injected in the right tibia plateau. Animals were subsequently randomized and treated with or without DOX. At the end of the experiment, mice were evaluated ex vivo for presence of spontaneous metastases in inner organs. Pie charts depict percentage of metastasis-free organs (blue) in each condition. **j** Growth of EwS subcutaneous xenografts of TC-71 and SK-N-MC cells containing a DOX-inducible re-expression construct for TCF7L1 deletion mutant ΔHMGB (arrow indicates start of DOX treatment). Data are represented as means ($n = 7$ animals/group). Two-sided Mann-Whitney test.
**Fig. 2** (See legend on previous page.)

**a** TCF7L1 expression
- Locomotion
- Negative
- Rac GTPase binding
- Neg. reg. actin filament depolymerization
- Necrosis
- Mitochondrial protein complex
- Mitochondrial gene expression
- RNA phosphodiester bond hydrolysis
- Cell adhesion
- Extracellular matrix structure

NES = 2

**b** Transwell assay
- Migration through membrane (%)
- TC-71
- SK-N-MC
- DOX (-)
- DOX (+)
- P = 0.0001
- P = 0.0006

**c** Single-cell 3D tracing assay
- Migrated distance (μm)
- TC-71
- SK-N-MC
- DOX (-)
- DOX (+)
- P = 0.0001
- P = 0.0001

**d** Orthotopic injection (i.o.)
- Randomization
- Spontaneous metastasis
- Percentage of metastasis-free organs
- DOX (-)
- DOX (+)
- n = 120
- n = 60

- Total number of macro-metastases across organs

- Liver
- Lungs
- Kidneys

27%

27%

67%

**e** Histological metastases area (%)
- Liver
- Lung
- Kidney

**f** Relative TCF7L1 expression
- Primary (n=100)
- Metastasis (n=100)
- P = 0.001

**g** Patients

**h** Relative tumorigenicity (% to DOX (-))
- TC-71
- SK-N-MC
- Empty
- Empty
- ΔCTNNB
- ΔC4M6-1
- ΔHMG
- ΔHMG
- DOX (-)
- DOX (+)
- P = 0.0288
- P = 0.0437
- P = 0.0375
- P = 0.00003
- P = 0.0025
- P = 0.0033

**i** Relative migration through membrane (%)
- TC-71
- SK-N-MC
- ΔHMG
- ΔHMG
- DOX (-)
- DOX (+)
- P = 0.2224
- P = 0.4873

**j** Tumor volume (mm³)
- SK-N-MC ΔHMG
- DOX
- P = 0.7048
re-expression of wildtype TCF7L1 in both EwS cell lines tested, but remain unaltered in the DNA-binding-domain deletion mutant (ΔHMG), further suggesting that DNA-binding of TCF7L1 is required to suppress the tumorigenic and migratory phenotype of EwS cells.

Conclusions

Previous studies proposed a binary model of EWSR1-FLI1-high promoting proliferation and EWSR1-FLI1-low promoting metastasis [19] following a traditional epithelial-mesenchymal-transition concept. However, our findings support an integrative concept of the ‘migratory stem cell’ [20]: EwS cells may reside in a ‘metastable’ state where all EwS cells of a tumor may be equipped with both proliferative and migratory capacities [21]. Upon different intrinsic/extrinsic cues (of which EWSR1-FLI1 may not be the only important component) these cells could underlie a certain accentuation.

In this context, we demonstrated that TCF7L1 is a critical mediator of metastasis in EwS, which may be utilized as a prognostic biomarker. Since we could detect a strong inverse correlation of TCF7L1 levels with patients’ overall survival in both the mRNA- as well as in the TMA-cohort, we believe that the most straightforward possibility to translate TCF7L1 into a routine clinical setting would be the IHC detection and semi-quantitative evaluation of TCF7L1 in primary biopsies. This technique is readily available, inexpensive, and the conditions of IHC staining of TCF7L1 in EwS tumors have been established in the current study, which would offer the possibility of further evaluating the prognostic value of TCF7L1 in ongoing and prospective clinical trials. In addition, it would be highly interesting to explore whether TCF7L1 may serve as a prognostic biomarker in other (EWSR1-rearranged) mesenchymal neoplasms, which is subject to future studies.

In summary, our study exemplifies the power of systems biology to decipher gene regulatory networks and to identify key players in the metastatic process, which may be highly relevant for, and translatable to, other oligomutated (pediatric) cancers.

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Authors’ contributions

F.C.A, M.M.L.K., and T.G.P.G. conceived the study. F.C.A. and T.G.P.G wrote the paper, and drafted the figures and tables. F.C.A. and M.M.L.K. carried out in vitro experiments. F.C.A, M.F.O., and T.G.P.G. performed bioinformatic and statistical analyses. F.C.A, M.M.L.K., J.L., T.L.B.H., J.M, R.I., and A.B. performed and/or coordinated in vivo experiments. S.K., M.F.O., K.C., M.U.C.G., G.A., S.B., C.A., M.M., C.F., A.M., S.O., L.R.-P., and M.K. contributed to experimental procedures. W.H., D.B. and U.T. provided clinical and/or statistical guidance. T.K., F.S., S.M.P., and F.B. provided laboratory infrastructure, contributed to RNA sequencing and/or provided histological guidance. T.G.P.G. supervised the study and data analysis. All authors read and approved the final manuscript.

Availability of data and materials

Original microarray data that support the findings of this study were deposited at the National Center for Biotechnology Information (NCBI) GEO and are accessible through the accession number GSE165929. Custom code is available at the National Center for Biotechnology Information (NCBI) GEO.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12943-021-01470-z.

Additional file 1:

Additional file 2:

Additional file 3:

Declarations

Ethics approval and consent to participate

Human tissue samples were retrieved from the tissue archives of the Institute of Pathology of the LMU Munich (Germany), the Bone Tumor Reference Center at the Institute of Pathology of the University Hospital of Basel, or the Gerhard-Domagk Institute of Pathology of the University of Münster (Germany) upon approval of the institutional review board. All patients provided informed consent. Tissue microarrays (TMAs) were stained and analyzed with approval of the ethics committee of the LMU Munich (approval no. 550–16 UE).

Animal experiments were approved by the government of Upper Bavaria and Northrhine and performed in accordance with ARRIVE guidelines, recommendations of the European Community (86/609/EEC), and United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines for the welfare and use of animals in cancer research.

Consent for publication

Not applicable.

Abbreviations

CCLE: Cancer Cell Line Encyclopedia; CTNNB1: Beta catenin; DOX: Doxycycline; DEG: Differentially expressed genes; EMT: Epithelial to mesenchymal transition; ETS: E26 transformation specific; EwS: Ewing sarcoma; EWSR1: Ewing sarcoma breakpoint region 1; ERG: ETS transcription factor ERG; FDR: False discovery rate; FET: FUS, EWSR1, TAF15; FC: Fold change; FFPE: Formalin-fixed and paraffin-embedded; FLI1: Friend leukemia virus integration 1; GO: Gene ontology; GSEA: Gene set enrichment analysis; HE: Hematoxylin Eosin; HMG: High mobility group; iHIC: Immunohistochemistry; iIF: Immune reactive score; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NES: Normalized enrichment score; NSG: NOD/SCID/gamma; TCF3: T-cell factor 3; TCF7L1: Transcription factor 7 like 1; TF: Transcription factor; TMA: Tissue microarray; ORF: Open reading frame; WGCNA: Weighted gene co-expression network analysis; Wnt: Wingless/Integrated.
Competing interests
TGPG serves as honorary consultant for Boehringer-Ingelheim International GmbH. All other authors declare no conflict of interest.

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