nature portfolio

Peer Review File

Small round cell sarcoma tumoroid biobank reveals CIC::DUX4 sarcoma vulnerability to MCL-1 inhibition

Corresponding Author: Professor Hans Clevers

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This study by Ringnalda et al describes the generation of a biobank of small round cell sarcomas established from patient samples or PDXs. The long-term passaged tumoroids they report capture features of the tumors they are derived from and could be used for drug screening.
This resource holds potential for downstream studies and, if sharable, could positively impact the rare cancer community. The manuscript could benefit from some clarifications, changes in visualizations, inclusion of critical quantitative analyses, and aligning terminology and references with current standards in the field. I commend the authors for addressing the challenge of cardiotoxicity associated with MCL1 inhibitors in the discussion.

Major points:

The appropriate terminology to use is not “Ewing-like tumors” but rather “undifferentiated small round cell sarcomas” or, possibly, “small round cell sarcomas” of which Ewing is one entity, and to which other fusion-driven, round cell tumors belong to. Please refer to and cite Dehner et al, Human Pathology 2024. Citing from the NCCN Bone Cancer Evidence Blocks 1.2025: “Other primary round cell sarcoma of bone was previously referred to as Ewing-like sarcoma, which is terminology that is no longer included in the WHO classification.” As such, the title and text throughout should replace the outdated terminology and reflect the standard of the field.

Line 33-34: “Currently, there is a lack of preclinical cell models that can predict drug response of these entities in vitro.” This is not accurate, there are a number of reported models, but most relevant to this study, patient-derived tumor organoid models of Ewing, CIC-rearranged sarcoma, DSRCTs etc have been recently reported in Al Shihabi et al, Cell Stem Cell 2024. Using similar molecular approaches, those authors have demonstrated fidelity to tumor of origin as well as performed high-throughput scale drug screens, including of longitudinal samples. This study should be mentioned appropriately.

Line 100-106: This result section should clearly separate PDOs from PDXOs, as these are anticipated to have different success rates etc. How many samples are derived PDOs vs PDXOs, and whether this is reflected in any difference in take rates should be reported up front.
Overall, it is unclear how many individual patients were included in this study – Supplementary table 1 seems to list 17 individual IDs. How many patients, how many samples per patient and how many PDXs were included should be clearly reported in the results and tables. Are the 36 reported overall samples? Patients? Patients+PDXs?

While the result section mentions that “Fresh PDX tumor specimens were
obtained from the Innovative Therapies for Children with Cancer (ITCCP4) consortium (Supplementary Table 1).”, there is no mention of ITCCP4 in Supplementary Table 1. Nor is obvious which samples are from patient tumors vs PDXs from the table.

Line 112-113: “The derivation efficiency was higher for treatment naïve (11 out of 11, 100%) than for relapse and chemotherapy-treated samples (16 out of113 20, 80%).” What is the breakdown by tumor type? Should be listed whether the failed samples belong to a specific histology.

Line 122: “Amongst the ES tumoroids,EWSR1::ERG and EWSR1::FEV tumoroids contained more dark cells and displayed

a less uniform shape, compared to the EWSR1::FLI1 tumoroids (Fig. 1d)." Firstly, there is no Figure 1D. Even if looking at Figure 1C, this is not obvious from the images, the author should provide a quantification. What are these "dark cells"? Similarly, the description of BCOR organoids is qualitative – unclear how the authors determined that there were "less dense pockets in the center of the tumoroid"? Was this by H&E of paraffin-embedded organoids?

While the quality of the images in Figure 2C makes it hard to fully interpret, there seems to be fairly large differences in staining between tumor vs tumoroid for ES-093 and 96 and 101- How are these explained? There are similar issues of possible discordances (pending higher quality images / color correction) with Supplementary Figure 2 for cases 10 and 77.

Figure 3B should include side-by-side comparisons of Circos plots of pairs of tumors vs tumoroids.

Similarity of pairs (tumoroid vs tumor) by WGS and RNA-seq needs to be globally quantified. Pearson correlation coefficients need to be plotted or reported in table format. This will allow a reader to clearly and unambiguously compare the primary samples and derivative tumoroids.

Line 272: "ETV5, a known target of Trametinib". Trametinib is a selective inhibitor of MEK, key components of the MAPK/ERK signaling pathway. While ETV5 is a downstream target within this pathway, Trametinib does not directly target ETV5. ETV5 modulates sensitivity to trametinib but is not a known target, which should be amended in the text.

Line 303: ". Tumoroids were established from both primary and metastatic disease timepoints." For all 14 patients?

While the authors state in line 287 that "These pathways could be used for specific
treatment of these tumors" including MAPK in CIC-rearranged sarcomas, they then report at line 335 that "We also found that CIC::DUX4 sarcomas should have high MAPK pathway activity (Supplementary Fig. 4a), but we did not notice an increased sensitivity of this group to compounds in CIC::DUX4 tumoroids compared to ES tumoroids". This is frankly not completely unexpected due to lack of clinically validated biomarkers for many of these agents – yet, the claim pathways can be used for treatment should be dimmed.

Line 381: "This is the first study that describes the establishment of a broad panel of ES and ELS patient-derived tumoroids, including those with rare fusions such as EWSR1::FEV, EWSR1::ERG and CIC::DUX4, which have, to our knowledge, not been described previously." As discussed above, other large studies have reported of patient-derived tumor organoids from EwS and other small blue round tumors harboring rare fusions. This study reports on the generation of established, long-term passaged organoid models so after contextualizing, this aspect should be highlighted instead.

Line 409: "Vice versa, some tumoroids carried additional somatic variants
compared to the matching tumor, e.g., in sample ES-041 (TP53 mutant) and ES-057." This finding does not seem to appear in the result section? Emergence of de novo TP53 mutations during long term passaging has been reported during establishment, long term passaging and immortalization of other cell lines and models, and should be mentioned as a possibility.

Minor points:

Line 165: "expect" should be "except"

Figure 3A: I suggest that the authors consider diversifying the panel of colors used so that different categories are more obviously separated. It appears that PDXs for which no organoid is derived (or sequenced?) are included in Figure 3. It may be clearer to limit to pairwise comparisons here.

Figure 3 uses both the conventional organoid nomenclature (3A) as well as tumoroid (3B). It also includes "-T" and "-O" and "-Tum" and "-Org" in 3A/D and 3E respectively. For clarity to the reader, the names used should be consistent. If they elect to use "tumoroid", the -T (=tumor) will be easily confused for -T (=tumoroid), so would require a different naming strategy.

The driver translocation should be labeled in Figure 3B.

Figure 4 B and C also should include different colors for tumoroid / PDX / tumor as they are very similar to the heatmap gradation. I recommend removing the grey boxes outlines for readability.

Reviewer #2

(Remarks to the Author)
In this manuscript, Ringnalda et al. present the development of tumoroids for Ewing sarcomas and for CIC- and BCOR-rearranged sarcomas, which they used to screen a small panel of drugs. They then identify MCL inhibitors to be nicely specific for CIC-rearranged sarcomas. Patient affected by a small blue round cell sarcoma (encompassing the tumor types above) are indeed in need of specific treatments. As these tumors are rare (even ultra-rare for some of the subtypes) it is quite difficult to establish cell lines to test hypothesis. The work presented here is therefore of a tremendous importance for the researchers in the field as the authors present methods and culture conditions to establish, quite efficiently, organoid models that they show to be quite representative of the original tumor. This work merit to be published, but not in the actual

version of the manuscript. I have indeed comments that need to be address before publication.

Major comments:
The term of Ewing-like sarcomas (ELS) is not used anymore, not recognized in the latest WHO classification and not suitable. This “old” entity is in fact several small, different, tumor entities. In the introduction and discussions, there are therefore some generalizations that are not necessarily true. For example, line 81 (page 2) indicates that ELS “show little to no response to chemotherapy”. While this is true for CIC-rearranged sarcomas, it is not for BCOR-rearranged sarcomas for which the patients are better responding than Ewing sarcoma patients. Similarly, the first sentence of the discussion (line 365 page 9) “ES and ELS are highly aggressive tumors that predominantly affect children and young adults“ may be true for BCOR- and CIC-rearranged sarcomas, but not for EWSR1::PATZ1 or EWSR1::NFATc2 sarcomas that fall also under this ELS type. The term of ELS should then be removed from the whole text and replaced by CIC- or BCOR-rearranged sarcomas.
It is quite difficult to know how many samples from how many different patients are included in this study. From supp table 1 it seems that there are 33 samples coming from 17 patients, right? Page 3 Line 111: 36 cases are indicated amongst which 32 tumoroids where established ??? In supp table 3 in Samples ID and M-Labels are not corresponding to the supp table 1. Maybe there is some mix up as well in supp table 2, the relapse of M369AAA was not cultured in the same medium than the primary(STS2 and STS1)? There are 2 ES-016 samples, one from the primary, one from a metastatic lesion, which is very confusing (correct “leasion” in “lesion” in Supp table 1). There is an ES-020T in fig 4 heatmaps that is never anywhere else…. A clear, single, supplementary table describing the clinical data of the patients, the different samples used from each patient’s tumor and then the organoid/pdx attempts with the derived ones together with the culture medium used, should be provided. By the way, I am surprised to see in supp table 1 a sample (ES-094) that have both an EWSR1::ERG and a FUS::FEV. To my knowledge there is no case in the literature of an ES with 2 fusions. Is it an error? Did the author validate this?
I have some difficulties with the statistics through the paper, either with the WGS SNV/CNA or with the expression profiles when the authors are comparing subtypes of tumors against each other’s. Indeed, the statistics may be completely skewed by the number of initial tumors. The authors are actually comparing several samples of the same original tumors and they end up analysis 2 BCOR::CCNB3 or 3 CIC::DUX4 against 12 Ewing sarcomas…. I think that the goal of this paper is to show that the derived organoids are similar to the tumors and could be used as surrogates for treatment, which is really great. But everything related to the comparison of the subtypes between them should really be moderated as the confirmation of what is already known (the author do not actually discover new things amongst the different tumors types) is true for the organoids as well.
Resolution of the Fig2 and supp Fig2 should be drastically increased to be able to see anything. WT1 seems to be expressed everywhere except in the ES-096 samples… For the BCOR-rearranged sarcomas and tumoroids, a BCOR staining should be shown.
The demonstration that the tumoroids are resembling the tumors is weak and should be reinforced. A figure showing the CNV / CNA / Fusion similarities/differences between tumors and their derived tumoroids should be provided, as a supplementary figure for each available O/T couples and for a few examples in a main figure.
In Fig 3 while there is consistency in many O/T couples, one can see also strong differences in several as well (ES-055,ES-046,ES-071,ES-090…). It therefore seems that O/T couples are not that obviously similar!
I do not get the scale of the Fig3c and Fig3d. What do you call “cellular prevalence”? Shouldn’t it represent the fraction of the clones you identified over the whole population? If you sum the prevalence of Fig3d shouldn’t you get the same sum than fig 3c?
How were ordered the heatmaps of fig 4? Could you please show the dendrograms (for the columns)?
Lines 268-271: “While most identified genes were known targets of the different tumors, like NKX2.2 and ETS1 for EWSR1::FLI1 and CIC::DUX4, respectively, we also identified new targets. For example, MAPT…. ETV5…”: Well, see Tim Triche paper, back in 2005 for the 1st one (10.1146/annurev-pathol-011110-130237) and many for the 2nd, including ref 6. As stated above, this entire paragraph (Identification of differentially expressed genes) should be either removed or written differently, as it is not in the scope of the paper to find DEG or pathways between CIC or BCOR tumors, but to demonstrate that the same DEG or pathways are modulated in the organoids.
Looking at the status of TP53 while screening for drugs is really important. It seems that both the EWSR1::ERG and EWSR1::FEV sample are TP53 mutated. Could this explain why they behave differently than EWSR1::FLI1? When testing the MTOR inhibitors could you stratify the ES, not by the fusion but by TP53 status? In a general manner, it is well acknowledged now by the community that Ewing sarcoma are defined by the presence of an EWSR1/FUS::(PEA3 ETS family members) fusion and that the fusions behave strictly similarly. What is important though, are secondary events.
The finding that MCL1 inhibitors are effective in CIC-rearranged sarcomas is really great!

