Mesenchymal tumor organoid models recapitulate rhabdomyosarcoma subtypes

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Abstract

Rhabdomyosarcomas (RMS) are mesenchyme-derived tumors and the most common childhood soft tissue sarcomas. Treatment is intense, with a nevertheless poor prognosis for high-risk patients. Discovery of new therapies would benefit from additional preclinical models. Here, we describe the generation of a collection of pediatric RMS tumor organoid (tumoroid) models (success rate of 41%) comprising all major subtypes. For aggressive tumors, tumoroid models can often be established within 4–8 weeks, indicating the feasibility of personalized drug screening. Molecular, genetic, and histological characterization show that the models closely resemble the original tumors, with genetic stability over extended culture periods of up to 6 months. Importantly, drug screening reflects established sensitivities and the models can be modified by CRISPR/Cas9 with TP53 knockout in an embryonal RMS model resulting in replicative stress drug sensitivity. Tumors of mesenchymal origin can therefore be used to generate organoid models, relevant for a variety of preclinical and clinical research questions.

Keywords: rhabdomyosarcoma; mesenchymal; tumoroid; drug screening; CRISPR/Cas9

Subject Categories: Cancer; Methods & Resources

Introduction

Rhabdomyosarcoma (RMS) is a type of malignant tumor of mesenchymal origin (Yang et al., 2014) and forms the most common soft tissue sarcoma in children and adolescents (Li et al., 2008). Historically, RMS has been divided into two main subtypes based on histology. Whereas embryonal RMS (eRMS) displays cellular heterogeneity and hallmarks of immature skeletal myoblasts (Patton & Horn, 1962), alveolar RMS (aRMS) cells are distributed around an open central space, thereby resembling pulmonary alveoli (Enterline & Horn, 1958). eRMS is more frequently observed in children under 10, accounting for two-thirds of all RMS cases, and generally has a better prognosis than aRMS, which is more common in adolescents and young adults (Perez et al., 2011). In aRMS, a sole genetic driver alteration is usually observed, caused by a chromosomal translocation resulting in a fusion gene between either PAX3 or PAX7 and FOXO1. In contrast, eRMS is genetically more heterogeneous, harboring mutations in several common oncogenes or tumor suppressor genes (Shern et al., 2014). Other subtypes of RMS have recently been recognized (WHO, 2020). RMS treatment is guided by protocols developed by multinational collaborative groups and includes systemic chemotherapy in addition to local therapy (radiotherapy and/or surgery; Skapek et al., 2019). The prognosis of RMS has improved over the last decades (Bisogno et al., 2019). For patients with high-risk, refractory, or relapsed disease, prognosis remains poor however, despite an immense treatment burden (Pappo et al., 1999; Mascarenhas et al., 2019). Thus, development of new therapeutic options is of critical importance for these patients.
Development of such treatment options requires in vitro models and may therefore benefit from application of organoid technology. The basis of this technology is that given a suitable growth environment, tissue stem cells self-renew as well as give rise to natural progeny which organize according to their preferred growth modality without the need for artificial cell immortalization. The technology was first established in healthy epithelial tissue from mouse small intestine (Sato et al., 2009) and soon adapted to various other healthy and diseased epithelial tissues, including cancer (Clevers, 2016). Tumor organoid (tumoroid) systems are proving useful in cancer research as they display genetic stability over extended culture periods, retaining the molecular characteristics of the tumor they are derived from. While dedicated co-culturing tumoroid systems of tumor and nontumor cells are starting to be developed (Yuki et al., 2020), the majority of tumoroid systems consist only of tumor cells. Tumoroid models can be expanded, facilitating high-throughput screening approaches such as small molecule or CRISPR/Cas9-knockout screening (Bleis et al., 2019).

To date, tumoroid approaches have been primarily applied to cancers derived from epithelial cells (i.e., carcinomas). Recent studies demonstrate that deriving tumoroid models from nonepithelial cancer is feasible but this has as yet not been achieved for pure mesenchymal cancers (Fusco et al., 2019; Jacob et al., 2020; Saltzman et al., 2020; Abdullah et al., 2021; Yamazaki et al., 2021). Application to tumors of mesenchymal origin such as RMS would be of obvious benefit. Tumoroid models of pediatric nephroblastoma (Wilm’s tumor) have been described, which, depending on the subtype, can contain stromal cells (Calandrini et al., 2020). In addition, cells derived from synovial sarcoma and other adult soft tissue sarcomas can grow to a limited extent on fetal calf serum, which, although undefined in terms of the required essential growth factors, also indicates feasibility (Brodin et al., 2019; Boulay et al., 2021). Furthermore, in vitro propagation of RMS tumor cells derived from patient-derived xenograft (PDX) mouse models has recently been shown (Manzella et al., 2020). Although these results are encouraging, no directly patient-derived collection of tumoroid models of malignant tumors of pure mesenchymal origin (i.e., sarcomas) has been generated and studied after growth for extensive periods in well-defined media components. In this study, we therefore set out to develop and apply approaches for generating a collection of tumoroid models that covers the major RMS subtypes, a pediatric cancer of mesenchymal origin with poor outcome for high-risk patients. Besides generating and extensively characterizing the tumoroid collection, we also investigated applicability for drug screening and genetic modification (Fig 1A).

