



Single-Cell RNA Sequencing of Ewing Sarcoma Tumors Demonstrates Transcriptional Heterogeneity and Clonal Evolution

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ABSTRACT

Purpose: Ewing sarcoma is the second most common bone cancer in children, accounting for 2% of pediatric cancer diagnoses. Patients who present with metastatic disease at the time of diagnosis have a dismal prognosis compared with the >70% 5-year survival of those with localized disease. Novel therapeutic approaches that can impact metastatic disease are desperately needed, as well as a deeper understanding of the heterogeneity of Ewing sarcoma tumors.

Experimental Design: In this study, we utilized single-cell RNA sequencing to characterize the transcriptional landscape of primary Ewing sarcoma tumors and the surrounding tumor microenvironment in a cohort of seven untreated patients with Ewing sarcoma, as well as in circulating tumor cells (CTC). A potential CTC therapeutic target was evaluated through immunofluorescence of fixed CTCs from a separate cohort.

Results: Primary tumor samples demonstrate a heterogeneous transcriptional landscape with several conserved gene expression programs, including those composed of genes related to proliferation and Ewing sarcoma gene targets, which were found to correlate with overall survival. Copy-number analysis identified subclonal evolution within patients prior to treatment. Analyses of the immune microenvironment reveal an immunosuppressive microenvironment with complex intercellular communication among the tumor and immune cells. Single-cell RNA sequencing and immunofluorescence of CTCs at the time of diagnosis identified TSPAN8 as a potential therapeutic target.

Conclusions: Ewing sarcoma tumors demonstrate significant transcriptional heterogeneity as well as a complex immunosuppressive microenvironment. This work evaluates several proposed targets that warrant further exploration as novel therapeutic strategies.

Introduction

While the intensification of conventional multimodal therapy has significantly improved the survival rate for localized Ewing sarcoma from less than 10% in the surgery-only era to approximately 70%, many patients still die from metastatic disease (1). Even those with initial metastatic disease can achieve a radiographic remission; however, metastatic relapse after a remission frequently occurs, demonstrating that an initial good response to conventional therapy does not guarantee a cure.

While the Ewing sarcoma genomic landscape involves few mutations, the pathognomonic $t(11; 22)$ *EWS-FLI1* translocation drives 90% of the disease (2–5). By studying *EWS-FLI1* transcriptional regulation, more than 1,000 genes have been identified as being directly or indirectly induced or repressed (6–9). While these results have spurred a significant effort focused on *EWS-FLI1*-induced genes that might be targeted to inhibit tumor progression, clinical translation has been difficult (10, 11). *EWS-FLI1* expression is arguably essential to the survival of Ewing sarcoma cells, but emerging experimental and clinical evidence suggests that Ewing sarcoma tumors have significant intratumoral *EWS-FLI1* transcriptional heterogeneity (12, 13). A major unmet need is to better understand tumor heterogeneity and how it potentially contributes to poor prognosis, with the goal of identifying therapeutic targets that are essential for metastasis and recurrence.

The understanding of the Ewing sarcoma tumor microenvironment (TME) is still evolving. Previous work in this cancer type has suggested that the TME is infiltrated by immunosuppressive myeloid cells, functionally impaired antigen-presenting cells, and T cells (14, 15). Ewing sarcoma tumor cells have been shown to lack expression of HLA class I molecules, potentially leading to decreased T-cell recognition (14, 16).

Despite the advances in the investigation of Ewing sarcoma tumor biology, clinical support and translation have been poor. For instance, the knowledge of intratumoral transcriptional activity in untreated patient tumors is limited, and we know very little about how this may drive treatment resistance or clonal evolution. In this study, we used single-cell RNA sequencing (scRNA-seq) to

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Translational Relevance

Five-year survival of patients diagnosed with localized Ewing sarcoma has improved to 70%, but novel treatments have limited success beyond standard surgery, radiotherapy, and chemotherapy regimens. In this study, we have used single-cell RNA sequencing to analyze untreated Ewing sarcoma primary and circulating tumor cells to better understand the biology while evaluating potential therapeutic targets. We characterize tumor heterogeneity by identifying clonal expansion prior to treatment and gene expression programs that correlate with overall survival. While evaluating dozens of proposed therapeutic targets, we find that *TSPAN8* is highly and broadly expressed in both primary and difficult-to-treat circulating tumor cells, making it an intriguing target for Ewing sarcoma across multiple disease stages. Additionally, this single-cell resource provides vast data for future exploration.

investigate the transcriptional heterogeneity of untreated tumors of patients with Ewing sarcoma, as well as circulating tumor cells (CTC). We found that patient tumors contain heterogeneous clusters of tumor cells with distinct genetic expression programs, which correlate with patient outcomes. Evaluation of primary tumors, CTCs, and the TME revealed several potential therapeutic targets.

Materials and Methods

Tumor collection and processing

This study was approved by the Colorado Multiple Institutional Review Board (COMIRB) and was conducted in accordance with the Ethical Principles and Guidelines for the Protection of Human Subjects of Research (Belmont Report). Patients with newly diagnosed Ewing sarcoma at Children's Hospital Colorado were included, and written informed consent and assent (as appropriate) were obtained from the patient or the guardian of the patient for the COMIRB-approved protocol (COMIRB 00-206), in which tumor and blood samples were collected. Age, sex, and disease status were collected from all patients. Tumor analysis was performed under the COMIRB-approved protocol, COMIRB 22-1456. Untreated primary tumor samples from biopsies were minced and immediately cryopreserved viably using DMSO/FBS (10:90) medium in a Corning CoolCell LX container, chilled at $-1^{\circ}\text{C}/\text{minute}$ until reaching -80°C , followed by archival storage at -150°C , according to the 10x Genomics protocol and published methods (17–19). Frozen tumor tissue was thawed at 37°C and gently dissociated using an optimized collagenase IV/DNase I/RPMI digestion protocol. Tumor-digest suspensions were strained through a $70\text{ }\mu\text{m}$ filter membrane and washed. Red blood cells were depleted using the ErythroClear Red Blood Cell Depletion Kit (STEMCELL Technologies). The resultant single-cell suspension was sorted for viable cells using DAPI staining and FACS (Beckman Coulter MoFlo XDP). The enriched tumor single-cell suspensions were loaded into the Chromium Next GEM Single Cell 3' platform (10x Genomics) with a target cell recovery of 4,000 cells maximum at the Genomics Shared Resource of the University of Colorado Cancer Center. Following GEM capture and barcoded cDNA library construction, libraries

were sequenced at a targeted depth of 50,000 reads per cell using an Illumina NextSeq 2000.