Minor comments.
Resolutions of the figures are really poor (probably due to the pdf conversion) and the police size too small, barely readable (see the boxplots of Fig 5 as an example)
Line 124 (page 3) Fig.1d should be 1c
Line 147 to 150 (page 4): as there is no data shown for the PCR, it should not be in the result section but in the material and methods.
Line 211 (page5) Fig.3d should be 3e
Line 228 (page 5) Supp fig 3b does not show fusions and there is no supplemental figure showing the fusions
Color code of Fig 4B is poor, it is difficult to see which is tumor or tumoroid. Also please choose a better color scaling for the expression values (same remark apply for panel C and D) such as in fig 5?
Line 773 (page 17) . The aligner is STAR, and the tool for fusion detection is STAR-fusion. Line 775 it is indicated that “Fusion were manually assessed”, what exactly is

Reviewer #3

(Remarks to the Author)
The manuscript by Ringnalda and colleagues explores the use of tumoroids as to perform functional assays and evaluate potential treatments for ES and ELS. While the manuscript is well written and technically well described, we include a few comments to improve the manuscript

Major comments:

- There is no validation for the drug screening predictions made with the tumoroids. This should be tested and included in the manuscript.
- There is no indication of the time points used for the WGS analyses. Did the authors check if tumoroid passages affects WGS data?
-The authors identified new pathways that could be targeted therapeutically in ES and ELS. However, they did not validate these observations by Western Blot analyses and they did not test specific inhibitors in these tumoroids. For example, no MAPK inhibitors such as trametinib were tested in CIC:DUX4 models.

Minor comments:

- In the line 334 the authors mention an increase in mRNA of MCL1 and BCL2 and they claim that this is the reason why there is a higher effect of S63845 and AZD5991. The authors should check the protein levels of MCL1 and NOXA.
- To our knowledge these BH3 mimetics do not inhibit BCL2 (line 334).
- In order to prove more detailed information on the activity of MCL1 inhibitors some cell death assays should be performed as CellTiter-Glo measures ATP production (basically measures the cell number) but not cell death specifically.We suggest Annexin / PI cell death analyses.
- Line 421: MCL1 instead of MCL.
- BH3 mimetics such as S63845 act fast. Did the authors tested these agents at shorter timepoints?

Reviewer #4

(Remarks to the Author)
General comments: This is a well-written, scientifically rich manuscript highlighting the usefulness of tumor organoids (i.e., tumoroids) in assessing ex vivo drug sensitivity for ES and other small round blue cell malignancies. The authors demonstrate that drug sensitivity differs by sarcoma subtype and identify MCL-1 inhibitors as a potential candidate for CIC::DUX4 sarcomas. I enumerate below constructive comments that may further enhance their work:

1. Though used historically in the literature when ES couldn't be readily distinguished from other small round blue cell sarcomas, there's a growing consensus that "Ewing-like-sarcoma" or "Ewing family of tumors" terminology shouldn't continue—see WHO 5th edition. While BCOR::CCNB3 (BCS) and CIC::DUX4 (CDS) display some morphological similarity to ES, the similarities stop there. The latter sarcomas have entirely different translocations that function differently than EWS::FLI1, vastly different survival rates, and as the authors suggest, unique drug sensitivities in their tumoroid models. I'd recommend using small round blue cell sarcomas (SRBCS), or since their work doesn't include DSRCT or RMS, simply BCS and CDS.
2. Lines 37-39: The statement that tumoroids retain key properties matching patient tumors isn't entirely true since the PDXs and tumoroids separated from human tumors in Figure 4, Panel A. Also, it's nebulous what key properties the authors are referring to.
3. There also seem to be significant differences in the IHC images between tissue and tumoroids in Figure 2, panels B & C. The tissue images for ES-053-BCS are missing in panels B & C. The authors might choose to quantify the expression of CD99 and WT1, respectively, using any number of spatial imaging-based software (e.g., Visiopharm, Halo, etc.), allowing the readers to evaluate the degree of similarity objectively.
4. Line 65: ETS binds "GGAA" microsatellites, not "CCAA" repeats.
5. Lines 74-75. The authors make my point that ES, CDS, and BCS are pretty different and were mainly clustered together with ES by mistake when pathologists only had immunohistochemistry to guide them. The authors might highlight here that CDS and BCS were often inadvertently treated on ES trials when they were considered atypical EWS::FLI1 negative ES.
6. It is interesting that there is a Sarcoma Culture Medium. Could the authors clarify its usage and how it was developed? Is it only for tumoroids or was it also used to establish cell lines?
7. Is there a rationale for why treatment-naïve would establish better than chemotherapy-treated samples?
8. Lines 84-94: The authors might wish to provide a few reasons why cell lines poorly predict clinical drug response, better making the case where tumoroids offer an improvement. For example, many have shown that cells cultured as monolayers with non-physiological stiffness lose fidelity to human tumors. Further, unlike tumoroids, cell lines are removed from heterotypic cells and devoid of native ECM. Finally, the mutation rate in cell lines, often propagated for decades in the lab, are substantially higher than their human tumor counterparts.
9. Are there cells from the tumor microenvironment present in the tumoroids? If they exist, are they gone by passage 20? I assume so. Does this affect the drug efficacy across the passages?
10. It is interesting that the different translocation-positive sarcomas demonstrate different tumoroid phenotype. Do the CIC::DUX4 cell profile have more cell-cell junctions than ES tumoroids?
11. Line 123: It's unclear what the authors mean by "dark cells," which seems like non-standard terminology.
12. Given the widespread availability of proteomic technologies (e.g., cyclic IHC, RPPA, etc.), it would have been nice to

better characterize protein expression along several key cell signaling cascades matching the pathways studied for tumoroid drug sensitivity (e.g., mTOR inhibitors, MCL1).
13. Line 328: While the authors mention that mTOR inhibitors affect the ES fusion protein (FP) expression, they may also wish to reference literature demonstrating that ES FP activates the IGF/PI3K/mTOR cascade via upregulation of IGF-1 and down-regulation of IGF-BP3, that together activate the upper portion of the pathway.
14. Lines 382-384: Sarcoma tumoroids have been established in large-scale by another group at UCLA. The authors should discuss this work in the context of their own manuscript. What are the major differences in the establishment of the tumoroids? Are there differences in subtypes studied in either paper?
15. Al Shihabi, Ahmad, et al. "The landscape of drug sensitivity and resistance in sarcoma." Cell stem cell (2024).
16. Is there a difference in response to drugs developed from tumoroids of different stages of diagnoses? Or even treatment-naïve vs chemotherapy-treated?
17. Are there differences in sensitivity to MCL1 inhibitors for 2D cell lines vs tumoroids in CIC::DUX4? Are the tumoroids easier to establish than the cell lines?
18. Lines 387-388: While potentially valid, it's an overstatement to suggest that tumoroid models are representative preclinical models, at least for drug sensitivity. To make this claim, the authors should have demonstrated concordance in drug sensitivity between tumoroids and PDXs or, preferably, patients treated with some of the same drugs.
19. Supplemental Fig 1: It's unclear if each dot represents a tumoroid on the plate or different plates of tumoroids. It might be helpful to include a similar graph grouping growth rates by sarcoma subtype as panel B.
20. Supp Fig 5: It might be helpful to color code the Y-axis by general drug mechanism of action. I don't see classic PI3K-Akt inhibitors listed. Also interesting is that Regorafenib showed no effectiveness in ES, which seems to go against the ES arm of the REGO-Bone study.
21. Another major discussion point is the drug response or utility of PDX, 2D cultures, and the patient derived tumoroids. PDX are still the gold-standard preclinical data used to support new clinical trials but the authors have not discussed the advantages of the tumoroids compared to PDX.
22. Is there a protocol for cryopreservation of the tumoroids? Are re-established tumoroids affected by the cryopreservation process (growth rate, mutational burden, drug response)?
23. Please include data tables associated with the heatmaps. It is unclear which drugs are where in the heatmaps. Some are called out like MCL1 inhibitors or mTOR inhibitors, but others are not in figure 5.

Minor Points:
1. Figure 1: The font on panel B is unreadable, and there's room to increase it without affecting the overall figure size. The axis in panel B has no label. Does this represent days or months?
2. Figure 2: Images missing for ES-053. Also, the naming (e.g., ES-053) implies ES. Instead, I strongly suggest the authors relabel the samples by CDS or BCS instead of ES whenever appropriate for all figures.
3. Figure 4. The color difference of Type in Panel B is barely detectable. Suggest using a different color pattern to make it easier for the readers.
4. Figure 4 font and symbols are too small to see. Panel A symbols are difficult to distinguish at the current size without zooming in. It would be nice to connect figure 4D to 5B. Otherwise, it is difficult to understand if these drugs are having on-target effect or have any efficacy due to the expression of these drug targets.
5. Fonts in Figure 5 are too small to read. Panel H it may be unnecessary to call out the individual tumoroids and keep it only as the translocation type. A larger figure can be included in the supplementary with the individual sample types called out.
6. Figure 5. As above, suggest relabeling ES-086, ES-087, and other samples that harbor a CIC::DUX4 rearrangement as CDS-086, CDS-087, etc. Take the same approach for BCOR-rearranged tumors, using "BCS" or "BCK" where appropriate. If the authors push back against sample renaming, at the very least, they should consistently name the samples as "ES-" vs. "ELS-". More precise labeling will make it easier for readers and will be important when depositing data in GEO.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #6

(Remarks to the Author)
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Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I thank the authors for addressing most of my comments. I have the following clarification, as one of the studies referenced in

this new version has been incorrectly addressed:

“Although several short-term pre-clinical models of ES have been established, such as cell lines and patient-derived tumor xenografts (PDX)16,17. Using cell lines has several disadvantges, including a recent study has described a screening platform for SRCS using short-term in vitro cultures16.”

This is incorrect. The study from Al Shihabi et al (now ref 16) is establishing patient-derived tumor organoids from clinical samples directly – no cell lines or xenografts. I would ask that the authors correctly reference the paper, instead of mentioning it is using cell lines which is misrepresenting the work entirely.

Reviewer #2

(Remarks to the Author)
Thanks to the authors for answering most of the reviewers’ comments and modifying the manuscript accordingly. While the authors answered most of my comments, I still have minor comments; in order of the manuscript:

It is still unclear which samples was processed by which techniques (WGS,WGS,RNAseq….). Again, a single spreadsheet with all the information would be much clearer, like:
Patient ID Tumor Sample Tumoroid
ID tech ID Tech
M1 ES-001TT WGS,WXS,RNA ES-001TO RNA
M1 ES-001TT WGS,WXS,RNA ES-002TO WGS
M2 … … .. ..

Line 87 : CCNB3 instead of CNNB3
Line 192-4 : ”We created mutation matrices and used these to calculate Jaccard’s distance between the samples. This revealed that the tumoroids genetically resemble their tumor counterparts (Fig. 3b, Supplementary Fig. 3b-c)”. Fig 3b shows circos plots for a single Tumor-tumoroid couple and with the exception of the CNV, it clearly shows strong differences in SNVs and translocations that do not support the “resemblance”. I acknowledge the efforts made by the authors to present the resemblance between tumoroïds and their respective tumor sample using Jaccard’s distance and discussing some observed dissimilarities but I would have also liked the authors to comment on some couples that apparently seems not similar (high Jaccard distance as seen in the heatmap) (TT and TO of BRS-057, ES-041 or ES-071 for example).
Supp Fig3b: I guess that the circos of the BRS-057TO is “corrupted” because it loses all the translocations, including the oncogenic one…
Line 195-6: “Also, several SRCS tumoroids showed CNAs such as gain of 1q, 12 and 8, and loss of 9 (Supplementary Figure 3a-b)”. I do not see any samples in the figures with a loss of chr9.
Line 219: “As shown in Fig.3b,” should probably be Fig 3c and supp Fig 3a.
Line 258: Supp Fig 3e is referred to for the first time after supp fig 3f, please reorder
Fig 4c: What is the color code (Expression, ChIP peak heights)?
Lines 390 and 400: Fig 5c should rather be Supp Fig 5d or Fig 5H, no?
Supp Fig5 d&e: names of samples were not corrected (all ES-XXX)
Figure 5a: Color codes for fusions in heatmaps do not correspond between the legend and the heatmap itself (pink and orange were inverted). It would be easier for the reader that the color codes stay similars between all the figures (including the supplementary figures): Ex: BCOR::CCNB3 is red in Fig4a, black in Fig 4b&c, purple in Supp Fig 4b, blue in Supp Fig 5c….
The organization of the figures corresponding to the drug screening (Fig 5 and Supp Fig 5) is confusing as supp fig 5c is (wrongly) referred to throughout this section and that Fig 5H is identical to supp fig 5E (which is never referenced in the main text)
Supp Fig6a: Could the authors also present the expression value of MCL1 and NOXA for the tumor tissue samples?
For the final experiment (looking for apoptosis, Supp Fig 7) FACS profiles (which could be quantified) should be presented instead of cell imaging. Moreover, could the authors comment on why the ES-046 cells seem to grow as adherent cells and not as tumoroids?
In the last sentences of the conclusion (lines 522-23) the authors may comment on the combination of MCL1 inhibitors with other drugs that has been investigated in Ewing sarcomas (doi 10.1016/j.canlet.2022.216028).
Lines 1002 & 1145: There is no “s” in “Jaccard”

Reviewer #3

(Remarks to the Author)
The authors answered all my comments. I would like to congratulate them for this interesting study.

Reviewer #4

(Remarks to the Author)
The authors have adequately addressed my concerns.