Results

A protocol to collect and process RMS tumor samples for tumoroid model establishment and propagation

Before starting to generate a collection of RMS tumoroid models, we first optimized sample acquisition and logistics between surgery, pathology, and organoid culture labs (Materials and Methods). In parallel to optimizing sample acquisition, we also optimized sample processing, including testing different formulations of growth media by a combination of systematic and trial and error approaches (Discussion). RMS tumor samples are diverse. Most samples are small needle biopsies (i.e., 16-gauge tru-cut), as large resection specimens are mostly restricted to pretreated RMS or to treatment-naive paratesticular fusion-negative eRMS (FN-eRMS). In addition, a subset of samples (4% here) are not solid, being acquired as bone marrow aspirates of infiltrating tumor cells (Fig 1B). Samples are plated as minced pieces embedded in a droplet of extracellular matrix (ECM) substitute (Basement-Membrane Extract, BME) and as single-cell suspensions in BME-supplemented medium. Outgrowth of tumor cells to tumoroid models can occur from both modalities. In the case of successful outgrowth of initially plated cells, cells organize as two-dimensional monolayers (Fig 1C). This appears to be the cells’ preferred growth modality, as plating them as single-cell suspensions in BME droplets results in cells escaping the surrounding matrix and sinking to the bottom of the culture plate from which they continue to grow in a monolayer. Therefore, cells are further propagated and expanded in this way. We considered an RMS tumoroid model to be successfully established if, over the course of culturing, the expression of specific tumor markers is retained and the culture expansion is at least sufficient for drug screening, all as described below (Table 1).

Early detection of tumor cells during culturing

Tumors consist of a variety of different cell types. These include normal cell types that can grow as well or even better in the provided culture conditions, possibly outcompeting tumor cells (Dijkstra et al., 2020). It would therefore be useful to test for the presence of tumor cells early during culturing to omit the unnecessary propagation of cultures lacking any. At early time points, material is limited, impacting the range of applicable assays. The establishment protocol therefore utilizes an RT-qPCR assay after the first or second passage of cells with probes for standard RMS histopathology markers, that is, DES, MYOG, MYOD1 (WHO, 2020), and the fusion transcript in fusion-positive RMS (FP-RMS; Ponce-Castañeda et al., 2014). We considered a sample positive for tumor cells if at least one of the three genes, plus for FP-RMS the fusion transcript, tests positive. All samples that successfully yield tumoroid models, show positivity for at least one marker gene at this stage, while most models (17 out of 19) are positive for all three marker genes and the fusion transcript if applicable (Fig 2A and B). The RT-qPCR-based approach is therefore a useful tool to determine feasibility at an early stage.

RMS tumoroid models retain marker protein expression and display heterogeneity in gene expression

A hallmark of RMS tumors is the expression of proteins associated with nonterminally differentiated muscle (i.e., Desmin, Myogenin, and MYOD1). Expression of these proteins differs between RMS subtypes (Dias et al., 2000) and can be associated with prognosis (Heerema-McKenney et al., 2008). To properly reflect the original tumors, RMS tumoroid models should therefore retain the expression patterns of these proteins. The RMS tumoroid establishment protocol therefore includes a morphological (H&E) and immunohistochemical (IHC) assessment at the time of successful establishment (i.e., drug screening). To enable comparison between tumoroid models and the tumors they were derived from, models are grown
Figure 1. A collection of RMS tumoroid models that represent the diverse clinical presentation of RMS.

A Tumor organoid (tumoroid) pipeline. WGS, whole-genome sequencing; RNA-seq, mRNA sequencing; liq N2, liquid nitrogen.

B Overview of available RMS tumoroid models in the collection separated by primary versus metastatic site and exact tumor location. The color of the inner circle indicates the histological subtype while the color of the outer circle indicates the presence or absence of a fusion transcript. Letters within the circle indicate disease instance. Asterisks mark tumoroid models derived from the same patient but from distinct tumor samples.

C Brightfield microscopy images of two representative RMS tumoroid models from a fusion-negative embryonal and a PAX3-FOXO1 fusion-positive alveolar tumoroid model grown in a two-dimensional monolayer in two magnifications as indicated by the scale bars.
Table 1. Overview of available RMS tumoroid models in the collection. Patients are numbered to allow identification of RMS tumoroid models derived from the same patient. For additional clinical parameters see Dataset EV1.

| Tumoroid model | Patient | Patient birth year | Histology | Fusion transcript | Patient sex | Disease instance of tumoroid establishment | Body site of sample used for tumoroid establishment |
|----------------|---------|--------------------|-----------|------------------|-------------|-------------------------------------------|--------------------------------------------------|
| RMS001        | 1       | 2001               | Embryonal | PAX3-FOXO1       | Male        | First relapse                             | Arm                                              |
| RMS002        | 2       | 2000               | Embryonal | PAX3-FOXO1       | Male        | Second relapse                            | Abdomen                                         |
| RMS003        | 3       | 2001               | Embryonal | PAX3-FOXO1       | Male        | Second relapse                            | Bone marrow metastasis (clavicle)                |
| RMS004        | 4       | 2001               | Embryonal | PAX3-FOXO1       | Male        | First relapse                             | Kidney                                          |
| RMS005        | 5       | 2003               | Embryonal | PAX3-FOXO1       | Male        | Primary disease                           | Bone marrow metastasis                           |
| RMS006        | 6       | 2004               | Embryonal | PAX3-FOXO1       | Male        | Second relapse                            | Calf                                            |
| RMS007        | 7       | 2004               | Embryonal | PAX3-FOXO1       | Female      | Second relapse                            | Subcutaneous metastasis                         |
| RMS008        | 8       | 2018               | Embryonal | PAX3-FOXO1       | Female      | Primary disease                           | Arm                                              |
| RMS009        | 9       | 2010               | Embryonal | PAX7-FOXO1       | Female      | Second relapse                            | Lymph node metastasis (groin)                   |
| RMS010        | 10      | 2011               | Embryonal | PAX7-FOXO1       | Male        | First relapse                             | Cerebral metastasis                             |
| RMS011        | 11      | 2014               | Embryonal | PAX7-FOXO1       | Female      | Primary disease                           | Leg                                              |
| RMS012        | 12      | 2003               | Embryonal | Negative         | Male        | Primary disease                           | Paratesticular                                  |
| RMS013        | 13      | 2013               | Embryonal | Negative         | Male        | Primary disease                           | Paratesticular                                  |
| RMS006        | 14      | 2014               | Embryonal | Negative         | Male        | Primary disease                           | Paratesticular                                  |
| RMS007        | 15      | 2005               | Embryonal | PAX3-WWTR1       | Male        | Primary disease                           | Shoulder                                        |