Blood collection and processing

At the time of diagnosis, peripheral whole blood was collected using EDTA (K2) BCTs and immediately passed through an $8\text{ }\mu\text{m}$ pore microfilter membrane (CellSieve) at a rate of $5\text{ mL}/\text{minute}$ on a KD Scientific LEGATO 110 syringe pump. Captured cells were washed from the membrane using RPMI 1640–10% FBS and immediately preserved with DMSO/FBS (10:90) medium in a Corning CoolCell LX container, chilled at $-1^{\circ}\text{C}/\text{minute}$ until reaching -80°C , followed by archival storage at -150°C (17). Frozen cells were thawed at 37°C and treated using the ErythroClear Red Blood Cell Depletion Kit (STEMCELL Technologies). Cells were constructed into barcoded single-cell cDNA libraries and sequenced as described above.

scRNA-seq processing

Cell Ranger (version 6.0.1; ref. 20) was used to process fastq files into cell and gene count tables using unique molecular identifiers (UMI) aligning to GRCh38 (compiled by 10x Genomics as 2020-A). Downstream quality control and analysis utilized the Seurat (version 4.3.0; ref. 21) pipeline. Cell Ranger–filtered data were further processed by removing genes identified in fewer than 10 cells. Cell barcodes were removed if they had greater than 50,000 UMIs, greater than 7,000 genes, greater than 20% of UMIs from mitochondria, or fewer than 200 genes. Cell doublets were identified and removed using the scDblFinder R package (version 1.6.0; ref. 22). These filtering criteria resulted in 13,367 cells across all samples, totaling 22,639 genes.

The primary and CTC-enriched blood samples were further processed independently. The gene counts were normalized using Seurat by dividing each cell's total counts and multiplying by 10,000, followed by natural-log transformation. The top 2,000 most variable genes were scaled with total UMIs, and the percentage of mitochondria was regressed out. Clusters were identified as cell types through multiple methods. The top 100 enriched genes by cluster were used as input for overrepresentation analysis (23) with gene sets from the MSigDB C8 collection (24), the PanglaoDB (25), and select Ewing sarcoma–related gene sets. Similar enrichment analysis was performed for further indicated comparisons. The SCEVAN R package (version 1.0.1) was used to confirm the identification of tumor cells (26). Canonical cell type markers were plotted in Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) space and using dot plots. Gene set enrichment analysis was performed on fold change using the fgsea R package (version 1.18.0; bioRxiv 2021.02.01.060012). Myeloid and T cells in the primary samples were made into subsets and analyzed separately.

Copy-number alterations in scRNA-seq

Copy-number alterations (CNA) were estimated in primary and CTC-enriched blood samples using the inferCNV R package (version 1.11.2; RRID:SCR_021140). In each data subset, cells defined as Ewing were input as the tumor, whereas all other cells were identified as normal. Following analysis of the dataset of primary samples, two samples were analyzed further as they appeared to have subclusters. Discrete subclusters were identified using random trees in which branches were manually trimmed to fewer clusters according to the hierarchy. Phylogenetic trees were produced with the uphyloplot2 Python package (version 2.3), with major chromosomal alterations labeled (27).

Nonnegative matrix factorization in scRNA-seq

Intratumor gene expression programs (GEP) were identified using consensus nonnegative matrix factorization (28). The Ewing sarcoma cells of each primary tumor were processed separately while including only genes expressed in all samples (12,000 genes). Consensus nonnegative matrix factorization was run per sample with the 2,000 most variant genes using raw counts, first with k ranging from 2 to 10 and 50 iterations to select a k per sample with high stability and a low error rate. After k selection, each sample was run a final time with 200 iterations.

Sample-level GEPs were defined as the top 50 correlated genes per program. Ewing cells from all samples were scored using AddModuleScore (21), followed by z -score transformation of each program's score and labeling the top program per cell as the program with the highest score. Merged GEPs were identified by correlating sample-level programs using the cell scores. Each merged GEP was defined by the aggregate of genes from the appropriate sample-level programs. Genes spanning multiple merged GEPs were removed. Merged GEPs were defined by overrepresentation analysis as described above (23). Cells were scored by merged GEPs as described above. UMAP plots were visualized after integrating the samples with the Reciprocal Principal Component Analysis (RPCA) method (21).

VIPER (version 1.26.0; ref. 29) was run on the cell-level merged GEP labels to identify transcriptional associations from the high-confidence transcription factors (A, B, and C) in the DoRothEA database (version 1.4.2; ref. 30). Top regulators were identified by those with the highest difference in mean enrichment between a merged GEP and the rest of the cells. Cells were also scored using the top independent components identified in Ewing sarcoma xenografts (12). These scores were compared with those from merged GEPs using the Pearson correlation test.

Evaluation of merged GEPs in Ewing sarcoma microarray datasets

Two publicly available Ewing sarcoma microarray datasets were analyzed using the merged GEPs. GSE17679 cohort B contains 44 samples and served as the discovery cohort, whereas GSE63157 served as the validation cohort, containing 85 samples (31, 32). Both datasets were normalized with *rma* (33). The most variable probe set was chosen for each gene. The genes for each merged GEP were used to subset the GSE17679 dataset. Z -score transformation across each gene was performed, and the samples were scored by the mean expression of the transformed values. Samples were divided into two groups using the median score for each merged GEP. Kaplan–Meier plots and log-rank tests were performed using the *survminer* (version 0.4.9; RRID:SCR_021094) and *survival* R packages (version 3.2-13; RRID:SCR_0211237). Heatmaps were generated with the *ComplexHeatmap* R package (version 2.8.0; RRID:SCR_017270). The merged GEPs demonstrating a correlation with survival were tested in a similar manner in GSE63157.

Cell communication in scRNA-seq

Cell communication between ligands and receptors in the cell types of each primary sample was predicted using the CellChat R package (version 1.6.1; RRID:SCR_021946). Select interesting interactions were highlighted based on the significant interactions across multiple samples. Ligand and receptor expression was plotted across cell types by patient sample.

FLI1 ChIP-seq in Ewing sarcoma cell lines

Publicly available FLI1 ChIP-seq data in Ewing sarcoma cell lines (GSE176400) were analyzed for binding near the gene *TSPAN8* (34). Illumina adapters and low-quality reads were filtered out using BBDuk (version 38.87; <http://jgi.doe.gov/data-and-tools/bb-tools>). Bowtie 2 (version 2.3.4.3; ref. 35) was used to align the sequencing reads to the hg38 reference human genome. Samtools (version 1.11; ref. 36) was used to select the mapped reads (*samtools* view -b -q 30) and sort the bam files. PCR duplicates were removed using the Picard MarkDuplicates tool (RRID:SCR_006525). The normalization ratio for each sample was calculated by dividing the number of uniquely mapped human reads of the sample with the lowest number of reads by the number of uniquely mapped human reads of each sample. These normalization ratios were used to randomly subsample reads to obtain the same number of reads for each sample using *samtools* view -s. Bedtools genomecov (RRID:SCR_006646) was used to create bedGraph files from the bam files (37). Normalized BigWig files were created using deepTools bamCoverage (38) and visualized using the trackplot R package (version 1.4.00; ref. 39).