Reviewer #5

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):
This study by Ringnalda et al describes the generation of a biobank of small round cell sarcomas established from patient samples or PDXs. The long-term passaged tumoroids they report capture features of the tumors they are derived from and could be used for drug screening. This resource holds potential for downstream studies and, if sharable, could positively impact the rare cancer community. The manuscript could benefit from some clarifications, changes in visualizations, inclusion of critical quantitative analyses, and aligning terminology and references with current standards in the field. I commend the authors for addressing the challenge of cardiotoxicity associated with MCL1 inhibitors in the discussion.

Response 1: We are thankful for the reviewer's insightful suggestions and appreciate the opportunity to emphasize the unique contributions of our study. Our institute has embarked on the creation of a pediatric cancer organoid biobank with the intention that the organoid lines that we describe are indeed shareable (of course under appropriate medical-ethical considerations).

Major points:
1.1 *The appropriate terminology to use is not "Ewing-like tumors" but rather "undifferentiated small round cell sarcomas" or, possibly, "small round cell sarcomas" of which Ewing is one entity, and to which other fusion-driven, round cell tumors belong to. Please refer to and cite Dehner et al, Human Pathology 2024. Citing from the NCCN Bone Cancer Evidence Blocks 1.2025: "Other primary round cell sarcoma of bone was previously referred to as Ewing-like sarcoma, which is terminology that is no longer included in the WHO classification." As such, the title and text throughout should replace the outdated terminology and reflect the standard of the field.*

Response 1.1:
Thank you for pointing this out. We now use "small round cell sarcomas" throughout the manuscript and refer to Ewing sarcomas (ES), CIC::DUX4 sarcomas (CDS) and BCOR-rearranged sarcomas (BRS) for the specific entities. We also now refer to the indicated literature in line 70.

1.2 *Line 33-34: "Currently, there is a lack of preclinical cell models that can predict drug response of these entities in vitro." This is not accurate, there are a number of reported models, but most relevant to this study, patient-derived tumor organoid models of Ewing, CIC-rearranged sarcoma, DSRCTs etc have been recently reported in Al Shihabi et al, Cell Stem Cell 2024. Using similar molecular approaches, those authors have demonstrated fidelity to tumor of origin as well as performed high-throughput scale drug screens, including of longitudinal samples. This study should be mentioned appropriately.*

Response 1.2: The recent paper by Al Shihabi *et al.* has now been added as a reference to the manuscript. Although they use similar nomenclature as we do, in particular

referring to tumor organoid (tumoroid) models, we would like to point out some important differences between the models that were described by Al Shihabi *et al*. compared to the models in our manuscript. Most importantly, the tumoroid models in our manuscript can be cultured and expanded long-term. As the original 'inventors' of tissue-derived organoid technology, we consider a culture to be a successful patient-derived tumoroid model when we have been able to expand it for at least 5 passages and have confirmed that the tumor driver, in this case the fusion gene, is still present in the model. We would like to point out that Al Shihabi *et al*. have performed all their analyses with short-term cultures, being established from tumor tissue 3 days prior to drug treatment. Because our tumoroids models are expandable, we were able to test over 200 drugs in duplicate in 6-8 concentrations, compared to one fixed concentration (1 µM) that was tested by Al Shihabi *et al*. Furthermore, this expansion of tumoroid lines allows us to perform repeat measurements to for validation experiments, to directly compare Omics-data to that of the original cancer and to share our well-characterized models with the wider research community.

We removed "*Currently, there is a lack of preclinical cell models that can predict drug response of these entities in vitro.*"

To refer to the manuscript by Al Shihabi *et al.*, we have added the following text in the manuscript in line 100-102: "a recent study has described a screening platform for SRCS using short-term *in vitro* cultures."

1.3 *Line 100-106: This result section should clearly separate PDOs from PDXOs, as these are anticipated to have different success rates etc. How many samples are derived PDOs vs PDXOs, and whether this is reflected in any difference in take rates should be reported up front. Overall, it is unclear how many individual patients were included in this study – Supplementary table 1 seems to list 17 individual IDs. How many patients, how many samples per patient and how many PDXs were included should be clearly reported in the results and tables. Are the 36 reported overall samples? Patients? Patients+PDXs?*

Response 1.3: Well taken. For clarification, we have now added additional columns named "Patient number" and "Origin" to the Supplementary Table 1, to distinguish patient-derived samples from PDX-derived samples, as well as how many samples per patient were received. In lines 132-139, we specify more clearly which tumoroids were established from PDX, patient-derived naïve and patient-derived post-chemo samples: "In 33 out of 37 (89%) samples, tumoroids were successfully established (**Fig. 1b, Supplementary Table 1**). The derivation efficiency was higher for treatment-naïve (10 out of 10, 100%) than for relapse and chemotherapy-treated samples (16 out of 20, 80%). Especially the ES chemotherapy-treated samples mainly consisted of necrotic tissue, which could explain the lower derivation efficiency. For PDX-derived tumoroids, we successfully established tumoroid lines in 7 out of 7 (100%) samples.".

1.4 *While the result section mentions that "Fresh PDX tumor specimens were obtained from the Innovative Therapies for Children with Cancer (ITCCP4) consortium*

(Supplementary Table 1).", there is no mention of ITCCP4 in Supplementary Table 1. Nor is obvious which samples are from patient tumors vs PDXs from the table.

Response 1.4: This was indeed an unintended omission in Supplementary Table 1. We have now added the names of the institutes that developed the PDX model in this table and added "(ITCC-P4)" behind each institute name. Regarding the difference between patient tumors and PDX material, we added an additional column to Supplementary Table 1, called "Origin", see also response 1.3.

1.5 *Line 112-113: "The derivation efficiency was higher for treatment naïve (11 out of 11, 100%) than for relapse and chemotherapy-treated samples (16 out of113 20, 80%)." What is the breakdown by tumor type? Should be listed whether the failed samples belong to a specific histology.*

Response 1.5: The samples that failed were all tumor samples from resection timepoints, and all originating from EWSR1::FLI1 samples, see also response 1.3. The patients of all these samples had recently undergone chemotherapy, and since Ewing sarcoma patients in general respond well to this treatment, the tumor tissue that we received was presumably not viable enough to result in a successful tumoroid culture.

To indicate this in the manuscript, we added the following part in lines 135-136: "Especially the ES chemotherapy-treated samples mainly consisted of necrotic tissue, which could explain the lower derivation efficiency."

1.6 *Line 122: "Amongst the ES tumoroids, EWSR1::ERG and EWSR1::FEV tumoroids contained more dark cells and displayed a less uniform shape, compared to the EWSR1::FLI1 tumoroids (Fig. 1d)." Firstly, there is no Figure 1D. Even if looking at Figure 1C, this is not obvious from the images, the author should provide a quantification. What are these "dark cells"? Similarly, the description of BCOR organoids is qualitative – unclear how the authors determined that there were "less dense pockets in the center of the tumoroid"? Was this by H&E of paraffin-embedded organoids?*

Response 1.6: Thank you for noticing that Fig. 1d is not there, and the reference in fact should be to Fig. 1c, we corrected this in the text in line 151. For the terminology "dark cells", we amended the sentence as followed: "Amongst the ES tumoroids, EWSR1::ERG and EWSR1::FEV tumoroids contained more compact cell structures, indicated by a darker appearance in the center of the tumoroid. They also displayed a less uniform shape compared to the EWSR1::FLI1 tumoroids (**Fig. 1c**)." Regarding the BCOR::CCNB3 tumoroids that were described as having "less dense pockets" in line 158-160, we adjusted the sentence as followed: "with some less dense pockets in the center of the tumoroids. BCOR staining showed that the core of the BRS tumoroids contained fewer cells/displayed a lower cell density compared to other fusion positive sarcomas (**Supplementary Fig. 2b**)."

1.7 *While the quality of the images in Figure 2C makes it hard to fully interpret, there seems to be fairly large differences in staining between tumor vs tumoroid for ES-093 and 96 and 101- How are these explained? There are similar issues of possible discordances (pending higher quality images / color correction) with Supplementary Figure 2 for cases 10 and 77.*

Response 1.7: We now added additional zoom-in pictures of the different stainings to show more specific details in these different tumor types. The differences in staining between ES-093, ES-096 (Ewing sarcoma) and ES-101 (CIC::DUX4 sarcoma, – renamed sample to CDS-101), demonstrates the specific nuclear WT1 protein expression in CDS-101 (CIC::DUX4 sarcoma) (**Fig. 2c**). For sample ES-093 (**Fig. 2c**), we also noticed a positive membrane stain, which we consider to be non-specific given that WT1 is a nuclear factor.. To specify that the WT1 is supposed to be nuclear, we amended the sentence about WT1 expression in CDS, which now reads: ". A hallmark of CDS is the strong nuclear expression of Wilms tumor 1 (WT1) (**Fig. 2c**) and ETS variant transcription factor 4 (ETV4) (**Supplementary Fig. 2a**)."
For Supplementary Fig. 2a we have also added zoom-in pictures of the ETV4 protein staining.

1.8 *Figure 3B should include side-by-side comparisons of Circos plots of pairs of tumors vs tumoroids.*

Response 1.8: Thank you for this suggestion. For a side-by-side comparison, we have now added the circos plots of matched tumors for which WGS data were available in Fig. 3b and Supplementary Fig. 3b-c. For relapse samples, no whole genome sequencing (WGS) data was available, only whole exome sequencing data. Since circos plots only provide a valuable image when using the same type of data input for all samples, we omitted these from the figure. We also added a side-by-side comparison of copy numbers of two tumor-tumoroid samples where we obtained WGS (**Supplementary Fig. 3d**)

1.9 *Similarity of pairs (tumoroid vs tumor) by WGS and RNA-seq needs to be globally quantified. Pearson correlation coefficients need to be plotted or reported in table format. This will allow a reader to clearly and unambiguously compare the primary samples and derivative tumoroids.*

Response 1.9: We added a sample distance table based on the DNA sequencing using Jaccards distance (Supplementary Fig. 3c). We also added a sample distance table based on the RNA expression using eucledian distance (Fig. 4c). All samples were clustered using unsupervised clustering.

1.10 *Line 272: "ETV5, a known target of Trametinib". Trametinib is a selective inhibitor of MEK, key components of the MAPK/ERK signaling pathway. While ETV5 is a downstream*

target within this pathway, Trametinib does not directly target ETV5. ETV5 modulates sensitivity to trametinib but is not a known target, which should be amended in the text.

Response 1.10: Thank you for this critical remark, we changed this sentence as followed in line 332: "*ETV5*, which is downstream of the target of Trametinib[7,45,46], could be identified as a DEG that was regulated specifically by the CIC::DUX4 fusion protein.". We also addressed this later in the paper in line 396-399: "We also confirmed that CDS tumoroids have high MAPK phosphatase (MKPs) expression (**Supplementary Fig. 4b**), which may contribute to the MAPK/MEK inhibitor resistance that we observed in CDS tumoroids compared to ES tumoroids (**Supplementary Fig. 5c**)[41,45].", where we then refer to the paper describing this link by Lin et al.

1.11 *Line 303: ". Tumoroids were established from both primary and metastatic disease timepoints." For all 14 patients?*

Response 1.11: No, tumoroids were established from primary as well relapse/metastatic timepoints for 10 patients. This information has been added to Supplementary Table 1.

1.12 *While the authors state in line 287 that "These pathways could be used for specific treatment of these tumors" including MAPK in CIC-rearranged sarcomas, they then report at line 335 that "We also found that CIC::DUX4 sarcomas should have high MAPK pathway activity (Supplementary Fig. 4a), but we did not notice an increased sensitivity of this group to compounds in CIC::DUX4 tumoroids compared to ES tumoroids". This is frankly not completely unexpected due to lack of clinically validated biomarkers for many of these agents – yet, the claim pathways can be used for treatment should be dimmed.*

Response 1.12: We agree that this claim is perhaps stated too boldly, and therefore removed the sentence "These pathways could be used for specific treatment of these tumors" from the text.

1.13 *Line 381: "This is the first study that describes the establishment of a broad panel of ES and ELS patient-derived tumoroids, including those with rare fusions such as EWSR1::FEV, EWSR1::ERG and CIC::DUX4, which have, to our knowledge, not been described previously." As discussed above, other large studies have reported of patient-derived tumor organoids from EwS and other small blue round tumors harboring rare fusions. This study reports on the generation of established, long-term passaged organoid models so after contextualizing, this aspect should be highlighted instead.*

Response 1.13: We corrected this sentence in line 455-457 into: "This study describes the establishment of a broad panel of ES, CDS and BRS patient-derived tumoroids which can be passaged and maintained long-term, including those with rare fusions such as EWSR1::FEV, EWSR1::ERG and CIC::DUX4."