as three-dimensional spheres to mimic the three-dimensional architecture of tumors (Fig 2C). When evaluated in this manner, RMS tumoroid models show retained expression patterns of Desmin, Myogenin, and MYOD1 at the time of successful establishment (i.e., drug screening), comparable to parental tissue. Cellular morphology is also retained, with aRMS models displaying homogenous, primitive cells, with large nuclei, and minimal cytoplasm, and eRMS models displaying more heterogeneous tumor cells with variable maturation (Figs 2C and EV1A), as in the tumors (, 2020). Lastly, additional H&E and IHC staining performed on two different passages of two tumoroid models (i.e., after the acquisition of the tumor sample and before the drug screening was performed;
Fig EV2A) show a high concordance concerning the heterogeneity of marker protein expression when compared to the primary tumor and the tumoroid model at the time of drug screening (Fig 2C). This indicates that there are no major changes concerning the composition of tumor cells acquired over the course of expanding the culture for drug screening.
In line with the heterogeneous expression of these marker proteins, single-cell RNA sequencing (scRNA-seq) shows that RMS tumoroid models retain heterogeneity in the expression of the corresponding genes (i.e., MYOG, MYOD1, and DES; Fig 2D). Importantly, this heterogeneity is not due to differences in cell cycle activity as differential gene expression patterns do not correspond with the expression of the cell cycle marker MKI67 (Fig 2D). Single-cell RNA-seq inferred per cell copy-number plots also suggest genomic heterogeneity, and furthermore show that RMS tumoroid models consist only of tumor cells as all analyzed cells in the samples show copy-number alterations in agreement with whole-genome sequencing inferred copy-number plots (Fig EV2B). In summary, RMS tumoroid models retain histopathological hallmarks of RMS tumors as well as display transcriptional heterogeneity in line with heterogeneous protein expression at the time of drug screening, giving a first indication that they reflect the tumors they were derived from to a high extent.

**A collection of RMS tumoroid models that represent the diverse clinical presentation of RMS**

Having established protocols for the acquisition, processing, initial growth, and characterization of RMS tumoroid models, we applied this to 46 consecutive samples from pediatric RMS patients treated in the Netherlands, resulting in a collection of 19 RMS tumoroid models (41% efficiency) (Table 1). These models are derived from tumors comprising both main histological subtypes (embryonal and alveolar), different fusion types (fusion positive PAX3-FOXO1, PAX7-FOXO1, a novel fusion PAX3-WWTR1, as well as fusion negative), various risk groups, locations, and are derived from primary as well as relapsed disease (Fig 1B and Dataset EV1). Outgrowth of cells from highly aggressive RMS subtypes shows a higher success rate, as indicated by 83% successful establishment for FP-RMS versus 16% for FN-RMS, and 61% success in relapsed, versus 30% in primary RMS tumors, respectively. There was no statistically significant difference in the success rate between recently treated and untreated tumors (33% versus 43%, respectively, \( P = 0.59 \), chi-square test). Similar differences between relapsed and primary tumors in establishment rates have been observed for orthotopic PDX models (O-PDX) of RMS (Stewart et al., 2017). Compared to O-PDX models of RMS, the overall success rate is lower for establishing RMS tumoroid models (41% versus 65%, respectively). While the success rate is lower, RMS tumoroid models can be more rapidly established. Engraftment of O-PDX RMS models takes 1–5.5 months before tumor growth is first observed in mice, while RMS tumoroid models can be fully established, that is, characterized and subjected to drug screening, in as little as 27 days for highly aggressive tumors. Overall, the median time from acquisition of the tumor sample to successful drug screening was 81 days (with seven models being screened in less than 2 months). This indicates that our approach could potentially be applied in a personalized medicine setting where it is crucial to obtain results as fast as possible to provide information on treatment options.

A subset of the tumoroid models were derived from the same patient, but at different points during treatment and/or from different body sites (marked with one or more asterisks in Fig 1B). This potentially facilitates studies of tumor evolution or acquired treatment resistance. Furthermore, the collection contains a model of an eRMS with a fusion between PAX3 and WWTR1, which has not previously been described in RMS. Such fusions have been reported as rare events in bifenotypic sinonasal sarcomas, which usually harbor PAX3-MAML3 fusions (Le Loarer et al., 2019). Taken together, the protocol efficiently yields tumoroid models from highly aggressive as well as from extremely rare RMS subtypes, resulting in an initial collection covering a broad spectrum of subtypes.

**RMS tumoroid models molecularly resemble the tumor they are derived from**

Given that the present tumoroid collection is the first to be established from tumors of purely mesenchymal origin, we asked to what extent the models further resemble the tumors they were derived from besides the retained hallmark protein expression levels and patterns described above. To this end, whole-genome sequencing (WGS) and bulk mRNA sequencing (RNA-seq) of the tumor and tumoroid model at the time of drug screening (i.e., successful establishment), were performed.