Whole-genome sequencing

Whole-genome sequencing (WGS) was performed on the seven primary tumor samples. Fastq files were processed with the nf-core/sarek pipeline (40). Mutations were evaluated using Mutect2 and annotated using Ensembl Variant Effect Predictor (VEP) (41). Genes and mutations were filtered to those previously identified in multiple Ewing sarcoma samples (3, 4) and those that passed Mutect2 and VEP filters (42). *EWSR1* translocations were identified using TIDIT (43), followed by annotation with SnpEff (RRID:SCR_005191; ref. 44). An OncoPrint was generated using the ComplexHeatmap R package (45).

TSPAN8 staining in CTCs

Archival fixed CTC slides for TSPAN8 restaining were obtained from the National Pediatric Cancer Foundation study MCC20320. Previously stained and imaged CTC slides from this study were screened for slides with >20 CTCs identified. The identified six CTC slides then underwent the QUAR-R restaining technique as previously described (46). Briefly, slides were soaked in PBS, and filters were demounted. Filters were washed, incubated with borohydride solution, washed, and then incubated with Tris (46, 47). After incubation, the filters were washed and stained with anti-TSPAN8 tagged with Alexa Fluor 488 at 2 μ g/mL (R&D Systems, clone 458,811). The stain was applied to the filters for 1 hour. Filters were washed and mounted on a fresh microscope slide with DAPI. CTCs were then re-read utilizing an Olympus BX54WI fluorescent microscope with Carl ZEISS AxioCam. Zen 2011 Blue software was used to measure the TSPAN8 expression on each cell as previously described (46, 47).

Cell lines

Breast cancer cell line MCF7 (RRID:CVCL_0031) and colon cancer cell line HT-29 (RRID:CVCL_0320) were procured from the ATCC and used as positive and negative controls for TSPAN8 immunofluorescent staining. Cell lines were cultured between 4 and 10 passages from the date of thaw and tested for mycoplasma by the ATCC Mycoplasma PCR testing service prior to all experiments. Due to the short culture period following receipt from the ATCC, cell line reauthentication was not performed.

Data availability

Due to specific patient privacy concerns, the University of Colorado Regulatory Compliance does not permit the public sharing of these raw fastq files. However, we are committed to collaborative data analysis, and more information and mechanisms for data access can be obtained by contacting the corresponding author. Cell Ranger unfiltered and filtered barcode counts as well as filtered gene counts and cell labels are available on Gene Expression Omnibus as GSE277083. The code used for the bulk of the scRNA-seq analysis is available on GitHub: https://github.com/Aeg22/ews_scrnaseq.

Results

scRNA-seq reveals transcriptional heterogeneity in Ewing sarcoma primary tumors

To characterize the intratumoral landscape of Ewing sarcoma, we performed droplet-based scRNA-seq and WGS on biopsies of primary tumor samples from seven patients with newly diagnosed and untreated Ewing sarcoma (Supplementary Fig. S1A). Six of the tumors had an *EWS-FLI1* translocation, whereas the other contained *EWS-ERG* (Table 1). Additionally, three tumors had mutations in previously associated Ewing sarcoma–altered genes (3, 4), including one with the most frequently mutated gene, *STAG2*. The WGS also identified several common CNAs, demonstrating the clinical relevance of these samples (Supplementary Fig. S1B and S1C). After doublet removal and filtering of low-quality cells, the scRNA-seq dataset consisted of 6,228 cells with a median of 13,791 UMIs per cell and a median of 3,058 genes per cell (Supplementary Fig. S1D and S1E; Supplementary Table S1).

Single cells were visualized using UMAP followed by unbiased clustering. We classified nine major cell types (Fig. 1A–D; Supplementary Fig. S2A and S2B; Supplementary Table S2). In addition to Ewing sarcoma, we found the TME to consist of myeloid cells, endothelial cells, T cells, B cells, osteoclasts, and matrix and inflammatory cancer-associated fibroblasts (CAF). Many of these cell types were present in all tumor samples although inflammatory CAFs were only observed in patient 001.

Ewing sarcoma cells were identified through several means. These cells expressed high levels of canonical Ewing sarcoma–associated genes such as *MAPT*, *KDSR*, *NKX2-2*, *NR0B1*, *STEAP1*, and *PRKCB* (Fig. 1C and D; Supplementary Fig. S2B; refs. 48–53). The classification of these cells as malignant was further supported by SCEVAN and the enrichment of several *EWS-FLI1* signature gene sets (Supplementary Fig. S2C and S2D). Among the Ewing sarcoma cells, a proliferating population was identified by the expression of *MKI67* and *PCNA* (Fig. 1C and D).

A benefit of scRNA-seq is the ability to characterize several forms of tumor heterogeneity. In this study, we utilized inferCNV to estimate genome-wide CNAs across the seven primary tumors (Fig. 1E; Supplementary Fig. S2E; ref. 19). Previous Ewing sarcoma studies and our WGS have identified several common CNAs, including gains in chr 1q, chr 2, chr 5, chr 7, chr 8, chr 12, and chr 20 and loss of chr 9p and chr 16q (4, 54–59). We identified several of these alterations, such as gains of chr1q, chr2, chr5, chr8, chr12, and chr20. These findings closely corroborated with the clinical karyotyping of these primary tumors when available (Table 1) and the findings from the WGS (Supplementary Fig. S1C).

Finally, we explored the expression of previously reported pre-clinical and clinical therapeutic targets for Ewing sarcoma in our dataset (Fig. 1F). TME-, immunotherapeutic-, and cell signaling-related targets were plotted across cell types and within tumor cells

by sample. Genes comprising the surfaceome of Ewing sarcoma, as proposed immunotherapeutic targets, were also evaluated (Supplementary Fig. S2F; ref. 60). We found that compared with other cell types in the primary tumor, Ewing sarcoma cells across all samples express moderate-to-high levels of *PAPPA* but comparatively low levels of most HLA genes. We show that several proposed immunotherapeutic targets, such as *ADGRG2* and *KDSR*, are uniquely and highly expressed in Ewing sarcoma, whereas others, such as *CD99* and *STEAP1*, are also highly expressed in CAFs (Fig. 1F; Supplementary Fig. S2F; refs. 60, 61). However, mRNA expression of these targets is not always ubiquitous across all tumors, as demonstrated by *IL1RAP* and *LINGO1*. Several of the proposed cell-signaling targets are also expressed at varying intertumoral levels, including *CDK4*, *PARP1*, and *DDX3X*, demonstrating a potential explanation for heterogeneous responses to treatments with single agents (62).