1.14 *Line 409: "Vice versa, some tumoroids carried additional somatic variants compared to the matching tumor, e.g., in sample ES-041 (TP53 mutant) and ES-057." This finding does not seem to appear in the result section? Emergence of de novo TP53 mutations during long term passaging has been reported during establishment, long term passaging and immortalization of other cell lines and models, and should be mentioned as a possibility.*

Response 1.14: In a few cases we found additional alterations in the tumoroid models, which indeed can occur due to in vitro propagation or alternatively can result from a genetic alteration present in only part of the tumor due to genetic intratumor heterogeneity. We amended this in the text as followed in line 198-200: "In some samples (e.g., ES-027) additional mutations were detected in the tumoroid samples compared to their matching tumor sample. This could be an indication of de novo mutations or clonal selection."
We have re-written part of the discussion to reflect this, the paragraph in lines 477-492 now reads: "Although the tumoroids closely resembled their matching tumors in many aspects, we also observed some differences. For instance, some somatic variants that were present in the tumor were not detected in the matching tumoroids, e.g., a *MYC* gene amplification in sample CDS-045. Vice versa, some tumoroids carried additional somatic variants compared to the matching tumor, e.g., in sample ES-041 (*TP53* mutant) and BRS-057. We reason that there are several explanations for this. First, the tumor tissue pieces used for diagnostic sequencing and for tumoroid derivation are different and there could be regional differences. In another study, we have analyzed clonal selection and tumor heterogeneity in three primary, previously untreated colon cancers[66]. We found genetic differences even in tumor cells which are closely related within the same tumor. We assume the same could hold true for ES, CDS and BRS tumors. Second, discrepancies can result from clonal outgrowth of mutations in cells that were already present, but in too low numbers to be detected. Third, additional de novo mutations might have occurred while culturing. Overall, we see more similarities than differences, meaning that these tumoroids can provide useful information, e.g., about tumor-specific drug sensitivities."

Minor points:

1.15 *Line 165: "expect" should be "except"*

Response 1.15: We have corrected this in the text.

1.16 *Figure 3A: I suggest that the authors consider diversifying the panel of colors used so that different categories are more obviously separated. It appears that PDXs for which no organoid is derived (or sequenced?) are included in Figure 3. It may be clearer to limit to pairwise comparisons here.*

Response 1.16: The color setting was chosen to be clearly visible for color-blind people. Changing to a wider range would diminish the visibility for color-blind people, as tested by author G. van Son (who happens to be red-green color-blind). For PDX samples, no germline sequencing data or sample was available, diminishing the value of doing WGS. To clearly make a pairwise comparison between tumor and tumoroid we made a sample distance table of all samples for which we had sequencing data available (WGS/WXS), including the PDX tumoroids samples that were submitted for WGS. We only performed this on a CDS tumoroid sample, CDS-086. We visualize the mutational information we have available for the other PDX samples as informed by the ITCCP4 consortium. To clearly address this difference in data source, we added the following text in the figure caption: "For samples for which no sequencing data was available, the mutational information provided by the ITCC-P4 consortium is indicated (+)."

1.17 *Figure 3 uses both the conventional organoid nomenclature (3A) as well as tumoroid (3B). It also includes "-T" and "-O" and "-Tum" and "-Org" in 3A/D and 3E respectively. For clarity to the reader, the names used should be consistent. If they elect to use "tumoroid", the -T (=tumor) will be easily confused for -T (=tumoroid), so would require a different naming strategy. The driver translocation should be labeled in Figure 3B.*

Response 1.17: Well taken. We changed T and O to TT and TO, corresponding to Tumor Tissue and TumorOid. We also added driver translocation labels to Fig. 3b.

1.18 *Figure 4 B and C also should include different colors for tumoroid / PDX / tumor as they are very similar to the heatmap gradation. I recommend removing the grey boxes outlines for readability.*

Response 1.18: We changed the colors in Fig. 4b and Fig. 4c for sample type Tumoroid/PDX/Tumor. The gray boxes were made thinner to improve visibility in the heatmaps in Fig. 4b and 4c and for consistency also in Supplementary Fig. 3 and 4.

Reviewer #2 (Remarks to the Author):

In this manuscript, Ringnalda et al. present the development of tumoroids for Ewing sarcomas and for CIC- and BCOR-rearranged sarcomas, which they used to screen a small panel of drugs. They then identify MCL inhibitors to be nicely specific for CIC-rearranged sarcomas. Patient affected by a small blue round cell sarcoma (encompassing the tumor types above) are indeed in need of specific treatments. As these tumors are rare (even ultra-rare for some of the subtypes) it is quite difficult to establish cell lines to test hypothesis. The work presented here is therefore of a tremendous importance for the researchers in the field as the authors present methods and culture conditions to establish, quite efficiently, organoid models that they show to be quite representative of the original tumor. This work merit to be published, but not in the actual version of the manuscript. I have indeed comments that need to be address before publication.

Response 2: We thank the reviewer for taking the time to help improve our manuscript.

Major comments:

2.1 *The term of Ewing-like sarcomas (ELS) is not used anymore, not recognized in the latest WHO classification and not suitable. This "old" entity is in fact several small, different, tumor entities. In the introduction and discussions, there are therefore some generalizations that are not necessarily true. For example, line 81 (page 2) indicates that ELS "show little to no response to chemotherapy". While this is true for CIC-rearranged sarcomas, it is not for BCOR-rearranged sarcomas for which the patients are better responding than Ewing sarcoma patients.*

Response 2.1: Thank you for pointing this out. We now use "small round cell sarcomas" throughout the manuscript and refer to Ewing sarcomas (ES), CIC::DUX4 sarcomas (CDS) and BCOR-rearranged sarcomas (BRS) for the specific entities. ELS have been changed to CDS and BRS. Thus, we have changed "ELS show little to no response to chemotherapy" to "CDS sarcomas show little to no response to chemotherapy" in line 96.

2.2 *Similarly, the first sentence of the discussion (line 365 page 9) "ES and ELS are highly aggressive tumors that predominantly affect children and young adults" may be true for BCOR- and CIC-rearranged sarcomas, but not for EWSR1::PATZ1 or EWSR1::NFATc2 sarcomas that fall also under this ELS type. The term of ELS should then be removed from the whole text and replaced by CIC- or BCOR-rearranged sarcomas.*

Response 2.2: We changed "ES and ELSare highly aggressive tumors that predominantly affect children and young adults" to: "ES, CDS and BRS are highly aggressive tumors that mainly affect children and young adults."

2.3 *It is quite difficult to know how many samples from how many different patients are included in this study. From supp table 1 it seems that there are 33 samples coming from 17 patients, right? Page 3 Line 111: 36 cases are indicated amongst which 32 tumoroids where established ???*

Response 2.3: Well taken. To provide a clearer representation, we have adjusted Supplementary Table 1 and added additional columns named "Patient number" and "Origin". This distinguishes patient derived samples from PDX models and indicates how many samples per patient were processed. We adjusted the sentence in lines 131-137 as followed: "In 33 out of 37 (89%) samples, tumoroids were successfully established (**Fig. 1b, Supplementary Table 1**). The derivation efficiency was higher for treatment-naïve (10 out of 10, 100%) than for relapse and chemotherapy-treated samples (16 out of 20, 80%). Especially the ES chemotherapy-treated samples mainly consisted of necrotic tissue, which could explain the lower derivation efficiency. For PDX-derived tumoroids, we successfully established tumoroid lines in 7 out of 7 (100%) samples." All 37 samples are described in Supplementary Table 1

2.4 *In supp table 3 in Samples ID and M-Labels are not corresponding to the supp table 1. Maybe there is some mix up as well in supp table 2, the relapse of M369AAA was not cultured in the same medium than the primary (STS2 and STS1)? There are 2 ES-016 samples, one from the primary, one from a metastatic lesion, which is very confusing (correct "leasion" in "lesion" in Supp table 1).*

Response 2.4: Thank you for noticing, this was indeed a mix-up of some identifiers. This has been amended in Supplementary Table 1 and 3. We added the following sentence in line 138: "Different media conditions were tested for all new samples, the media conditions that resulted in tumoroid growth per sample are shown in **Supplementary Table 2**." In Supplementary Table 1 we corrected *"leasion" into "lesion"*.

2.5 *There is an ES-020T in fig 4 heatmaps that is never anywhere else.... A clear, single, supplementary table describing the clinical data of the patients, the different samples used from each patient's tumor and then the organoid/pdx attempts with the derived ones together with the culture medium used, should be provided.*

Response 2.5: We did not have tumor tissue available to establish a tumoroid model from this patient and therefore did not include it in the rest of the paper, but forgot to remove it from Fig. 4, thank you for noticing. For clarification, we have now added additional columns named "Patient number" and "Sample info", to Supplementary Table 1 to clarify which samples belong to the same patient. Regarding the medium conditions for the different tumoroid models, these are described in Supplementary Table 2 "Medium Tumoroids", see also response 2.4. Regarding the attempts for the establishment, see also response 2.3.

2.6 *By the way, I am surprised to see in supp table 1 a sample (ES-094) that have both an EWSR1::ERG and a FUS::FEV. To my knowledge there is no case in the literature of an ES with 2 fusions. Is it an error? Did the author validate this?*

Response 2.6: Thank you for pointing out this error, we have failed to filter this out when we finalized the manuscript before submission. This annotation came from the ITCC-P4 consortium at the time we received the PDX material, and was based on the original tumor from which the PDX models were generated. In our RNA analysis of PDX tumor and tumoroid we only detected EWSR1::ERG. We corrected this in Supplementary Table 1. We do not know why the PDX material differs from its ITCC-P4 annotation and have reported this to the ITCC-P4 consortium.

2.7 *I have some difficulties with the statistics through the paper, either with the WGS SNV/CNA or with the expression profiles when the authors are comparing subtypes of tumors against each other's. Indeed, the statistics may be completely skewed by the number of initial tumors. The authors are actually comparing several samples of the same original tumors and they end up analysis 2 BCOR::CCNB3 or 3 CIC::DUX4 against*

12 Ewing sarcomas…. I think that the goal of this paper is to show that the derived organoids are similar to the tumors and could be used as surrogates for treatment, which is really great. But everything related to the comparison of the subtypes between them should really be moderated as the confirmation of what is already known (the author do not actually discover new things amongst the different tumors types) is true for the organoids as well.

Response 2.7: We agree with the reviewer that the main goal of the paper is to show that the tumoroids resemble their matching tumors on a multi-omic level, and not to identify new differences between the different entities. This is why, when looking at expression profiles in Fig. 4B, we only focused on known marker genes to determine whether these are stably expressed in tumoroids versus tumors. We also looked at genes that are specifically expressed in each tumor entity (Fig. 4c), and here we combined it with publicly available chip-seq data to make the data more reliable. To put less emphasis on identifying differentially expressed genes, we changed the title of this paragraph to "Identification of fusion-protein driven transcription", and the first part of this paragraph in lines 299-308 to: "Next, we aimed to determine whether genes that are specifically expressed in each tumor entity and can possibly be attributed to the activity of the fusion oncoprotein, are also expressed in the matching tumoroids**.** In addition to tumoroid characterization, these genes might contain interesting entity-specific drug targets."
To further emphasize the similarities between tumoroids and matching tumors, we added a Jaccards distance table based on WGS (Supplementary Fig. 3c) and a Euclidian distance table based on the RNA expression (Supplementary Fig. 4b). We also added a comparison of copy numbers of two tumor-tumoroid samples for which we had WGS available for both tumor and tumoroid in Supplementary Fig. 3d.

2.8 *Resolution of the Fig2 and supp Fig2 should be drastically increased to be able to see anything. WT1 seems to be expressed everywhere except in the ES-096 samples… For the BCOR-rearranged sarcomas and tumoroids, a BCOR staining should be shown.*

Response 2.8:
We now added additional zoom-in pictures of the different stainings to show more specific details in these different tumor types. The differences in staining between ES-093, ES-096 (Ewing sarcoma) and ES-101 (CIC::DUX4 sarcoma, – renamed sample to CDS-101), demonstrates the specific nuclear WT1 protein expression in CDS-101 (CIC::DUX4 sarcoma) (**Fig. 2c**). For sample ES-093 (**Fig. 2c**), we also noticed a positive membrane stain, which we consider to be non-specific given that WT1 is a nuclear transcription factor. To specify that the WT1 is supposed to be nuclear, we amended the sentence about WT1 expression in CDS, which now reads in line 174: " A hallmark of CDS is the strong nuclear expression of Wilms tumor 1 (WT1) (**Fig. 2c**) and ETS variant transcription factor 4 (ETV4) (**Supplementary Fig. 2a**)." For Supplementary Fig. 2a we have also added zoom-in pictures of the ETV4 protein staining.
Additionally, we performed a BCOR staining on the BCOR-rearranged sarcomas and tumoroids and added the pictures to Supplementary Fig. 2b, including a negative staining control (liver).