Copy number profiles were first compared between tumors and tumoroid models showing that profiles are indeed highly concordant (Fig 3A). The detected copy number alterations included those commonly observed in RMS, with genomic gains in chromosome 8 in FG-RMS and gains in chromosome 1 and 12 in FP-RMS (Weber-Hall et al., 1996; Shern et al., 2014). In addition, copy number profiles of individual RMS tumors and derived tumoroid models show a high concordance (Fig EV3A and B). This indicates that the established models resemble the tumors they were derived from on a more global genomic level.

Various mutational processes are active in cells, which cause distinct somatic mutational signatures. These signatures are characterized by specific patterns of single base substitutions (SBS) in the context of their two flanking bases ( Alexandrov et al., 2020). The presence of certain somatic mutational signatures in a cell can be associated with the underlying mutational processes. These processes are not restricted to in vitro systems, but can also occur during culture (Petlija et al., 2019), forming a potential source of genomic destabilization. To test whether the somatic mutational signatures and thus the underlying mutational processes present in the tumors (“T”) are concordant with those in the derived tumoroid models (“O”), we first measured the relative contributions of different signatures per sample. The main signatures observed are signatures associated with cellular aging (SBS1 and SBS5), a signature associated with increased oxidative stress (SBS18; Alexandrov et al., 2020), and a signature associated with exposure to the chemotherapeutic Temozolomide (TMZ; Kucab et al., 2019) used in the treatment of RMS (Defachelles et al., 2021; Fig 3B). Signatures associated with cellular aging (SBS1 and SBS5) show a significant correlation with patient age only for SBS1 and only in RMS tumoroid models \( P = 0.02 \) but not tumors \( P = 0.17 \). Furthermore, FP-RMS tumors and tumoroid models show a significantly higher contribution of SBS1 (but not SBS5) to their overall somatic mutation frequency compared to FN-RMS tumors and tumoroid models \( P = 0.02 \) and \( P = 0.006 \), respectively. However, patients with FP-RMS tumors were older than patients with FN-RMS tumors (median 14 years versus median 6 years, respectively), which may have influenced this. Importantly, the signatures detected in the tumoroid models are highly concordant with those detected in the original
Figure 3. RMS tumoroid models molecularly resemble the tumor they are derived from.

A Copy number frequency plots of RMS tumors (upper row) and derived RMS tumoroid models (lower row) divided by fusion-status (columns). Chromosomes are annotated on the x-axis from left to right while the y-axis shows the percentage of samples in this group carrying a gain (red) or loss (blue) in this genomic region.

B Contribution of somatic mutational signatures per tumor and tumoroid model. SBS, single base substitution; TMZ, temozolomide; T, tumor; O, tumoroid.

C Table depicting pathogenic single-nucleotide variants (SNVs) in RMS tumors (T) and tumoroid models (O). Circle color indicates SNV type while circle size indicates variant allele fraction (VAF). Vertical dotted lines separate samples derived from individual patients. Highlighted are genes previously reported for this RMS subtype.

D Correlogram of bulk mRNA sequencing expression profiles of pediatric kidney tumors (controls) as well as RMS tumoroid models and RMS tumors. CCRCC, Clear Cell Renal Cell Carcinoma; CMN, Congenital Mesoblastic Nephroma; WT, Wilms’ Tumor; Cor, correlation.
tumors (Fig 3B). In line with this, no global differences in SBS profiles between RMS tumors and derived tumoroid models are detected when analyzed collectively (Fig EV3C). Lastly, we calculated the similarities of the individual SBS profiles of all tested tumor and tumoroid model samples. Tumoroid models cluster with the tumors they are derived from, indicating that the mutational landscape is retained in the models (Fig EV3D). Only the tumor and tumoroid model of RMS127 do not cluster closely, likely due to derivation from a bone marrow aspirate with low tumor cell infiltration (5–10% as estimated by pathology). Overall, there is a high concordance between the somatic mutational signatures of RMS tumors and the derived tumoroid models, again illustrating that the established models resemble the patient tumors.

While FP-RMS are driven by the prototypal fusion genes, FN-RMS are characterized by SNVs in known oncogenes such as TP53 or RAS family members (Shern et al., 2014). Currently available preclinical models of RMS do not cover the full spectrum of these SNVs, thus limiting the applicability to test novel targeted drugs in RMS. To investigate whether the established RMS tumoroid models harbor any of these SNVs and whether these were already present in the tumor, all SNVs predicted to be pathogenic for protein function and with a variant allele fraction (VAF) of above 0.3 in either tumor and/or tumoroid were evaluated. As already indicated by the mutational signature analyses, RMS tumoroid models retain SNVs present in the tumor to a high degree (Fig 3C). Similarly, samples from the same patient but from different sites or acquired from different relapses also show a high overlap in SNVs (Fig 3C). In agreement with a recent study investigating mutational frequency in different tumor types (Casey et al., 2020), the FN-RMS tumoroid models show a higher somatic mutation frequency than the FP-RMS tumoroid models (P = 0.02; Fig EV3E) indicating that the models are representative for this characteristic, as well.

Two of the FN-eRMS tumoroid models harbor previously described oncogenic mutations for FN-eRMS, that is, CTNNB1 (p.T41A) in RMS012 and FGFR1 (p.V550L) in RMS444 (Shern et al., 2014). To our knowledge, RMS012 is the first preclinical RMS model harboring this specific mutation. Moreover, one PAX3-FOXO1 FP-aRMS (RMS410) displays an oncogenic mutation in KRAS (p.G12A), which is uncommon in FP-RMS (Shern et al., 2014). Lastly, two FN-RMS tumoroid models (RMS007 and RMS012) display non-annotated frameshift mutations in the BCOR gene with a high VAF of above 0.9. Mutations in BCOR have been reported in RMS with a higher prevalence in FN-RMS compared to FP-RMS (Shern et al., 2014). The mutation analysis shows that RMS tumoroid models not only retain specific SNVs already present in the tumor but that these models also contain mutations for which no preclinical model was previously available.