Ewing sarcoma tumors demonstrate transcriptional heterogeneity

Next, we sought to define the heterogeneity of transcriptional programs in Ewing sarcoma tumors. The tumor cells identified in Fig. 1 were isolated, integrated across patients, and then subjected to nonnegative matrix factorization analysis in a sample-specific manner to identify intratumor GEPs (Supplementary Fig. S3A; ref. 28). This revealed a total of 25 GEPs (Supplementary Table S3), which were grouped into six main meta-programs through hierarchical clustering (Supplementary Fig. S3B; Supplementary Table S4). Most of the GEPs were represented in multiple tumors, suggesting that they represent diverse functions shared among patients. Based on the gene expression and gene set overrepresentation analysis within each merged GEP, we termed the six merged GEPs MAPK, EWS, Proliferation, UPR, Ribosome, and OxPhos (Fig. 2A and B; Supplementary Fig. S3C–S3E).

Potential regulators and associations with previous gene programs were further investigated. Transcriptional drivers of each program were explored with VIPER (29). Several drivers were as expected, such as *JUNB*, *FOXP1*, *E2F2*, *EPAS1*, and *ATF6*, associating with merged GEPs MAPK, OxPhos, Proliferation, Ribosome, and UPR, respectively (Supplementary Fig. S3F). The top drivers of the merged EWS GEP were identified as *ONCECUT1*, *MEIS1*, and *POU5F1*. The lack of enrichment of *FLI1* and *ERG* in the program is not surprising, considering the major role of *EWS-FLI1*–mediated transcriptional repression through the displacement of wild-type Erythroblast Transformation Specific (ETS) transcription factors (6). Meanwhile, *MEIS1* has been shown to cooperate with *EWS-FLI1* to drive its transcriptional profile (63). We also compared the merged GEPs to previously identified programs in scRNA-seq from Ewing sarcoma xenografts (12). Most merged GEPs closely correlated with programs in xenografts, including the merged EWS GEP and the Ewing sarcoma program from Aynaud and colleagues (12), demonstrating the similarity in programs between Ewing sarcoma primary tumors and model systems (Supplementary Fig. S3G).

EWS and Proliferation merged GEPs correlate with patient outcome

Next, we investigated whether genes enriched in each merged GEP correlate with overall survival in previously published datasets (31, 32). The two microarray datasets used in this analysis consisted of 44 Ewing sarcoma tumors, which included 32 primary tumors, five recurrences, and seven metastases (GSE17679; ref. 32), and 85 primary Ewing sarcoma tumors, which were collected from

Table 1. Clinical characteristics of patient samples submitted for scRNA-seq.

ID	Patient data	Location	Metastases	Fusion	Tumor karyotype	Recurrence	Overall survival (months)	Samples	
								Tumor	CTC blood
001	13-year M	L fibula	—	<i>EWS-ERG</i>	Loss of 20, gain of 21	—	12	+	+
002	19-year F	Pleural mass	+ (Spine)	<i>EWS-FLI1</i>	Not performed	+ (Local progression on therapy)		—	+
005	15-year M	R iliac crest	—	<i>EWS-FLI1</i>	Gain of 5, 8, 1q	+ (Local, 9 months off therapy)	29	+	+
006	8-year M	Paraspinal	—	<i>EWS-FLI1</i>	Failed	—		—	+
009	15-year F	Sacrum	+ (R humerus)	<i>EWS-FLI1</i>	Gain of 2, 4, 12, 16, 20, 21	+ (Local, 7 months off therapy)	24	—	+
010	15-year M	R humerus	—	<i>EWS-FLI1</i>	Failed	—		+	+
012	17-year F	Cutaneous (R thigh)	—	<i>EWS-FLI1</i>	Not performed	—	16	—	+
038	9-year M	L ileum	—	<i>EWS-FLI1</i>	Clone 1: No gains or deletions	+ (Metastatic, 3 months off therapy, lung)		+	+
051	19-year M	R ulna	+ (Liver, spine, sacrum, ilium, femurs)	<i>EWS-FLI1</i>	Clone 2: Gain of 8	+ (Metastatic, 2 months off therapy, extensive disease in bones and brain)	—	+	—
061	6-year M	R scapula	—	<i>EWS-FLI1</i>	Clone 1: Gain of 2, 8, 20 Clone 2: Gain of 2, 5, 8, 12, 13, 20	—		+	—
066	14-year F	L scapula	—	<i>EWS-FLI1</i>	Failed	—	—	+	—

patients enrolled in Children's Oncology Group (COG) and Euro Ewing trials (GSE63157; ref. 31). GSE17679 was used as a discovery cohort in which we found that patients with low merged GEP EWS scores or high proliferation scores had significantly poorer survival (Fig. 2C–F). The other four merged GEPs did not correlate with overall survival (Supplementary Fig. S4A–S4D).

We further evaluated merged GEP EWS and Proliferation in the validation cohort, GSE63157. Similar to GSE17679, patients with low merged GEP EWS scores had significantly poorer survival. While high merged GEP Proliferation scores correlated with poorer survival in GSE17679, they did not reach statistical significance in GSE63157 (Fig. 2G and H).

Intratumor subclonal evolution in Ewing sarcoma

As mentioned previously, we identified that most patient tumors were found to have distinct large chromosomal gains and losses that largely agreed with clinical findings as well as the WGS. However, other than a report utilizing molecular inversion probes, there has been little published on CNA subclones within Ewing sarcoma (64). We leveraged scRNA-seq to analyze individual cells to identify and investigate Ewing sarcoma subclones, which were found in tumors from patients 005 and 061.

In patient 005, two subclones were found to diverge following an early chr8 gain (Fig. 3A and B). One subclone, termed Subcluster.1.1, emerged with gains in chr1 and chr5 and a loss of chr11p, whereas a smaller cluster emerged with loss of chr1p and chr16q and gains in chr2 and chr9, suggesting both chromosomal gains and losses contribute to tumor evolution. These two subclones demonstrate significant transcriptional heterogeneity (Fig. 3C). Similarly, three subclones were identified within the tumor from patient 061 (Fig. 3D). Early alterations were defined by loss of chr1 and gains of chr2, chr8, and chr20. Subclones were identified by the additional gain of chr5 and heterogeneous loss and gain of chr12 (Fig. 3E). A slight gain of chr12 was observed in the 061 WGS (Supplementary