2.9 *The demonstration that the tumoroids are resembling the tumors is weak and should be reinforced. A figure showing the CNV / CNA / Fusion similarities/differences between tumors and their derived tumoroids should be provided, as a supplementary figure for each available O/T couples and for a few examples in a main figure.*

Response 2.9: We added a Jaccards distance table based on WGS in supplementary Fig.3c and a euclidian distance table based on the RNA expression in supplementary Fig. 4c. We also added a comparison of copy numbers of two tumor-tumoroid samples where we had WGS available for both tumor and tumoroid in Supplementary Fig. 3d. To show a clear overview of breakpoints per patient, we added a graphical overview in Supplementary Fig. 2c. We unfortunately do not have WGS of all samples, because most relapse samples in our center are sequenced with WXS. For PDX samples and for some samples where no germline sample is available, a fair comparison on SNVs would not be possible with these different data formats. We added a tumor/tumoroid circos plot comparison example in Fig. 3b.

2.10 *In Fig 3 while there is consistency in many O/T couples, one can see also strong differences in several as well (ES-055,ES-046,ES-071,ES-090...). It therefore seems that O/T couples are not that obviously similar!*

Response 2.10: In order to show similarity between tumor and tumoroid couples, we added a clustered sample distance table for both DNA and RNA in Supplementary Fig.3c and Fig.4c, respectively. This indicates that O/T couples are generally quite similar. In a few cases, we found additional alterations in the tumoroid models, which indeed can occur due to *in vitro* propagation or alternatively can result from a genetic alteration present in only part of the tumor due to genetic intratumor heterogeneity. We amended this in the text as followed in lines 198: “In some samples (e.g., ES-027) additional mutations were detected in the tumoroid samples compared to their matching tumor sample. This could be an indication of de novo mutations or clonal selection.”
We have re-written part of the discussion to reflect this, the paragraph in lines 477-492 now reads: “Although the tumoroids closely resembled their matching tumors in many aspects, we also observed some differences. For instance, some somatic variants that were present in the tumor were not detected in the matching tumoroids, e.g., a *MYC* gene amplification in sample CDS-045. Vice versa, some tumoroids carried additional somatic variants compared to the matching tumor, e.g., in sample ES-041 (*TP53* mutant) and BRS-057. We reason that there are several explanations for this. First, the tumor tissue pieces used for diagnostic sequencing and for tumoroid derivation are different and there could be regional differences. In another study, we have analyzed clonal selection and tumor heterogeneity in three primary, previously untreated colon cancers[66]. We found genetic differences even in tumor cells which are closely related within the same tumor. We assume the same could hold true for ES, CDS and BRS tumors. Second, discrepancies can result from clonal outgrowth of mutations in cells that were already present, but in too low numbers to be detected. Third, additional de novo mutations might have occurred while culturing. Overall, we see more similarities than differences, meaning

that these tumoroids can provide useful information, e.g., about tumor-specific drug sensitivities."

2.11 *I do not get the scale of the Fig3c and Fig3d. What do you call "cellular prevalence"? Shouldn't it represent the fraction of the clones you identified over the whole population? If you sum the prevalence of Fig3d shouldn't you get the same sum than fig 3c?*

Response 2.11: We understand the confusion, so we rewrote the paragraph describing these results and adapted figure Y-axis descriptions (Fig. 3c) and legends (Fig. 3d). Shortly, we are plotting the presence of clusters of mutations to investigate the mutational landscape of ES over different timepoints during the course of the disease in both tumor tissue and tumoroids. We now discus these results as follows in lines 231-253: "Next, we more deeply analyzed tumor tissue and matching tumoroid lines from the 3 different time points (ES-016, ES-055 and ES-080) of one ES patient to better understand the mutational composition at different timepoints. We clustered all mutations found over all samples based on similarity of VAF and allele-specific copy numbers. This resulted in 16 different clusters of mutations (**Fig. 3d and 3e**). We noticed that not all clusters are present in all samples, indicating clonal evolution. In addition, we noticed that different groups of mutations could be either timepoint-specific or sample-specific. For instance, cluster 10 is present in all samples except for the first tumor sample (ES-016-TT). Possibly, *in vitro*, we selected for a clone already harboring this group of mutations, much like it seems to have happened *in vivo*. We also noticed clusters of mutations that only appeared in a single tumoroid sample, like cluster 12, 13 and 2. These mutations are only present in tumoroid samples and are likely a result of clonal selection or de novo mutations. In the second relapse sample (ES-080), an additional *STAG2* mutation could be identified (**Fig. 3a**). We detected a *TP53* mutation in the first relapse sample (ES-055, tumor and tumoroid), but not in the second relapse sample (ES-080, tumor and tumoroids) (**Fig. 3a**). This already indicates that there is variation in tumor development and clonal selection *in vivo*, as also indicated by the different clusters of mutations that are present in the tumor samples (**Fig 3c**). Subsequently, we compared the mutational distribution of the tumoroids to their matching tumor samples. Although not identical, most clusters of mutations that were present in the tumors, were also present in the matching tumoroids, and vice versa (**Fig. 3d, Supplementary Fig. 3f**), with some exceptions that could be explained by either de novo mutations or clonal selection."

2.12 *How were ordered the heatmaps of fig 4? Could you please show the dendrograms (for the columns)?*

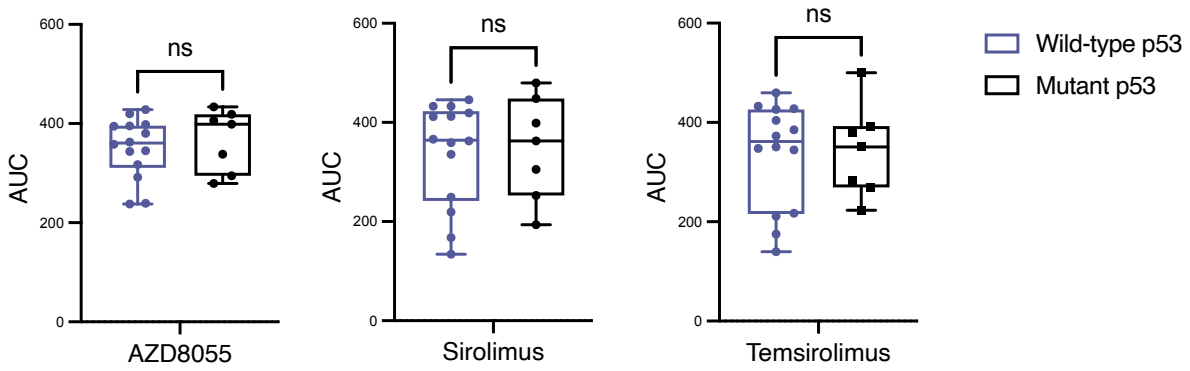
Response 2.12: The genes were selected as described in the text. Samples were ordered based on the fusion type. Samples and genes were not hierarchically clustered.

2.13 *Lines 268-271: "While most identified genes were known targets of the different tumors, like NKX2.2 and ETS1 for EWSR1::FLI1 and CIC::DUX4, respectively, we also identified new targets. For example, MAPT…. ETV5…": Well, see Tim Triche paper, back in 2005 for the 1st one (10.1146/annurev-pathol-011110-130237) and many for the 2nd, including ref 6. As stated above, this entire paragraph (Identification of differentially expressed genes) should be either removed or written differently, as it is not in the scope of the paper to find DEG or pathways between CIC or BCOR tumors, but to demonstrate that the same DEG or pathways are modulated in the organoids.*

Response 2.13: We changed line 318, where we discuss these targets, to: "Most identified genes were known targets of the different tumors," and we changed line 322 to: "ETV5, which is downstream of the target of Trametinib (MEK)", where we also added the suggested references. We agree with the reviewer that the identification of differentially expressed genes between different tumors is not the main focus of this study, and we further elaborate on this in a previous response (2.7). To make this more clear we changed the title of this paragraph to "Identification of fusion-protein driven transcription".

2.14 *Looking at the status of TP53 while screening for drugs is really important. It seems that both the EWSR1::ERG and EWSR1::FEV sample are TP53 mutated. Could this explain why they behave differently than EWSR1::FLI1? When testing the MTOR inhibitors could you stratify the ES, not by the fusion but by TP53 status?*
In a general manner, it is well acknowledged now by the community that Ewing sarcoma are defined by the presence of an EWSR1/FUS::(PEA3 ETS family members) fusion and that the fusions behave strictly similarly. What is important though, are secondary events.

Response 2.14: Thank you for this interesting question. To answer this, we performed some additional data analyses. When we compared the response to mTOR inhibitors between *TP53* mutant versus wildtype tumoroids in all ES samples (EWSR1::FLI1,EWSR1::ERG and EWSR1::FEV), we saw no significant difference, as shown in the figure below. We added the figure shown below to Supplementary Fig. 5a, and the following sentence to the manuscript in lines 377-379: "As the response to mTOR inhibition was independent of the *TP53* mutational status in ES tumoroids (**Supplementary Fig. 5a**), this response seems to be EWSR1::FLI1-specific.".



The finding that MCL1 inhibitors are effective in CIC-rearranged sarcomas is really great!

We are very happy with this enthusiastic response, thank you.

Minor comments.

2.15 *Resolutions of the figures are really poor (probably due to the pdf conversion) and the police size too small, barely readable (see the boxplots of Fig 5 as an example)*

Response 2.15: We understand the reviewer. To improve this, we now added additional zoom-in pictures of the different stainings to show more specific details in these different tumor types. For better readability, we adjusted all the font sizes in Fig.5.

2.16 *Line 124 (page 3) Fig.1d should be 1c*

Response 2.16: We corrected this in the text.

2.17 *Line 147 to 150 (page 4): as there is no data shown for the PCR, it should not be in the result section but in the material and methods.*

Response 2.17: We added an additional figure in Supplementary Fig. 2c that shows the breakpoints of each patient sample. We refer to this and added the following sentence in line 183-184: "An overview of genetic breakpoints is shown in **Supplementary Fig. 2c**."

2.18 *Line 211 (page5) Fig.3d should be 3e –*

Response 2.18: We corrected this in the text.

2.19 *Line 228 (page 5) Supp fig 3b does not show fusions and there is no supplemental figure showing the fusions*

Response 2.19: We added an additional figure (Supplementary Fig. 2c) that shows the breakpoints of each patient sample. We amended Supplementary Fig. 3b with circos plots, where the red line inside the circos plot represents the translocation, including the fusion name. Unfortunately, no RNAseq was available for BRS-057, so the translocation is not indicated in this sample.

2.20 *Color code of Fig 4B is poor, it is difficult to see which is tumor or tumoroid. Also please choose a better color scaling for the expression values (same remark apply for panel C and D) such as in fig 5?*

Response 2.20: We agree. The color code has been changed in all figures, to better distinguish the tumors from the tumoroids.

2.21 *Line 773 (page 17). The aligner is STAR, and the tool for fusion detection is STAR-fusion.*

Response 2.21: We corrected this in lines 864-865: "Reads were mapped to the human reference genome (GRCh38 and gencode version 32) by STAR (v.2.7.2d). Fusions were detected by STARfusion (v1.6.0) using standard settings.

2.22 *Line 775 it is indicated that "Fusion were manually assessed", what exactly is*

Response 2.22: Thank you for catching this, this comes from an earlier version of the manuscript. We removed *"Fusion were manually assessed" from* the text in the material and methods section, and amended the sentence as follows in lines 922-923: "Fusion transcripts were compared between tumor and tumoroid to confirm matching tumor/tumoroid pairs".

Reviewer #3 (Remarks to the Author):

The manuscript by Ringnalda and colleagues explores the use of tumoroids as to perform functional assays and evaluate potential treatments for ES and ELS. While the manuscript is well written and technically well described, we include a few comments to improve the manuscript

Major comments:

3.1 *There is no validation for the drug screening predictions made with the tumoroids. This should be tested and included in the manuscript.*

Response 3.1: Indeed, ideally, we would want to determine whether the drug responses in the tumoroids are correlated to the *in vivo* responses of their matching tumors. To some extent, we show that this is the case for chemotherapy agents such as Etoposide, Vincristine and Doxorubicin that are currently used in a clinical setting. These are in general more effective in ES patients than in CDS sarcoma patients, which we also see reflected in the Ewing sarcoma versus CDS sarcoma tumoroids (**Fig. 5c**).
We now refer to this in the text in lines 366-372 as follows: "The drugs used in these regimens and included in our drug library are marked in red in **Fig. 5c**. Although the response varied between the different tumoroid lines, the CDS tumoroids overall responded less to the chemotherapeutics when compared to the other groups (**Fig. 5c**). Previous retrospective studies have shown that CDS sarcomas are less chemo-sensitive than ES[14,50-52] which was reflected in our tumoroids, indicating that tumoroid drug response can be predictive of *in vivo* response, and highlighting the need for fusion gene-specific treatment options."
To elaborate on the predictive value of tumoroids in general, we added the following sentence to the discussion in lines 450-453: "Several studies have shown that tumoroids

of different cancer entities, including rectal cancer, colorectal cancer, lung cancer, head and neck cancer, pancreatic cancer, and ovarian cancer, can predict patient responses to radiation therapy, chemotherapy and targeted therapy."
To validate some of the drug screen results, we selected drugs that showed interesting responses and, in the case of MCL1 inhibitors, added additional drugs with the same mechanism of action. We then performed follow-up experiments with the CDS sarcoma tumoroid lines, as well as some ES tumoroid lines to confirm the specificity of MCL1 inhibitors to CDS sarcomas. We see the identification of these MCL1 inhibitors as a proof of concept for the type of research that can be done with this tumoroid biobank.