Tumors are composed of different tumor cell clones that can undergo processes such as clonal expansion, genetic diversification as well as clonal selection (Greaves & Maley, 2012). To assess to what degree established RMS tumoroid models reflect the clonal composition of the tumor they were derived from, we performed two analyses on the matching WGS data.

First, we compared the VAFs of SNVs in coding regions which showed no copy-number alterations or loss of heterozygosity (LOH; Fig EV4A). For the majority of samples, SNVs with a VAF of around 0.5 (indicating a major clone in the population given the filtering criteria for SNVs described above) can be detected in tumor and tumoroid, indicating that major clones are retained. Furthermore, the presence of SNVs with VAFs of below 0.5 indicates subclonal populations. Again, such SNVs with matching VAFs in tumor and tumoroid samples can be detected, indicating that subclonal populations are also largely retained in the established RMS tumoroid models. In addition, a subset of SNVs present in the tumoroid with a high VAF is not present in the tumor with the chosen cut-offs, indicating that our method may enrich for such clones. Importantly, none of these SNVs are found in reported oncogenic driver genes, indicating that these are likely passenger mutations.

In the second analysis performed on the matching WGS data, fractions of alternate alleles (B-alleles) were compared between matching tumors and tumoroid models (Fig EV4B). Importantly, patterns present in the tumor are retained in the derived tumoroid, indicating that the relative contributions of clones with varying B-allele fractions are retained in the established models. Minor shifts between the peaks are likely the result of the tumor sample being impure, that is, containing normal cells, whereas the tumoroid models consist purely of tumor cells.

Taken together, these analyses indicate that established RMS tumoroid models maintain the genetic characteristics including the clonal composition of the RMS tumor they were derived from to a large extent.

mRNA expression profiles of FN- and FP-RMS are fundamentally different, mainly due to the transcriptome-wide impact of the fusion transcript in FP-RMS (Wachtel et al., 2004). Additionally, the transcriptional program of in vitro cultured organoids is influenced by the culture conditions and can deviate from the transcriptional program of the parent tissue (Lu et al., 2021). Given that FN-RMS and FP-RMS tumoroid models are cultured in the same medium, we asked whether the transcriptional differences observed between the original tumors are retained in the models. Analysis of RNA-seq shows a high correlation between the expression profiles of RMS tumoroid models with the same fusion status (i.e., FP-RMS versus FN-RMS, Fig 3D), as has previously been shown for primary RMS tumor samples (Wachtel et al., 2004). Furthermore, correlation between the expression profiles of RMS tumor and tumoroid models of the same fusion-type is high, while correlation with control kidney tumors is low, showing that the fundamental differences in the expression profiles of FN- and FP-RMS are retained in culture (Fig 3D). In summary, the early tumor cell detection by RT-qPCR, the morphological and marker protein analyses, WGS for copy number profiles, for somatic mutual signatures, for individual SNVs, and for assessing the clonal composition, as well as the comparative transcriptomic analyses, indicate that the RMS tumoroid models resemble the original RMS tumor they are derived from.

Genetic and transcriptional stability of tumoroid models over time

We next asked whether the models remain genetically and transcriptionally stable after culture over extended periods. To investigate stability, a subset of RMS tumoroid models were kept in culture over 3–6 months until they reached passage 40 (“OL” for late passage, as compared to “O”, the standard passage analyzed here and sufficient for drug screening) and characterized again by WGS and RNA-seq. Furthermore, two independently derived tumoroid models (i.e., established from the same tumor piece of which a part was
cryo-preserved after the first tumoroid establishment) were included in this comparison (“O2”) to assess the robustness of the establishment protocol.

RMS tumoroid models show long-term propagation potential with five out of seven lines tested reaching passage 40 and two lines dropping out at passages 17 and 20, respectively. As drug screening is usually performed between passages 6 and 12, this shows that models can be readily used after such an initial screening. Comparison of individual copy number profiles of standard and late passage (Fig EV5A), as well as independently derived paired tumoroid models (Fig EV5B) shows no major copy number differences between the respective models. Furthermore, analysis of somatic mutational signatures shows that the contributions of identified somatic mutational signatures (Fig 4A) as well as the contributions of individual SBSs between models (Fig EV5C) are highly similar. Lastly, analysis based on SBS profiles shows clustering of models derived from individual patients (Fig EV5D). The exception to this is RMS335 “OL,” which may be due to a mutation in the DNA damage response gene ATM in both the tumor and the tumoroid sample, potentially resulting in the gain of new mutations during culturing. The similarity between SBS profiles of RMS335 “O” and “OL” was nevertheless high. Taken together, these analyses show that RMS tumoroid models generally remain stable, even over extended periods of culturing and that the establishment protocol is robust, yielding highly similar models when independently derived from a single tumor sample.

As before (see Fig 3C), individual pathogenic SNVs were also evaluated. Importantly, the majority of SNVs are retained after long-term culturing, with the models acquiring only a few additional SNVs (Fig 4B). In addition, the models independently derived from the same tumor show a high overlap of pathogenic SNVs (Fig 4B). Notably, the afore-mentioned oncogenic mutations in CTNNB1 and FGFR4 are detected in all related samples (Fig 4B). This further indicates that the models do not lose key mutations and that the establishment protocol results in the outgrowth of representative tumor cells harboring these mutations. RNA-seq expression profiles from the standard passage, late passage, as well as the independently derived tumoroid models were compared to test whether the core transcriptional program of RMS is retained. Principal component analysis shows that global expression profiles of tumoroid models derived from the same patient cluster together (Fig 4C). This suggests that our models are not only genetically, but also transcriptionally stable over time.