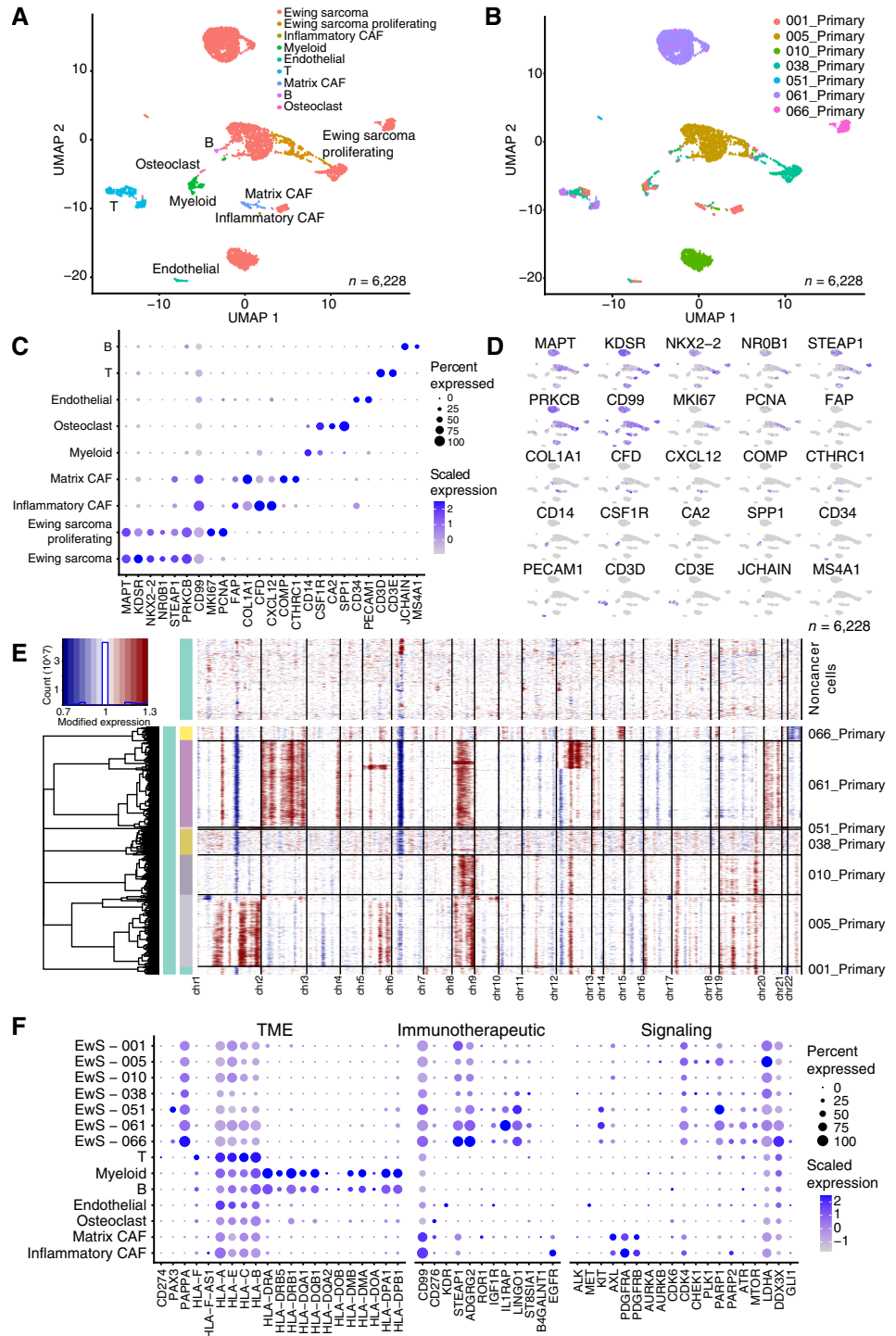
Fig. S1C), consistent with the two subclones identified in the scRNA-seq. Additionally, the clinical karyotyping performed on this tumor also identified a subclone with gains of chr5, chr12, and chr13, consistent with the aggregate of subclusters 1.2.1 and 1.2.2 (Table 1). Similar to patient 005, the patient 061 subclones also demonstrate transcriptional heterogeneity (Fig. 3F).

Immune infiltrates suggest an immunosuppressed TME

In Ewing sarcoma, the TME has been suggested to be proinflammatory with high chemokine expression (65). We investigated the composition and characteristics of the Ewing sarcoma TME, focusing on the relatively abundant T and myeloid cells (Fig. 4A–C). We were able to distinguish four T-cell subtypes within the TME, consisting of tissue resident memory, CD8⁺ effector, regulatory, and CD4⁺IL7R⁺ T cells (Fig. 4A; Supplementary Table S5; refs. 66, 67). While the majority of the T-cell subtypes did not express exhaustion markers such as *CTLA4*, *TIGIT*, *LAG3*, or *PDCD1* (gene encoding PD1; ref. 15), there are several factors suggesting an immunocompromised TME. The regulatory T cells expressed the inhibitory molecules *CTLA4* and *TIGIT*, which can contribute to inactive antigen-presenting cells (Fig. 4B; ref. 68). Ligands of CD161 (encoded by *KRB1*), *LLT1* (encoded by *CLEC2D*), and *CD69* (also known as *CLEC2C*) are expressed in the majority of the T-cell subtypes in the scRNA-seq data (Fig. 4B). Most studies in immune cells suggest that *LLT1*–*CD161* interactions lead to immunosuppressive environments, although the specific relationship in memory T cells remains to be seen (69). Additionally, *CD69* has been shown to inhibit the terminal differentiation of CD8⁺ T cells, which is consistent with the observed low *TOX* expression (Fig. 4B; ref. 70). *MIF* expression, along with the expression of the receptors *CD74* and *CXCR4*, was also identified in the Ewing sarcoma TME (Fig. 4B). While these interactions can lead to chemotaxis, *MIF* binding to *CD74* in T cells has been shown to reduce activation and proliferation (71–74).

Figure 1.

scRNA-seq of Ewing sarcoma (EWS) primary tumors identifies several cell types including cancer cells. UMAP plot labeled by cell type (A) or sample source (B). The expression of canonical marker genes across cell types is shown as a dot plot (C) or UMAP (D). E, Inferred CNAs in the Ewing sarcoma cells by sample, with all other cell types serving as control. F, Expression of select genes within Ewing sarcoma tumor cells from specific patients and aggregate cell types.



The myeloid population was characterized into noninflammatory CD1C⁺ dendritic cells, IL1B⁺ inflammatory monocytes, FCN1⁺ inflammatory monocytes, and CD14⁺ complement macrophages (Fig. 4D–F; Supplementary Table S6), some of which have been identified in the TME of Ewing sarcoma and other cancer types (15, 75–79). Interestingly, the CD14⁺ complement macrophages express *APOE*, *PLTP*, and *CD163*, which, along with *C1QC*,

indicate a strong anti-inflammatory phenotype. This could oppose the other tumor-infiltrating inflammatory myeloid cells (Fig. 4E). The myeloid cells described here express several markers that work in conjunction with T cells to contribute to an immunosuppressed environment. TIM3 (encoded by *HAVCR2*), which is moderately expressed in each subpopulation, has been shown to reduce the response to tumors (80). *CD80*, a ligand of