3.2 *There is no indication of the time points used for the WGS analyses. Did the authors check if tumoroid passages affects WGS data?*

Response 3.2: We added the range of passages that were used for WGS analyses to the methods section line 860 and in the results in line 142-145 as followed: "Upon establishment of a stable culture, cells were expanded for drug screen experiments, which were performed between passage 5 and 10. We collected DNA and RNA for further downstream analysis at the same timepoint".
Although we agree that performing WGS analyses on multiple passages would be interesting, WGS is still a very costly technique, so for financial reasons we chose to only perform WGS on one passage.

3.3 *The authors identified new pathways that could be targeted therapeutically in ES and ELS. However, they did not validate these observations by Western Blot analyses and they did not test specific inhibitors in these tumoroids. For example, no MAPK inhibitors such as trametinib were tested in CIC::DUX4 models.*

Response 3.3: Thank you for the suggestion. We added a Western blot analysis of MCL1 and NOXA, two proteins involved in the MCL1 pathway (Supplementary Fig.6b). We have adapted the text in the result section to reflect this in lines 389-396, which now reads: "Looking back at the transcriptome analysis, we noticed that CDS indeed expressed higher levels of *MCL1*, the target of these drugs, compared to the other groups (**Fig. 4d, Supplementary Fig.6a**). In contrast to the anti-apoptotic MCL1, NOXA is a pro-apoptotic protein that binds and neutralizes MCL1, and we found that *NOXA* mRNA expression is lower in CDS compared to ES (Supplementary Fig.6a). In a Western blot analysis, we saw a similar increase in MCL1 and NOXA ratio in two CDS compared to two ES tumoroid samples (Supplementary Fig. 6b)."
In the high-throughput drug screens, we tested MAPK inhibitors such as trametinib also in CDS models. We refer to these results in line 396-399: "We also confirmed that CDS tumoroids have high MAPK phosphatase (MKPs) expression (**Supplementary Fig. 4a**), which may contribute to the MAPK/MEK inhibitor resistance that we observed in CDS tumoroids compared to ES tumoroids (**Supplementary Fig. 5c**)."

Minor comments:

3.4 *In the line 334 the authors mention an increase in mRNA of MCL1 and BCL2 and they claim that this is the reason why there is a higher effect of S63845 and AZD5991. The authors should check the protein levels of MCL1 and NOXA.*

Response 3.4: Thank you for the suggestion. As indicated in the previous reply, we performed a Western blot analysis on MCL1 and NOXA levels in tumoroids and added the data to Supplementary Fig. 6b. We refer to these results in line 394-396: " In a Western blot analysis, we saw a similar trend in MCL1 and NOXA expression in two CDS compared to two ES tumoroid samples (Supplementary Fig. 6b)."

3.5 *To our knowledge these BH3 mimetics do not inhibit BCL2 (line 334).*

Response 3.5: We corrected this sentence and removed BCL2 from the text (line 390). The corrected sentence in line 389 -391 as follows:" Looking back at the transcriptome analysis, we noticed that CDS indeed expressed higher levels of *MCL1*, the target of these drugs, compared to the other groups (**Fig. 4d**)"

3.6 *In order to prove more detailed information on the activity of MCL1 inhibitors some cell death assays should be performed as CellTiter-Glo measures ATP production (basically measures the cell number) but not cell death specifically. We suggest Annexin / PI cell death analyses.*

Response 3.6: We agree with the reviewer that using assays like CellTiter-Glo does not give any information about cell death specifically. We therefore performed additional imaging experiments where we analysed Annexin V (for early apoptosis) and PI (for cell death in general) in CIC::DUX4 and EWSR1:FLI1 tumoroids during MIK665 treatment. We added this analysis to Supplementary Fig. 7 and refer to the results in lines 416-420 as followed: "
To determine whether the MCL1 inhibition reduces cell viability in CDS tumoroids by inducing apoptosis, we performed Annexin V and PI imaging experiments upon treatment with the MCL1 inhibitor MIK665. This confirmed that MCL1 inhibition induced apoptosis already after 1 hour in CDS tumoroids but not in ES tumoroids (**Supplementary Fig. 7**)."

3.7 *Line 421: MCL1 instead of MCL.*

Response 3.7: We corrected this.

3.8 *BH3 mimetics such as S63845 act fast. Did the authors tested these agents at shorter timepoints?*

Response 3.8: We acknowledge the limitation of having only 1 timepoint in our drug screen. To address this, we analysed Annexin V and PI immunofluorescent signal using

live imaging at different timepoints, 1, 3, 6 hours, respectively. We refer to these results in lines 416-420 as follows: “To determine whether the MCL1 inhibition reduces cell viability in CDS tumoroids by inducing apoptosis, we performed Annexin V and PI imaging experiments upon treatment with the MCL1 inhibitor MIK665. This confirmed that MCL1 inhibition induced apoptosis already after 1 hour in CDS tumoroids but not in ES tumoroids (**Supplementary Fig. 7**)”, see also response 3.6.

Reviewer #4 (Remarks to the Author):

General comments: This is a well-written, scientifically rich manuscript highlighting the usefulness of tumor organoids (i.e., tumoroids) in assessing ex vivo drug sensitivity for ES and other small round blue cell malignancies. The authors demonstrate that drug sensitivity differs by sarcoma subtype and identify MCL-1 inhibitors as a potential candidate for CIC::DUX4 sarcomas. I enumerate below constructive comments that may further enhance their work:

4.1. *Though used historically in the literature when ES couldn’t be readily distinguished from other small round blue cell sarcomas, there’s a growing consensus that “Ewing-like-sarcoma” or “Ewing family of tumors” terminology shouldn’t continue—see WHO 5th edition. While BCOR::CCNB3 (BCS) and CIC::DUX4 (CDS) display some morphological similarity to ES, the similarities stop there. The latter sarcomas have entirely different translocations that function differently than EWS::FLI1, vastly different survival rates, and as the authors suggest, unique drug sensitivities in their tumoroid models. I’d recommend using small round blue cell sarcomas (SRBCS), or since their work doesn’t include DSRCT or RMS, simply BCS and CDS.*

Response 4.1: Thank you for pointing this out. We now use “small round cell sarcomas” throughout the manuscript and refer to Ewing sarcomas (ES), CIC::DUX4 sarcomas (CDS) and BCOR-rearranged sarcomas (BRS) for the specific entities.

4.2. *Lines 37-39: The statement that tumoroids retain key properties matching patient tumors isn’t entirely true since the PDXs and tumoroids separated from human tumors in Figure 4, Panel A. Also, it’s nebulous what key properties the authors are referring to.*

Response 4.2: To better describe on which key properties we focus when comparing tumor with tumoroid, in line 50-52 in the abstract has been reformulated to: “Using histology, whole genome sequencing and RNA sequencing, we demonstrate that these tumoroids retain histological characteristics, known marker gene expression and chromosomal rearangements of their matching patient tumors”. Furthermore, we would like to emphasize that in Fig. 4a, principal component 2 (PC2), which is mainly driven by the difference between tumor and tumoroids, indicates only 13% variance.

4.3. *There also seem to be significant differences in the IHC images between tissue and tumoroids in Figure 2, panels B & C. The tissue images for ES-053-BCS are missing in panels B & C. The authors might choose to quantify the expression of CD99 and WT1, respectively, using any number of spatial imaging-based software (e.g., Visiopharm, Halo, etc.), allowing the readers to evaluate the degree of similarity objectively.*

Response 4.3: Unfortunately, we were unable to obtain additional tissue of ES-053 (now renamed to BRS-053), so could not perform stainings on this tumor sample. To avoid confusion, we decided to remove this sample from Figure 2. We use histological images mainly to determine whether a marker is expressed or not and whether we see the expected subcellular localization (e.g., nuclear staining for WT1). We hope that the improved quality of the IHC images and the addition of zoomed areas will help the reader to better assess the stainings.

4. *Line 65: ETS binds "GGAA" microsatellites, not "CCAA" repeats.*

Response 4.4: Thank for noticing, we have corrected this in the text line 80.

4.5. *Lines 74-75. The authors make my point that ES, CDS, and BCS are pretty different and were mainly clustered together with ES by mistake when pathologists only had immunohistochemistry to guide them. The authors might highlight here that CDS and BCS were often inadvertently treated on ES trials when they were considered atypical EWS::FLI1 negative ES.*

Response 4.5: We totally agree with the reviewer. We also highlighted this important statement a few times in the manuscript and as followed in the abstract in line 43-45 and in the introduction in line 89 -90: "Despite being different in many aspects, including genetics, possible cell-of-origin, and pathology, patients with any of these entities all receive the same therapeutic regimen".

4.6. *It is interesting that there is a Sarcoma Culture Medium. Could the authors clarify its usage and how it was developed? Is it only for tumoroids or was it also used to establish cell lines?*

Response 4.6: The term Sarcoma Culture Medium (SCM) is indeed used to refer to the different types of medium in which the tumoroids were cultured. The basic components**,** Advanced DMEM/F-12 supplemented with P/S, HEPES, Glutamax, B27, N2 and N-Acetyl Cysteine, originate from the early days of organoid establishment in 2009 (Sato et al., 2009). With this medium as a basis, we test a lot of different growth factors. When we find conditions in which cells can be expanded, we check whether we're actually growing tumor cells by checking the presence of the tumor-specific fusion gene. To refer to the establishment of the SCM, we added the following part in the result section in lines 123-128: "As a starting point, we used a basic organoid culture medium (Advanced DMEM/F12 supplemented with penicillin/streptomycin, HEPES, Glutamax, N-2, B27 without vitamin A and n-acetyl-cysteine). Using a trial and error-based approach, we empirically tested growth factors which eventually led to the development of three types

of Sarcoma Culture Medium (SCM), all including EGF, FGF2 and IGF1, but in different concentrations (**Fig. 1a; Supplementary Table 2**)."
In this study, we did not determine if our Sarcoma Culture Medium would support cell line establishment.

4.7. *Is there a rationale for why treatment-naïve would establish better than chemotherapy-treated samples?*

Response 4.7: We find that chemotherapy treated samples often contain necrotic areas, which to our idea is the result of effective therapy. We added the following sentence (line 135-136): "Especially the ES chemotherapy-treated samples mainly consisted of necrotic tissue, which could explain the lower derivation efficiency.". These necrotic areas were mainly seen in Ewing sarcoma patients that received chemotherapy rounds (VIDE or VDC/IE) and less so in CIC::DUX4 sarcoma patients, which is consistent with the fact that the CIC::DUX4 sarcoma patients respond less well to this treatment regime.

4.8. *Lines 84-94: The authors might wish to provide a few reasons why cell lines poorly predict clinical drug response, better making the case where tumoroids offer an improvement. For example, many have shown that cells cultured as monolayers with non-physiological stiffness lose fidelity to human tumors. Further, unlike tumoroids, cell lines are removed from heterotypic cells and devoid of native ECM. Finally, the mutation rate in cell lines, often propagated for decades in the lab, are substantially higher than their human tumor counterparts.*

Respons 4.8: We have referred to a few additional references in the text and added the following sentences (lines 105-111): "These tumoroid models retain key properties of the original tumors, including cellular heterogeneity. Several studies have shown that tumoroids are valuable tools to detect entity- and patient-specific drug sensitivities with higher fidelity than regular cell lines. Patient-derived tumoroids can also be used to dissect the transforming mechanism in detail or to study the consequences of oncogenic mutations and investigate tumor cell heterogeneity."