RMS tumoroid drug screening reflects established drug sensitivities

Having established that the RMS tumoroid models resemble the tumors they are derived from with stability during prolonged culturing, their suitability for research was further investigated in two specific ways, that is, via drug screening and CRISPR/Cas9 genome editing. Development of novel RMS treatments has been hampered by a lack of preclinical models that can efficiently be subjected to drug screening. To be of use for such screening approaches, it is imperative that new models reflect drug sensitivities already known for these tumors. This was investigated with a custom pediatric cancer library of 165 compounds, comprising standard of care chemotherapeutics as well as early-phase clinical trial targeted compounds. To mimic the three-dimensional configuration of tumors, cell plating was optimized so that tumoroid cells form homogenous 3D spheres in 384-well plates. The protocol also included prior growth curve determination of the number of cells that must be plated for each individual tumoroid model to prevent overgrowth during screening (Materials and Methods).

Vincristine and actinomycin D are routinely used in RMS treatment and indeed show broad efficacy in all models tested (Fig 5A, bottom highlighted box). Furthermore, the proteasome inhibitor bortezomib shows a similarly high efficacy in all tested models. This is in line with previous studies showing that both major histological RMS subtypes are susceptible to bortezomib treatment in vitro (Bersani et al., 2008) and in vivo (Manzella et al., 2020), suggesting that RMS tumoroid models indeed reflect drug sensitivities known for RMS tumors.

Clustering of RMS tumoroid models based on drug efficacy shows two main groups, comprising 12 and 4 models, respectively, with one unclustered model (RMS000FLV). This model nevertheless shows high sensitivity to the afore-mentioned drugs (bottom of Fig 5A). The outlier behavior is not caused by differences in growth during the experiment (see Table EV1) and may be explained by the fact that this is the only treatment-naïve FP-aRMS model in the collection (Fig 1B). The group of four models, that contains all successfully screened FN-RMS tumoroid models, is more sensitive to all tested inhibitors of MEK/ERK (MAPK signaling pathway) as well as the two inhibitors of γ-secretase (NOTCH signaling pathway) in the drug panel, when compared to the other group containing only FP-RMS tumoroid models (Fig 5A, top highlighted box, and Fig 5B and C). Importantly, sensitivity of FN-RMS against inhibitors of MAPK and NOTCH signaling has previously been reported (Belyea et al., 2011; Yohe et al., 2018). This group also contains the FP-aRMS tumoroid model RMS000HQC which shows a very low expression of its fusion transcript (Fig 2B), which potentially resulted in it displaying sensitivities more commonly observed in FN-RMS. Taken together, our results indicate that drug sensitivities observed in RMS tumoroid models reflect those known in primary RMS tumors. This shows the potential these models hold for testing novel drugs. As drug screening could be performed as early as 27 days after sample acquisition, with a median time to drug screening of 81 days, this highlights their relevance for personalized approaches.

RMS tumoroid models can be molecularly edited using CRISPR/Cas9

The applicability of preclinical models would be further enhanced by the possibility of genetic modification. To test this, we used CRISPR/Cas9 (Jinek et al., 2012) to knock out the well-known tumor suppressor gene TP53. This choice is based on the recent report that loss of functional P53 protein confers a worse prognosis in RMS (Shern et al., 2021). RMS012 FN-eRMS tumoroid cells, with wild-type TP53 as determined by WGS, were transfected with a plasmid harboring a TP53 targeting sgRNA as well as Cas9. Successfully edited cells were selected with nutlin-3 (Drost et al., 2015; Fig 6A), resulting in cells with complete loss of P53 protein as confirmed by Western Blot (Fig 6B). Sanger sequencing shows a spectrum of Indels consistent with a polyclonal population of P53-deficient cells (Fig EV6A).

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Discussion

A tumoroid collection of purely mesenchymal origin

To date, organoid technology has primarily been employed to generate models of malignant tumors of epithelial origin (i.e., carcinomas; Bleijs et al., 2019). Feasibility to use this technology on nonepithelial cancer has only recently been shown (Fusco et al., 2019; Jacob et al., 2020; Saltsman et al., 2020; Abdullah et al., 2021; Yamazaki et al., 2021). Here, we extend the tumor organoid approach, demonstrating applicability to tumors of entirely mesenchymal origin (i.e., sarcomas), resulting in only the second thoroughly characterized tumoroid collection specific for pediatric cancer.

Two factors likely contributed to the delayed adaptation of organoid technology to sarcomas. First, the technology was developed for healthy epithelial tissue, followed later by translation to the corresponding cancer entity, as in the case of colorectal carcinoma (Sato et al., 2009, 2011; Van De Wetering et al., 2015). The cell of origin of...
Figure 5. RMS tumoroid drug screening reflects established drug sensitivities.

A Clustered heatmap of viability measurements per RMS tumoroid model (x axis) and drug (y axis), showing the Area Under the Curve (AUC) after treatment of the cells for 120 h with a dose-range of 0.1 nM to 10 μM. Low AUC (red) indicates high drug efficacy while high AUC (blue) indicates low drug efficacy. Annotated clusters of (3) MEK/ERK and γ-secretase inhibitors showing specific efficacy in RMS tumoroid models without (RMS007, RMS002, RMS444) or low (RMS000HQC) fusion transcript expression, and (2) drugs that show broad efficacy across RMS tumoroid models.

B Principal component analysis of drug screening AUC values of the RMS tumoroid models (RMS000FLV omitted due to outlier behavior as discussed in the main text). The ellipse indicates the cluster that shows specific sensitivity against MEK/ERK and γ-secretase inhibitors.