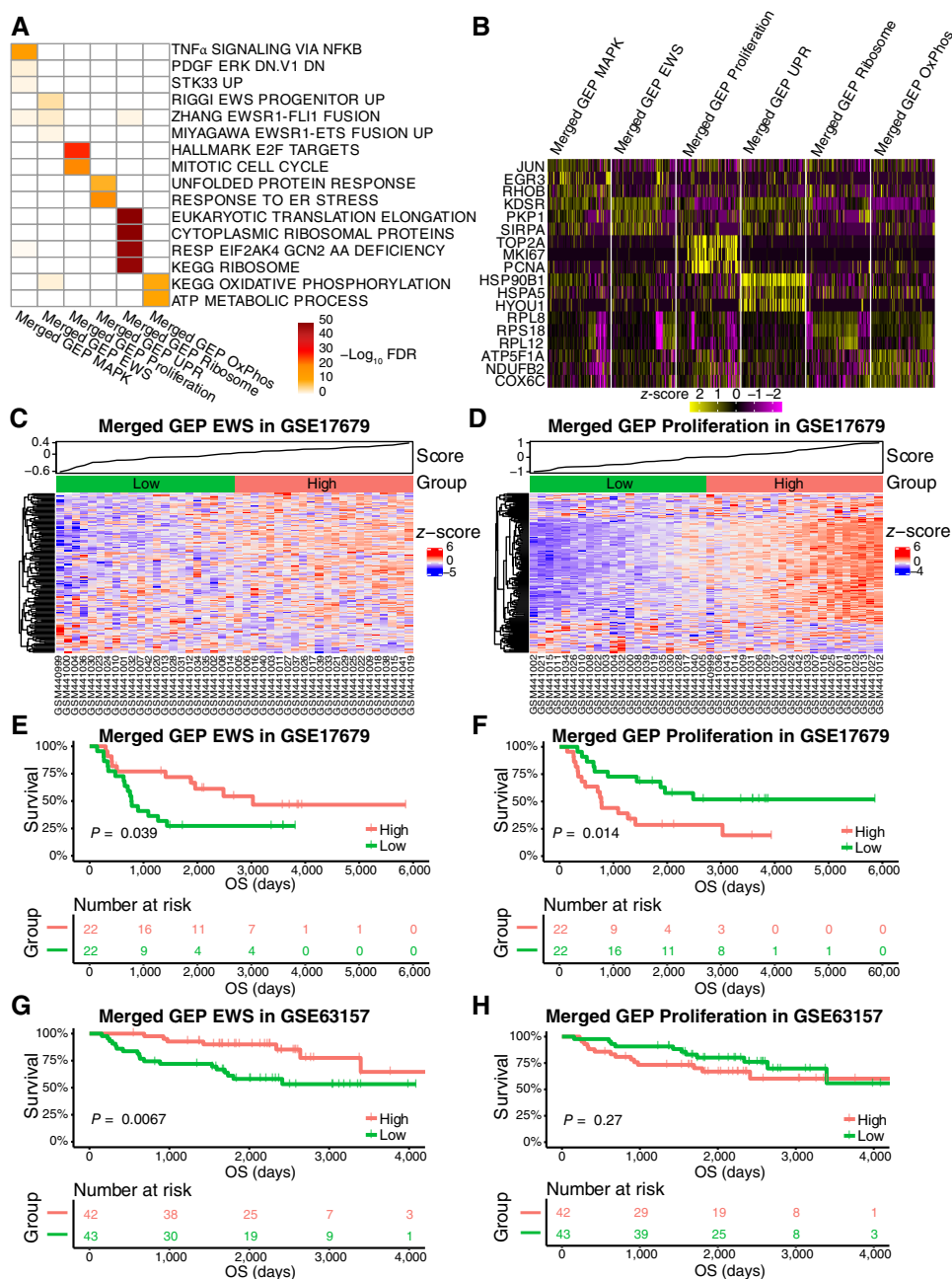


Figure 2.

Identification of GEPs in Ewing sarcoma (EWS) primary tumors and correlation to patient outcomes. **A**, Merged GEPs analyzed using over-representation analysis with the FDR of select gene sets displayed by the program. **B**, Heatmap of select genes with merged GEP groups downsampled to 100 cells, with yellow indicating higher expression. Heatmaps displaying the expression of the genes in the EWS (**C**) and Proliferation (**D**) merged GEPs in Ewing sarcoma tumors from GSE17679. Sample scores are plotted at the top with a median split used to classify samples as low (green) or high (red) for each program. Kaplan-Meier curves with a log-rank test comparing the overall survival between the low and high groups identified from EWS (**E**) or Proliferation (**F**) GEPs in GSE17679 and the validation Ewing sarcoma cohort, GSE63157 (**G** and **H**). OS, overall survival.

the T-cell stimulatory receptor *CD28*, is lowly expressed in the myeloid cells of our study, as well as in those from another Ewing sarcoma study, which suggests that dysfunctional myeloid cells may reduce the T-cell response (14). Finally, high *MIF* expression is similar to that in T cells that can reduce T-cell activation and proliferation (74). Overall, characterization of the myeloid and T cells in the Ewing sarcoma TME identifies several mechanisms of immunosuppression.

Diverse cell-intrinsic and cell-extrinsic interactions are possible in the Ewing sarcoma TME

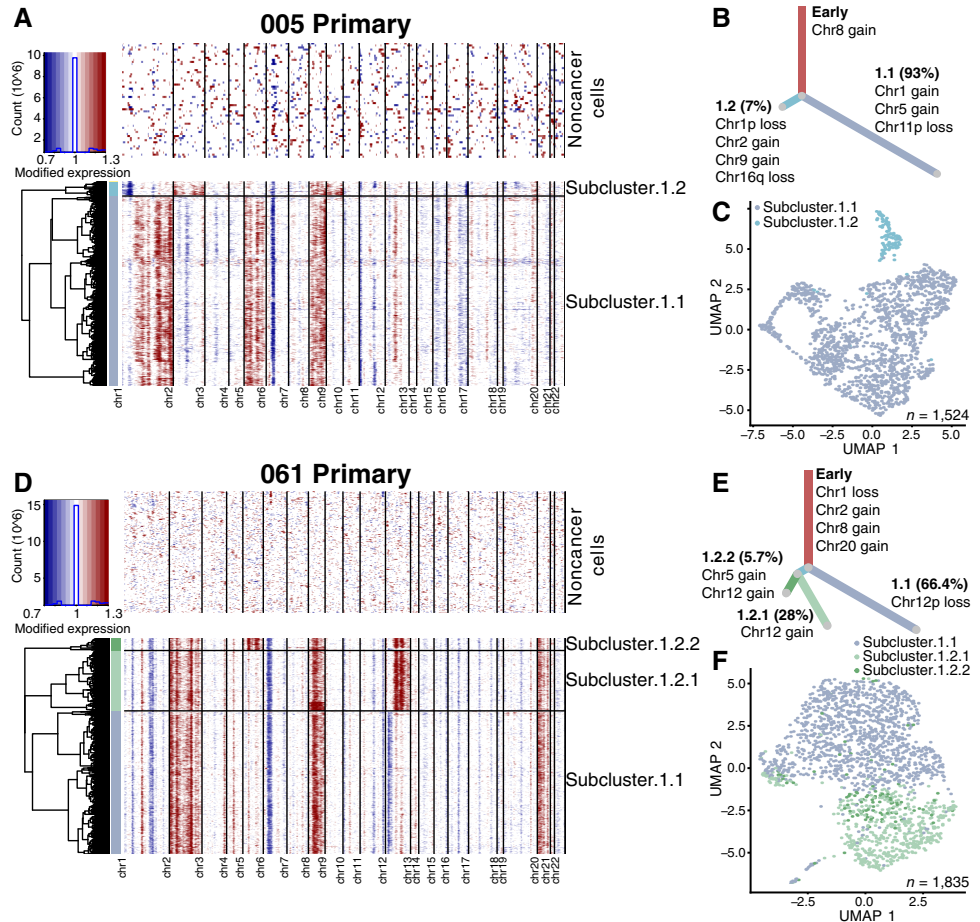
Following the determination of specific cell types in the TME, we next sought to identify potential cellular interactions using CellChat