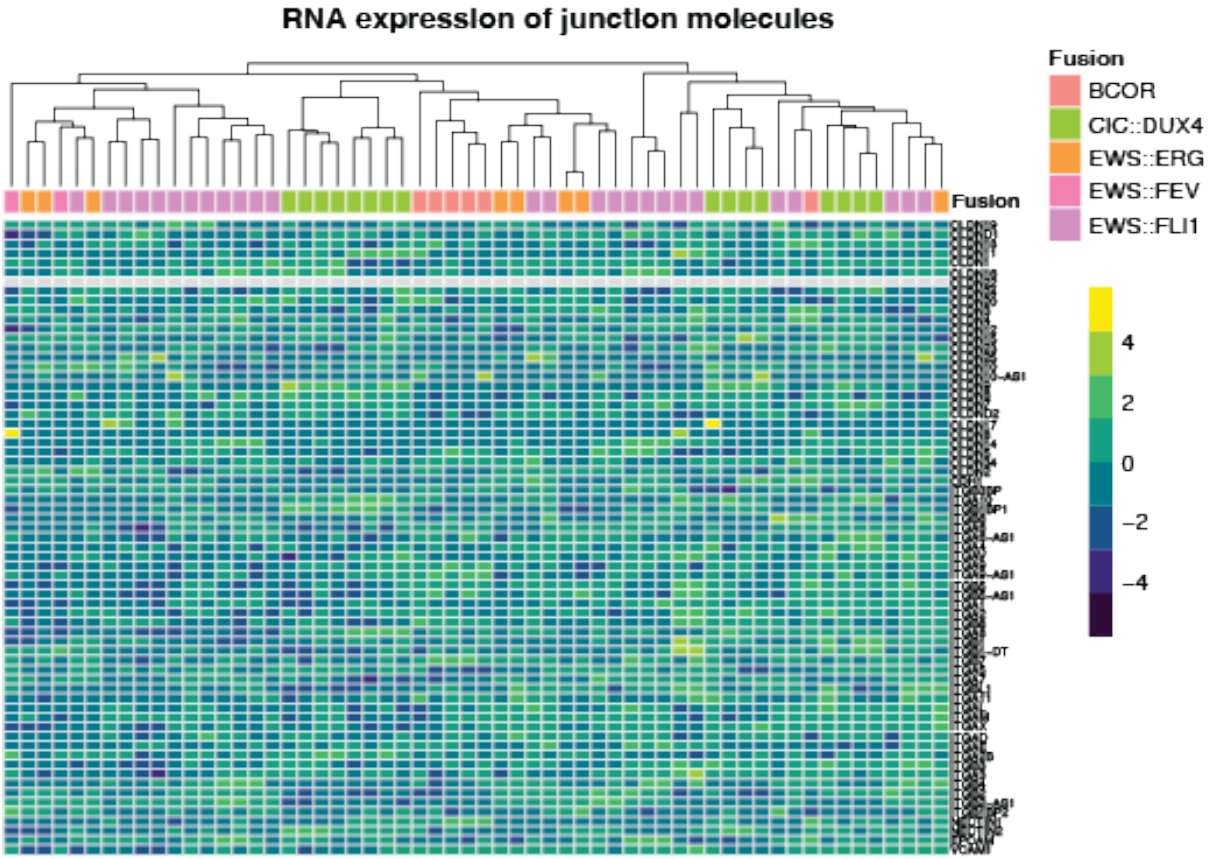
4.9. *Are there cells from the tumor microenvironment present in the tumoroids? If they exist, are they gone by passage 20? I assume so. Does this affect the drug efficacy across the passages?*

Response 4.9: The following holds for all types of tumoroids (as orginally developed in our lab), and was described by Sato et al. (Gastroenterology, 2011) and van de Wetering et al. (Cell, 2015). With the culture medium that we use, we know that only tumor cells willl expand long-term. Immune cells, stroma and blood vessels are not maintained. The drug screens have been performed between passages 5 and 10, by which time only tumor cells persist. Testing drugs that target the interplay between tumor cells and tumor microenvironment cells would require a more complex co-culture system which

contains tumoroids and cells from the tumor microenvironment. We haven't tested drug efficacy at different passages.

***4.10**. It is interesting that the different translocation-positive sarcomas demonstrate different tumoroid phenotype. Do the CIC::DUX4 cell profile have more cell-cell junctions than ES tumoroids?*

Respons 4.10: The idea that these phenotypic differences could be linked to cell-cell junctions is intriguing. We did not investigate this on protein level but we checked our RNA sequencing data for the expression of genes that are related to cell-cell junctions. There was no clear difference between the different sarcoma entities, see also attached figure below.



***4.11**. Line 123: It's unclear what the authors mean by "dark cells," which seems like non-standard terminology.*

Respons 4.11: This terminology was indeed mainly descriptive. We now rewrote this sentence, which now reads in line 153-156: "Amongst the ES tumoroids, EWSR1::ERG and EWSR1::FEV tumoroids contained more compact cell structures, indicated by a darker appearance in the center of the tumoroid. They also displayed a less uniform shape compared to the EWSR1::FLI1 tumoroids (**Fig. 1c**)."

4.12. *Given the widespread availability of proteomic technologies (e.g., cyclic IHC, RPPA, etc.), it would have been nice to better characterize protein expression along several key cell signaling cascades matching the pathways studied for tumoroid drug sensitivity (e.g., mTOR inhibitors, MCL1).*

Respons 4.12: To assess protein expression along the MCL1 signalling cascade, we performed a Western blot for MCL1 and NOXA in 2 CDS and 2 ES tumoroid lines. We refer to this in lines 394-396: "In a Western blot analysis, we saw a similar trend in MCL1 and NOXA expression in two CDS compared to two ES tumoroid samples (Supplementary Fig. 6b)."

4.13. *Line 328: While the authors mention that mTOR inhibitors affect the ES fusion protein (FP) expression, they may also wish to reference literature demonstrating that ES FP activates the IGF/PI3K/mTOR cascade via upregulation of IGF-1 and down-regulation of IGF-BP3, that together activate the upper portion of the pathway.*

Respons 4.13: Well taken. We have now added the following reference: Prieur A., Tirode F., Cohen P., and Delattre O., EWS/FLI-1 silencing and gene profiling of Ewing cells reveal downstream oncogenic pathways and a crucial role for repression of insulin-like growth factor binding protein 3, Molecular and Cellular Biology. (2004) 24, no. 16, 7275–7283, 2-s2.0-354302628, https://doi.org/10.1128/MCB.24.16.7275-7283.2004.

4.14. *Lines 382-384: Sarcoma tumoroids have been established in large-scale by another group at UCLA. The authors should discuss this work in the context of their own manuscript. What are the major differences in the establishment of the tumoroids? Are there differences in subtypes studied in either paper? Eg. Al Shihabi, Ahmad, et al. "The landscape of drug sensitivity and resistance in sarcoma." Cell stem cell (2024).*

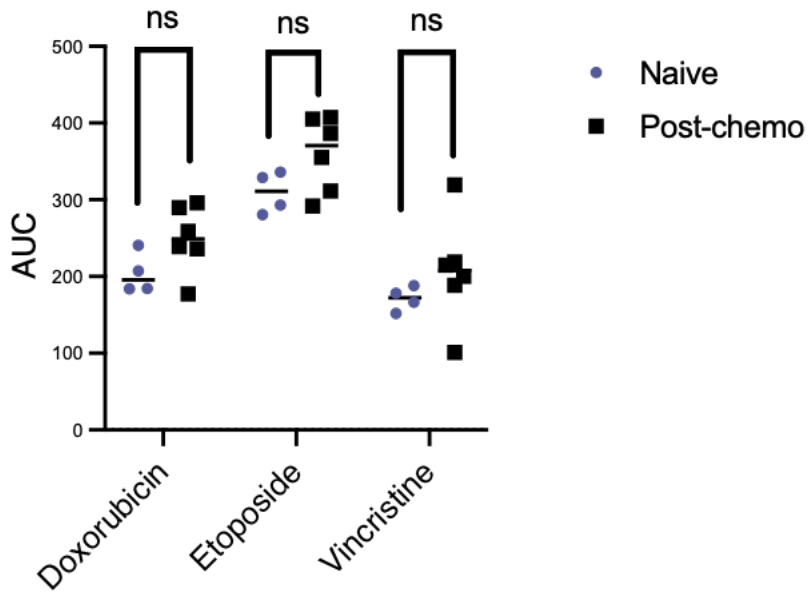
Response 4.14: The recent paper by Al Shihabi *et al*. has now been added as a reference to the manuscript. Although this study uses similar nomenclature to ours, referring to tumor organoid (tumoroid) models, we would like to point out some important differences between the models that were described by Al Shihabi *et al*. compared to the models in our manuscript. Most importantly, the tumoroid models in our manuscript can be cultured and expanded long-term. As the original 'inventors' of tissue-derived organoid technology (Sato et al, 2009; Sato et al 2011), we consider a culture to be a successful patient-derived tumoroid model when we have been able to expand it for at least 5 passages and have confirmed that the tumor driver, in this case the fusion gene, is still present in the model. We would like to point out that Al Shihabi *et al*. have performed all their analyses with short-term cultures, being established from tumor tissue 3 days prior to drug treatment. Because our tumoroids models are expandable, we were able to test over 200 drugs in duplicate in 6-8 concentrations, compared to one fixed concentration (1 µM) that was tested by Al Shihabi *et al*. Furthermore, this expansion of tumoroid lines allows us to perform repeat measurements to for validation experiments,

to directly compare Omics-data to that of the original cancer and to share our well-characterized models with the wider research community.

To refer to the manuscript by Al Shihabi *et al.*, we have added the following text in the manuscript: “Although several short-term pre-clinical models of ES have been established, such as cell lines and patient-derived tumor xenografts (PDX)[1,16], few models exist for other SRCS.”

4.15: *Is there a difference in response to drugs developed from tumoroids of different stages of diagnoses? Or even treatment-naïve vs chemotherapy-treated?*

Respons 4.15: To answer this question, we compared currently used drugs for EWSR1::FLI1 sarcoma treatment and specifically compared naïve and post-chemotherapy samples. We added this figure to Supplementary Fig. 5. We refer to this additional analysis in lines 383-388: “We also tested chemotherapeutics that are currently used in ES patients. All EWSR1::FLI1 tumoroids responded to currently used chemotherapy agents. We observed that EWSR1::FLI1 tumoroid lines derived from chemotherapy-treated tumors responded slightly less to chemotherapeutics than those derived from treatment-naïve samples. This difference was not significant, possibly due to the small sample size (**Supplementary Fig. 5b**).”



4.16*. Are there differences in sensitivity to MCL1 inhibitors for 2D cell lines vs tumoroids in CIC::DUX4? Are the tumoroids easier to establish than the cell lines?*

Respons 4.16: We agree that it would be interesting to determine whether there is a difference in drug sensitivity between CIC::DUX4 sarcoma 2D cell lines versus

tumoroids. However, the aim of our paper was to establish a small round cell sarcoma tumoroid biobank that can be used to assess drug sensitivity, and we feel that additionally testing 2D cell lines is out of scope for this paper. This would, however, be interesting to determine in future studies. As for the establishment of tumoroid lines, our protocol is very robust for treatment-naïve samples where we show a derivation efficiency of 100%. We've not made a direct comparison per tumor tissue how well we would be able to establish a tumoroid versus cell line.

4.17. *Lines 387-388: While potentially valid, it's an overstatement to suggest that tumoroid models are representative preclinical models, at least for drug sensitivity. To make this claim, the authors should have demonstrated concordance in drug sensitivity between tumoroids and PDXs or, preferably, patients treated with some of the same drugs.*

Respons 4.17: We agree that we should not overstate the predictive value of our model. To elaborate on the predictive value of tumoroids in general, we added the following sentence to the discussion in lines 451-454: "Several studies have shown that tumoroids of different cancer entities, including rectal cancer, colorectal cancer, lung cancer, head and neck cancer, pancreatic cancer, and ovarian cancer, can predict patient responses to radiation therapy, chemotherapy and targeted therapy."

4.18*. Supplemental Fig 1: It's unclear if each dot represents a tumoroid on the plate or different plates of tumoroids. It might be helpful to include a similar graph grouping growth rates by sarcoma subtype as panel B.*

Respons 4.18: We have added this information to Supplementary Fig.1a as followed "Each dot represents a different plate of tumoroids". In addition, to give more insight in the growth rates between sarcoma subtype, an additional panel has been added as Supplementary Fig.1b.

4.19. *Supp Fig 5: It might be helpful to color code the Y-axis by general drug mechanism of action. I don't see classic PI3K-Akt inhibitors listed. Also interesting is that Regorafenib showed no effectiveness in ES, which seems to go against the ES arm of the REGO-Bone study.*

Respons 4.19: We have color-coded the heatmaps y-axis by general drug mechanism as suggested.
In the REBOONE study, regorafenib has been shown to modestly delay progression-free survival is ES patients with metastatic disease. To discuss this in the manuscript, we added the following paragraph in the discussion in lines 495-500: In the REGOBONE study, regorafenib has been shown to modestly delay progression-free survival in ES patients with metastatic disease [67]. However, in our ES tumoroids, we did not observe any response to regorafenib. *In vivo*, regorafenib is heavily metabolized by the liver. Therefore,

not all active metabolites such as the M-2 and M-5 metabolites are present in our *in vitro* model, potentially explaining the lack of response to regorafenib in our ES tumoroids.

***4.20**. Another major discussion point is the drug response or utility of PDX, 2D cultures, and the patient derived tumoroids. PDX are still the gold-standard preclinical data used to support new clinical trials but the authors have not discussed the advantages of the tumoroids compared to PDX.*

Respons 4.20: We have added a section on the advances of using tumoroid models compared to PDX models in line 446-451 with additional references: "PDX models are currently the gold standard preclinical models, but these are time-consuming, take rates are generally low and they are very costly to produce and maintain. Tumoroid models can be established in a matter of weeks with high take rates, and because we can efficiently expand them, they are suitable for large-scale high-drug screening platforms".