C Principal component analysis of the top 25 contributing drugs that influence variance. The ellipse indicates the MEK/ERK and γ-secretase inhibitors as well as AZD4547 (RMS000FLV omitted as described above).

Source data are available online for this figure.
RMS is still not fully characterized (Hettmer & Wagers, 2010). Therefore, extensive culture optimization of healthy tissue first, with translation to cancerous tissue later, has not been feasible for RMS. Consequently, optimization had to be conducted on tumor samples, which are not readily available. Second, and related to the issue of tissue availability, sarcomas are far less common than carcinomas, accounting for less than 1% of all solid adult malignancies (Burningham et al., 2012). In pediatric cancer however, sarcomas are much more common, encompassing 21% of all solid tumors in children (Burningham et al., 2012). This further highlights the importance of the currently described approach and collection.

**Representation of a broad spectrum of RMS subtypes**

The tumoroid models include representatives of both major histological subtypes, all major fusion types, different age groups, both sexes, treatment-naive as well as pretreated, primary as well as metastatic tumors (Fig 1B, Table 1 and Dataset EV1). Rarer subtypes such as sclerosing/spindle-cell RMS (Rudzinski et al., 2015) will be exciting to include, as also indicated by a recent case report (Acanda De La Rocha et al., 2021). Compared to patient incidence rates (Glosli et al., 2021), the collection has an underrepresentation of head and neck RMS. Although RMS tumor samples from this region were acquired, models from such samples failed, regardless of subtype, clinical stage, sample quantity, or quality. Interestingly, this indicates that RMS arising in the head and neck may depend on specific factors that have not yet been identified. Studies in genetically engineered mice indicate that aberrant Hedgehog signaling can give rise to FN-RMS from nonmyogenic endothelial progenitors in the head and neck (Drummond et al., 2018). Here, activation of Hedgehog signaling by Smoothened agonists did not facilitate establishment of head and neck

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**Figure 6.** RMS tumoroid models can be molecularly edited using CRISPR/Cas9 with P53-deficient eRMS being more sensitive to the checkpoint kinase inhibitor prexasertib.

A Transfection and selection strategy to achieve TP53 knockout in a TP53 wild-type tumoroid model.

B Western Blot analysis of TP53 wild-type (WT) and knockout (KO) RMS tumoroid line RMS012. Histone 3 (H3) served as loading control.

C Dose-response curve of TP53 WT and KO cells treated with the Chk-1 inhibitor prexasertib. Thin lines with numbers indicate individual biological replicates (n = 3) while thick lines indicate fitted lines over all replicates. The statistical significance of the differences in fitted IC50 values between WT and KO were obtained using a two-sided t-test (P = 0.008).

Source data are available online for this figure.
RMS tumoroid models, indicating that additional factors may be necessary. Regardless of such future developments, the protocol described here yields models that can be rapidly established from a broad range of quite different RMS subtypes. **New models for RMS research**

Currently available preclinical models of RMS include conventional cell lines, various genetically engineered animal models (GEMs), as well as patient-derived xenograft models (PDX or O-PDX when transplanted orthotopically; Kashi *et al.*, 2015; Imle *et al.*, 2021). Each of these systems possesses specific benefits and drawbacks, resulting in suitability for different research questions or stages in drug development (Kim *et al.*, 2020). While conventional cell lines are easy to use, with low costs, large-scale screening potential, and ease of genetic modification, due to prolonged culturing they often do not recapitulate many basic features of the genetic and molecular background of the tumor they were derived from, thus possessing only limited predictive value (Hinson *et al.*, 2013). GEMs, on the other hand, are well-suited for cell of origin studies and can provide valuable insights into cancer onset mechanisms. GEMs are usually not suitable for high-throughput screening, mostly due to the low tumor penetrance or the intricacies of animal studies (Kersten *et al.*, 2017). In contrast to GEMs, in O-PDX models, tumor samples are transplanted onto immune-deficient animals (usually mice, although zebrafish has recently been reported (Yan *et al.*, 2019)). This allows for engraftment, growth, and later propagation of the tumor tissue from animal to animal. Tumors propagated in this manner are thought to be genetically stable over time and to reflect the patient tumor they were derived from, giving them predictive value concerning preclinical drug testing (Gao *et al.*, 2015). Disadvantages include the necessary use of animals as hosts and the tumor entity-specific engraftment time which can take up to several months (Pompili *et al.*, 2016). Furthermore, there is evidence that not all PDX models are genetically stable (Petljak *et al.*, 2019).