(81). We identified multiple ligand-receptor interactions involving Ewing sarcoma tumor cells (Supplementary Fig. S5A and S5B; Supplementary Table S7). Of the tumor-intrinsic interactions, the most commonly occurring across patient tumors were WNT5A-FZD1, NPY-NPY1R/NPY5R, and MDK-NCL. WNT5A has been previously reported as highly expressed in metastatic tumors and as a potential driver of ROR1 signaling in Ewing sarcoma cells (48, 82–84). While midkine has been found to be part of the Ewing sarcoma secretome, the mechanism of action, and whether NCL (Nucleolin) is a signaling receptor for midkine in Ewing sarcoma, is unknown (85).

Additionally, there are several tumor-immune interactions possible in multiple tumor samples (Supplementary Fig. S5A and S5B).

Figure 3.

Subclones were identified in Ewing sarcoma primary tumors by the analysis of CNAs. Ewing sarcoma primary tumors 005 (**A–C**) and 061 (**D–F**) displayed subclones. **A** and **D**, Inferred CNAs in subclones of each sample. **B** and **E**, Phylogenetic trees inferring the evolution of the subclones based on the CNAs, with the percentage of cells in each arm indicated. **C** and **F**, Subclones displayed as UMAP plots.



The most common across samples were interactions between MIF, expressed in Ewing sarcoma tumor cells, and CD44, CD74, and CXCR4, expressed in several different infiltrating immune cells. As noted above, MIF was commonly expressed in myeloid and T cells but has not been reported in Ewing sarcoma cells despite being highly expressed in several cancer types (86–91). In mouse models of soft-tissue sarcoma and melanoma, MIF has been reported to interact with CD74-expressing myeloid cells in the TME, causing remodeling to a protumor TME (92, 93).

In terms of immune-intrinsic interactions, only three interactions were identified as probable in at least three patient tumor samples, all occurring within tissue-resident memory T cells (Supplementary Fig. S5C and S5D). As noted earlier, the interactions involving the ligands MIF, LIT1, and CD69 can promote a protumor and immunosuppressed TME (69, 70, 92, 93).

Ewing sarcoma CTCs identified in peripheral blood

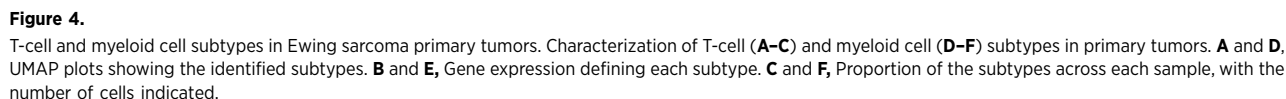
To further investigate mechanisms of metastatic progression, we next sought to identify and interrogate Ewing sarcoma CTCs using scRNA-seq. Eight patients had peripheral blood collected, including four patients whose primary tumors were also analyzed. After collection, blood samples were enriched for CTCs using CellSieve microfiltration membranes as previously described (Supplementary Fig. S6A; ref. 94). Following similar filtering to the primary tumors, 7,139 cells were identified in the blood samples, with a median of

1,055 UMIs per cell and a median of 489 genes per cell (Supplementary Table S1). Unbiased clustering revealed 10 distinct cell types, which were identified by the expression of canonical cell type markers (Fig. 5A–C; Supplementary Fig. S6B; Supplementary Table S8).

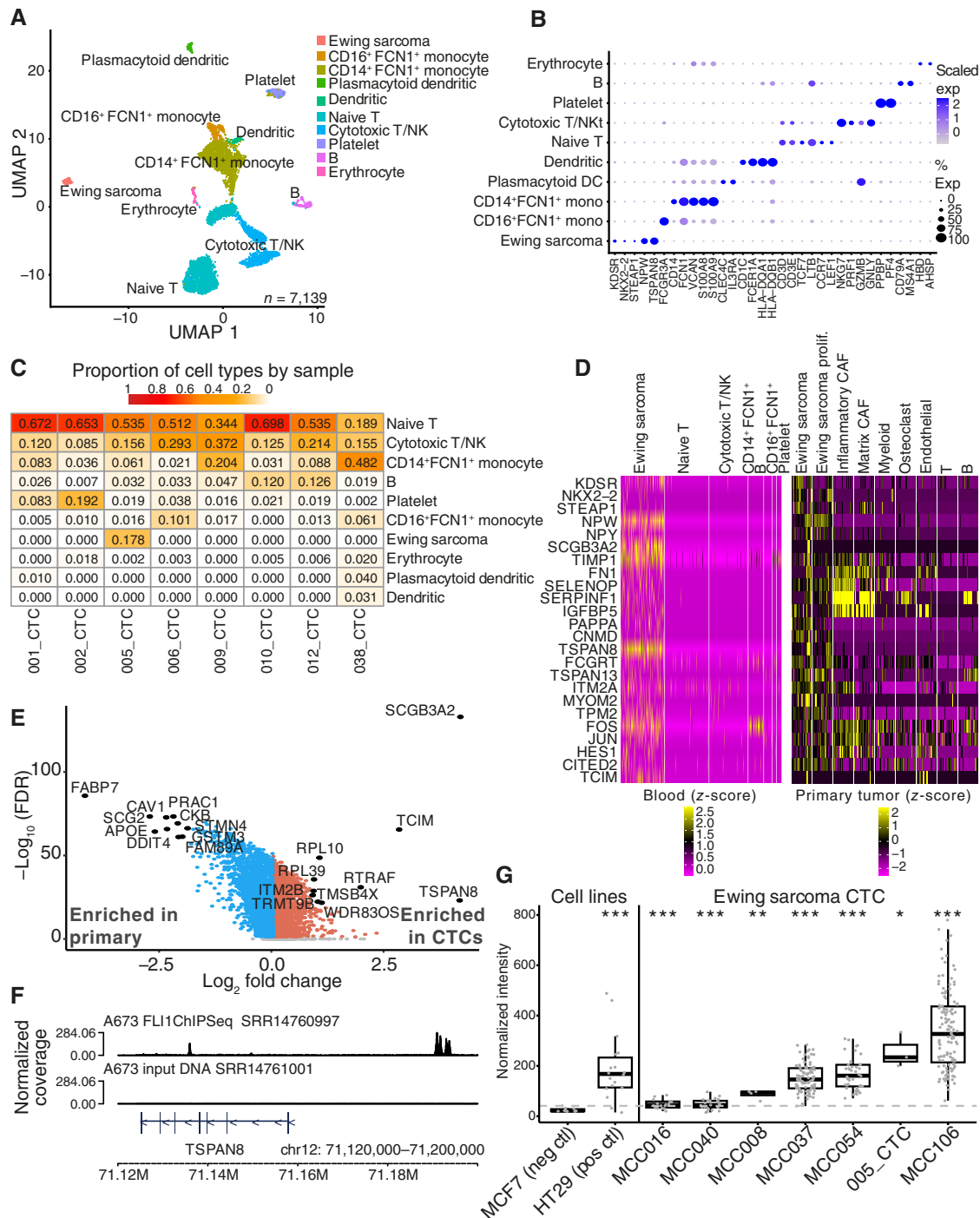
A cluster of cells were identified as Ewing sarcoma CTCs in the blood of patient 005 through several mechanisms. These cells expressed higher levels of canonical genes *KDSR*, *NKX2-2*, *STEAP1*, and *NPW* (Fig. 5B). The primary Ewing sarcoma and CTCs from patient 005 expressed similar genes more highly compared with other cell types (Fig. 5D; ref. 34). Finally, the Ewing sarcoma CTCs were also enriched in Ewing sarcoma-associated gene sets when compared with other blood cells (Supplementary Fig. S6C).