***4.21.** Is there a protocol for cryopreservation of the tumoroids? Are re-established tumoroids affected by the cryopreservation process (growth rate, mutational burden, drug response)?*

Respons 4.21: The tumoroids can indeed be cryopreserved and thawed again. We added a part in the materials and methods section where we describe how we cryopreserved the tumoroids in line 835-836 as follows: "Tumoroids were frozen down for long-term storage in CellBanker 2 (#11914, Amsbio).". In the results section we added the following text in line 148-150: " Tumoroids were viably frozen down for long-term storage. We did not observe a clear difference in growth after a freeze-thaw cycle.". We have not performed DNA analysis to study a possible effect on mutational burden or performed drug screens both before and after cryopreservation, this could be interesting to assess in a follow-up study.

***4.22**. Please include data tables associated with the heatmaps. It is unclear which drugs are where in the heatmaps. Some are called out like MCL1 inhibitors or mTOR inhibitors, but others are not in figure 5.*

Respons 4.22: Data tables associated with heatmaps are attached as Source data file.
Minor Points:

***1.** Figure 1: The font on panel B is unreadable, and there's room to increase it without affecting the overall figure size. The axis in panel B has no label. Does this represent days or months?*

Response 4.1: We adjusted Fig. 1b and added the label "Sample number" to the axis.

2. *Figure 2: Images missing for ES-053. Also, the naming (e.g., ES-053) implies ES. Instead, I strongly suggest the authors relabel the samples by CDS or BCS instead of ES whenever appropriate for all figures.*

Response 4.2: Unfortunately, for this sample there was no material available, so we were unable to perform the additional IHC stainings for this sample. To avoid any confusion, we decided to remove this sample from the IHC overview pictures. Regarding the sample names/labels in all figures, we adjusted this as follows: Ewing sarcoma (ES), CIC::DUX4 sarcoma (CDS) and BCOR-rearranged sarcoma (BRS).

3. *Figure 4. The color difference of Type in Panel B is barely detectable. Suggest using a different color pattern to make it easier for the readers.*

Response 4.3: We changed the colors of the different tumor types in Fig.4b.

4. *Figure 4 font and symbols are too small to see. Panel A symbols are difficult to distinguish at the current size without zooming in. It would be nice to connect figure 4D to 5B. Otherwise, it is difficult to understand if these drugs are having on-target effect or have any efficacy due to the expression of these drug targets.*

Response 4.4: We increased the size of the symbols in Fig. 4. We show drug response to targeted drugs in supplementary Fig 5c. Here we added a colorcoding to highlight the specific pathways these drugs target. This makes it easier to connect to the expression data shown in Fig. 4d.

5. *Fonts in Figure 5 are too small to read. Panel H it may be unnecessary to call out the individual tumoroids and keep it only as the translocation type. A larger figure can be included in the supplementary with the individual sample types called out.*

Response 4.5: We changed all fonts in Fig.5. In panel H we replaced all individual samples names to their translocation type. All individual samples names can be found in Supplementary Fig. 5c.

6*. Figure 5. As above, suggest relabeling ES-086, ES-087, and other samples that harbor a CIC::DUX4 rearrangement as CDS-086, CDS-087, etc. Take the same approach for BCOR-rearranged tumors, using "BCS" or "BCK" where appropriate. If the authors push back against sample renaming, at the very least, they should consistently name the samples as "ES-" vs. "ELS-". More precise labeling will make it easier for readers and will be important when depositing data in GEO.*

Response 4.6: We corrected the sample names/labels in all figures, we adjusted this as followed: Ewing sarcoma (ES), CIC::DUX4 sarcoma (CDS) and BCOR-rearranged sarcoma (BRS).

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

I thank the authors for addressing most of my comments. I have the following clarification, as one of the studies referenced in this new version has been incorrectly addressed:

"Although several short-term pre-clinical models of ES have been established, such as cell lines and patient-derived tumor xenografts (PDX)16,17. Using cell lines has several disadvantges, including a recent study has described a screening platform for SRCS using short-term in vitro cultures16."

This is incorrect. The study from Al Shihabi et al (now ref 16) is establishing patient-derived tumor organoids from clinical samples directly – no cell lines or xenografts. I would ask that the authors correctly reference the paper, instead of mentioning it is using cell lines which is misrepresenting the work entirely.

We thank the reviewer for their feedback and for this clarification, as the previous paragraph was indeed not written down correctly. We seem to have made an error in the previous revision process when adding and removing texts, as we are very much aware that the study from Al Shihabi et al. does not describe cell lines. We have adapted the paragraph, now in lines 97-98 to the following text: "Although several pre-clinical models of ES have been established, such as cell lines and patient-derived tumor xenografts (PDX)[16,17], few models exist for other SRCS. It's possible to use cell lines in long-term and high-throughput screening analyses, but they have several disadvantages, including clonal outgrowth and very low establishment success percentages[18]. A recent study has described a screening platform for SRCS using short-term patient-derived *in vitro* cultures[19]. For some pediatric tumor entities, including Wilms tumors, rhabdomyosarcomas, atypical teratoid/rhabdoid tumors and small cell carcinoma of the ovary, hypercalcemic type (SCCOHT), long-term patient-derived tumor organoid (tumoroid) models have been reported. These tumoroid models allow for sustained *in vitro* expansion and retain key properties of the original tumors, including cellular heterogeneity[18-22]. Several studies have shown that tumoroids are valuable tools to detect entity- and patient-specific drug sensitivities with higher fidelity than regular cell lines[23-31]. Patient-derived tumoroids can also be used to dissect the transforming mechanism in detail or to study the consequences of oncogenic mutations and investigate tumor cell heterogeneity[18,19]. In this study, we aim to establish a living tumoroid biobank of SRCS."

Reviewer #2 (Remarks to the Author):

Thanks to the authors for answering most of the reviewers' comments and modifying the manuscript accordingly. While the authors answered most of my comments, I still have minor comments; in order of the manuscript:

We thank the reviewer for a very thorough look through the manuscript which has allowed us to correct several mistakes that we had previously overlooked.

It is still unclear which samples was processed by which techniques (WGS,WGS,RNAseq….). Again, a single spreadsheet with all the information would be much clearer, like:
Patient ID Tumor Sample Tumoroid
ID tech ID Tech
M1 ES-001TT WGS,WXS,RNA ES-001TO RNA
M1 ES-001TT WGS,WXS,RNA ES-002TO WGS
M2 … …. .. ..

We agree that it would be clearer to add this information in a spreadsheet. We adapted Supplementary Table 1, and added Column "Analysis Tumor" and Column "Analysis Tumoroid", where we specified the techniques that were used for the individual samples.

Line 87 : CCNB3 instead of CNNB3

We corrected this the text.

Line 192-4 : "We created mutation matrices and used these to calculate Jaccard's distance between the samples. This revealed that the tumoroids genetically resemble their tumor counterparts (Fig. 3b, Supplementary Fig. 3b-c)".

Fig 3b shows circos plots for a single Tumor-tumoroid couple and with the exception of the CNV, it clearly shows strong differences in SNVs and translocations that do not support the "resemblance". I acknowledge the efforts made by the authors to present the resemblance between tumoroïds and their respective tumor sample using Jaccard's distance and discussing some observed dissimilarities but I would have also liked the authors to comment on some couples that apparently seems not similar (high Jaccard distance as seen in the heatmap) (TT and TO of BRS-057, ES-041 or ES-071 for example).

Certain TT-TO pairs indeed show relatively large Jaccard's distances in Fig. 3b, e.g., ES-042 and ES-071. This distance is caused by a difference in sequencing method, i.e., WXS versus WGS. We have now added a sentence to address this in the Result section in lines 196-199, which now reads: "Some TT-TO paired samples were sequenced with different techniques, i.e., WXS versus WGS. These techniques cover different parts of the genome and share fewer variants, which explains why some pairs show relatively large Jaccard's distances (e.g., ES-042 and ES-071)."

Supp Fig3b: I guess that the circos of the BRS-057TO is "corrupted" because it loses all the translocations, including the oncogenic one…

The circos plot of BRS-057 TO indeed shows no translocations, which is the case because we have no RNAseq data for this sample, and therefore no input that the circos plot pipeline can use to show the translocations. We added this information in the figure caption for Supplementary Fig. 3b. The previously mentioned addition of the techniques used per sample in Supplementary Table 1 will further clarify that RNAseq data was not available for this sample.

Line 195-6: "Also, several SRCS tumoroids showed CNAs such as gain of 1q, 12 and 8, and loss of 9 (Supplementary Figure 3a-b)". I do not see any samples in the figures with a loss of chr9.

Thank you for noticing, we corrrected this sentence in line 199-200, which now reads: "Also, several ES tumoroids showed CNAs on chromosomes 1, 8, 9 and 12 (Supplementary Fig. 3a-b),"

Line 219: "As shown in Fig.3b," should probably be Fig 3c and supp Fig 3a.

Indeed, we corrected this in the text.

Line 258: Supp Fig 3e is referred to for the first time after supp fig 3f, please reorder

We changed the order of the figures in Supplementary Fig. 3 and have corrected this in the text.

Fig 4c: What is the color code (Expression, ChIP peak heights)?

The color code in the scalebar indicates expression. To clarify, we have corrected the sentence in the legend text as followed: "Heatmap showing the expression of the top 20 differentially expressed genes per tumor entity that have a CHIPseq binding peak of their characteristic (fusion-)protein in close proximity to the transcription start site.

Lines 390 and 400: Fig 5c should rather be Supp Fig 5d or Fig 5H, no?

Indeed, we corrected this in line 393.

Supp Fig5 d&e: names of samples were not corrected (all ES-XXX)

We corrected this in Fig. 5g and Supplementary Fig. 5d-e

Figure 5a: Color codes for fusions in heatmaps do not correspond between the legend and the heatmap itself (pink and orange were inverted). It would be easier for the reader that the color codes stay similars between all the figures (including the supplementary figures): Ex: BCOR::CCNB3 is red in Fig4a, black in Fig 4b&c, purple in Supp Fig 4b, blue in Supp Fig 5c….

Having the color codes stay similar throughout all figures would indeed provide a better overview of our results and make it easier for the reader. We have made the required changes in Fig. 5g and Supplementary Fig. 5d-e

The organization of the figures corresponding to the drug screening (Fig 5 and Supp Fig 5) is confusing as supp fig 5c is (wrongly) referred to throughout this section and that Fig 5H is identical to supp fig 5E (which is never referenced in the main text)

We have corrected the reference to Supplementary Fig. 5c to Supplementary Fig. 5d-e. We have removed Supplementary Fig. 5e as this was indeed identical to the figure that was previously indicated as Fig. 5h. We additionally noticed that we skipped Fig. 5g in Fig. 5, so Fig. 5h has now been re-named Fig. 5g.

Supp Fig6a: Could the authors also present the expression value of MCL1 and NOXA for the tumor tissue samples?

Yes of course, we added the expression plots of the tumor tissue to the Supplementary Fig. 6a.

For the final experiment (looking for apoptosis, Supp Fig 7) FACS profiles (which could be quantified) should be presented instead of cell imaging. Moreover, could the authors comment on why the ES-046 cells seem to grow as adherent cells and not as tumoroids?

We indeed attempted to perform FACS in order to quantify the apoptotic signal. However, we noticed that the tumor cells did not respond well to the FACS procedure and many cells died because of the harsh dissociation methods that are required to make them single cells. We therefore chose to go for cell imaging, a technique that did not require harsh dissociation.
With respect to the adherent cells: we use a small percentage of BME (0.2%) in our culture medium, which will inherently provide a coating of the well that cells can easily adhere to. Although ES tumoroid lines in general grow as very nice spheres, they also don't mind to grow in 2D so we regularly see cells that adhere to the BME coating on the bottom of the well when using culture treated plates instead of suspension plates.

In the last sentences of the conclusion (lines 522-23) the authors may comment on the combination of MCL1 inhibitors with other drugs that has been investigated in Ewing sarcomas (doi 10.1016/j.canlet.2022.216028).

This is indeed an interesting paper. We have now included this in the Discussion in lines 521-525:
"In future studies, it would be interesting to perform combination screens to identify drugs with synergistic effects, which could lower required dosing and thereby reduce MCL-1-related toxicity. For example, a recent study in zebrafish xenografts showed that dual targeting of MCL-1 and BCL-X_L effectively targeted ES cells ."

Lines 1002 & 1145: There is no "s" in "Jaccard"

Thank you, we corrected this in line (Methods) and in legend text Supplementary Fig. 3 in line 1158.

Reviewer #3 (Remarks to the Author):

The authors answered all my comments. I would like to congratulate them for this

interesting study.

We thank the reviewer for their congratulations and again for critical analysis of our work which helped to improve the manuscript.

Reviewer #4 (Remarks to the Author):

The authors have adequately addressed my concerns.

We again thank the reviewer for critical analysis of our work which helped to improve the manuscript.

Reviewer #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We again thank the reviewer for critical analysis of our work which helped to improve the manuscript and hope that is was a useful training in peer review.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

We again thank the reviewer for critical analysis of our work which helped to improve the manuscript and hope that is was a useful training in peer review.