Since we had identified subclones within the primary tumor from patient 005 (Fig. 3A), we next assessed whether the CTCs represented either of those subclones. We were able to identify what appeared to be gains in chr8, chr9, and chr12, suggesting that the CTCs potentially represent both subclones found in the primary tumor, but the technical quality of the CTCs was not as high as in the primary, preventing a definitive conclusion (Supplementary Figs. S1E and S6D).

When comparing patient 005 primary Ewing sarcoma cells to CTCs, we found the primary cells to be more enriched with the merged GEP EWS program, which would be consistent with a previous finding in cell lines that a reduction of Ewing sarcoma activity enhances metastatic potential (Supplementary Fig. S6E; ref. 95). Interestingly, one of the most highly expressed genes in the CTCs compared with primary tumor cells



Much of what is known about the Ewing sarcoma TME is that it is often highly immunosuppressive, and this study corroborates many of those findings. We found tumor cells to express only low levels of HLA genes, consistent with previous work showing a lack of HLA expression by Ewing sarcoma cells, potentially leading to decreased T-cell recognition (16). We also found evidence that the myeloid cells of the Ewing sarcoma TME may limit T-cell activation through the expression of

**Figure 5.**

Circulating Ewing sarcoma cells identified in the blood of patient 005. Cell types identified in the blood samples displayed as a UMAP (A) and discriminating cell markers as a dot plot (B). C, Proportion of cell types in each sample. D, Gene expression of Ewing sarcoma-enriched genes displayed as a heatmap in blood (left) and primary tumor cell types from patient 005. E, Volcano plot highlighting genes significantly enriched in circulating compared to primary tumor cells within 005. F, ChIP-seq binding profile of FLI1 or input DNA near *TSPAN8* in the A673 cell line. G, *TSPAN8* staining in Ewing sarcoma cell lines and CTCs. The 005_CTC sample is the same source as the 005_CTC scRNA-seq sample. *P* values are from *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

TIM3 and the low expression of *CD80* (14, 80). The T-cell subtypes themselves also seem to support immunosuppression through potential LLT1-CD161, CD69-CD161, and MIF-CD74/CXCR4 interactions, all

of which have previous evidence of supporting protumor environments (69, 70, 74). We identified inflammatory CAFs, which can secrete CXCL12 to recruit regulatory T cells (105). Taken in

aggregate, our scRNA-seq analysis of the TME largely agrees with previous studies that the Ewing sarcoma TME is immunosuppressed, representing an area of need for future clinical development.

Detecting and analyzing circulating Ewing sarcoma cells has been challenging because of the low expression of epithelial markers used in typical carcinoma CTC detection (106–113). We have previously reported that CellSieve can detect CTCs in many patients with high-grade sarcoma, which also includes patients with Ewing sarcoma (94, 114). In this study, we identified a population of circulating cells that expressed many of the Ewing sarcoma markers from the primary cells as well as consistent gene set enrichment. Although not all canonical markers and CNAs were identified, this was attributed to poorer sequencing depth in these samples, and we labeled this population as Ewing sarcoma CTCs. We were able to molecularly characterize the transcriptome of a population of Ewing sarcoma CTCs for the first time. We identified that these CTCs in our study have less enrichment of GEP EWS compared with matched primary cells, supporting the finding in cell lines and xenografts that low EWS-FLI1 activity leads to more metastasis (95). Finally, we found that TSPAN8, a transmembrane protein that has been investigated as a potential cell surface target in other cancer types (115, 116), was highly enriched in the CTCs. Consistent with previous reports that investigated the surfaceome of Ewing sarcoma (60), we also found *TSPAN8* expression to not be restricted to the CTCs but also highly and broadly expressed in primary Ewing sarcoma tumors. Future studies should explore TSPAN8 as an antibody- or CAR T cell-based therapeutic target, as well as investigate the role of *TSPAN8* expression in tumor metastasis.

Authors' Disclosures

A.B. Duffy reports other support from Creatv MicroTech during the conduct of the study as well as other support from Creatv MicroTech outside the submitted work. C. Cost reports other support from Johnson and Johnson outside the submitted work. D.L. Adams reports other support from Creatv MicroTech during the

conduct of the study as well as other support from Creatv MicroTech outside the submitted work, and has a patent for Provisional Filed pending to Creatv MicroTech. No disclosures were reported by the other authors.

Authors' Contributions

A. Goodspeed: Data curation, software, formal analysis, investigation, visualization, methodology, writing—original draft, writing—review and editing. **A. Bodlak:** Investigation, methodology, writing—original draft. **A.B. Duffy:** Data curation, investigation, methodology. **S. Nelson-Taylor:** Investigation, methodology. **N. Oike:** Investigation, methodology. **T. Porfilio:** Investigation, methodology. **R. Shirai:** Investigation, methodology. **D. Walker:** Investigation, methodology. **A. Treece:** Resources. **J. Black:** Resources. **N. Donaldson:** Resources. **C. Cost:** Resources. **T. Garrington:** Resources. **B. Greffe:** Resources. **S. Luna-Fineman:** Resources. **J. Demedis:** Resources. **J. Lake:** Resources, investigation, methodology. **E.P. Danis:** Data curation, software, investigation, visualization, methodology. **M. Verneris:** Investigation. **D.L. Adams:** Data curation, investigation, visualization, methodology, writing—original draft. **M. Hayashi:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing.

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Note

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