The RMS tumoroid models combine several of the above-mentioned benefits. They can be rapidly established and expanded like conventional cell lines, enabling drug screening. Importantly, established drug sensitivities of RMS in general and of RMS subtypes specifically (i.e., inhibitors of MAPK and NOTCH signaling in FN-RMS), are retained in tumoroid models, showing their applicability as models in translational RMS research. In addition, tumoroid models in general are suitable for transplantation into mice (Fumagalli *et al.*, 2018; Grassi *et al.*, 2019; Dekkers *et al.*, 2021), which enables pharmacokinetic studies, further broadening their use in translational research. As with cell lines, molecular editing to mimic certain disease backgrounds is possible using CRISPR/Cas9. Unlike cell lines however, tumoroid models depend on defined media including recombinant growth factors as well as an ECM substitute, resulting in higher costs. RMS tumoroid models molecularly resemble the patient tumor they are derived from, with tumor sample-specific SNV patterns being retained in matching tumoroid models, thereby also recapitulating differences between tumor samples derived from the same patient (e.g., RMS108, RMS109, and RMS110 showing overlap but also differences in SNVs in the tumor samples which are retained in the corresponding tumoroid models). A subset of SNVs present in the tumor are not detected in the established RMS tumoroid which can be due to (i) this clone not being present in the tissue piece that was used for the establishment of the tumoroid, and/or (ii) the respective clone was present in the piece but got lost over the course of culturing and/or (iii) the sequencing depth for the tumoroid model was not sufficient to pick up this mutation (30X coverage in the tumoroid model versus 90X coverage in the tumor, respectively). RMS tumoroid models share this resemblance with the patient tumor they were derived from with O-PDX mouse models, while possessing the above-mentioned advantages of rapid establishment and expansion. Compared to O-PDX mouse models, establishment success is lower in RMS tumoroid models, indicating that niche factors in the host mice are important for facilitating establishment. This may be especially important in particular subtypes such as RMS from the head and neck region. On the other hand, RMS tumoroid models are less intricate in their propagation. Lastly, while GEMs are considered essential for cell of origin studies, recent advances have shown that by genetic editing, such studies can now also be performed in tumoroid models (Custers *et al.*, 2021). Lastly, the scRNA-seq-based analysis of two RMS tumoroid models indicates that tumor cell heterogeneity, an important hallmark of plasticity, are maintained *in vitro*. To fully assess to what extent this heterogeneity reflects the heterogeneity in the tumor the models were derived from, an extensive analysis of matching primary RMS tumor samples would be necessary, which will be exciting to explore in future studies.

In conclusion, we established a well-characterized, well-annotated collection of RMS tumoroid models, being the first such collection of tumoroid models derived from purely mesenchymal malignant tumors (i.e., sarcomas) and only the second comprehensive tumoroid model collection derived from pediatric cancer (Calandrini *et al.*, 2020). This collection contains all major subtypes of RMS and the models can be used for drug screening as well as molecular editing. An interactive, browser-based companion Shiny app (https://rmsdata.rms-biobank.eu/) that makes all the described data easily accessible, accompanies this paper. The RMS tumoroid models will be a useful complementary system to study the biology of RMS and to improve treatment.

**Materials and Methods**

**Tumor sample acquisition**

Tumor samples of RMS were obtained via an established tumor sample acquisition route from patients treated at the Emma Children’s Hospital Amsterdam (Amsterdam UMC; RMS006, RMS007, RMS013) or as part of the biobank initiative of the Princess Máxima Center for Pediatric Oncology, Utrecht, the Netherlands (PMC; remaining tumor samples). Ethics approval was granted for the biobanking initiative, and the PMC biobank committee granted approval for this project. All patients and/or their legal representatives signed informed consent to have tumor samples taken for biobank usage. Experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.

A subset of patients was furthermore enrolled in a local personalized medicine trial (i.e., iOTHER study) through which a subset of DNA and RNA specimens from RMS tumors were obtained.
Tumor sample preparation for establishment of RMS tumoroid models

Solid tumor samples (i.e., needle biopsies or resection specimens) were transferred to collection medium (see below) to retain viability of cells. After pathological examination, suitable samples (i.e., containing tumor cells) were processed in a sterile work environment as follows: the sample was transferred to a sterile dish and covered with a droplet of BM1* culture medium (see below) before being minced to fine pieces using scalpels. If the tumor sample was of sufficient size, a portion of this minced mixture was stored viably (see below). The remainder of the mixture was put on a prewet 70% ethanol. After 5 min, the mixture was spun down (300 g, 5 min, 4°C) and the supernatant was removed from the resulting pellet. If the pellet was of grey color (i.e., not containing a high percentage of red blood cells, RBCs), the pellet was resuspended in 1 ml of BM1* medium. If the pellet contained a high percentage of RBCs (i.e., by displaying a red stain), the cell pellet was resuspended in red blood cell lysis buffer (Roche) and incubated at room temperature for 5 min. Thereafter, the reaction was stopped by flushing the well or flask with collection medium and the resulting mixture was spun down again (as above). Again, supernatant was removed and now the pellet was resuspended in 1 ml of BM1* medium (without Basement Membrane Extract, BME, see below). In each case, cells were now counted using a TC20 Automated Cell Counter (BioRad) to get a rough estimate on viability and cell numbers. Cells were plated with a sufficient density (at least 10,000 viable (BioRad) to get a rough estimate on viability and cell numbers. Cells were plated with a sufficient density (at least 10,000 viable cells per 1 cm² of surface area), supplemented with 0.3–0.5% cold BME type 2 (see below).

A: The single-cell fraction was now spun down (300 g, 5 min, 4°C) and the supernatant was removed from the resulting pellet. If the pellet was of grey color (i.e., not containing a high percentage of red blood cells, RBCs), the pellet was resuspended in 1 ml of BM1* medium. If the pellet contained a high percentage of RBCs (i.e., by displaying a red stain), the cell pellet was resuspended in red blood cell lysis buffer (Roche) and incubated at room temperature for 5 min. Thereafter, the reaction was stopped by adding collection medium and the mixture was spun down again (as above). Again, supernatant was removed and now the pellet was resuspended in 1 ml of BM1* medium (without Basement Membrane Extract, BME, see below). In each case, cells were now counted using a TC20 Automated Cell Counter (BioRad) to get a rough estimate on viability and cell numbers. Cells were plated with a sufficient density (at least 10,000 viable cells per 1 cm² of surface area), supplemented with 0.3–0.5% cold BME type 2 (see below).

B: The strainer fraction was dissolved in pure cold BME (roughly one-third volume strainer fraction and two-third volume BME) and mixed thoroughly. Droplets of 5–10 μl of this mixture were formed on 24- or 48-well prewarmed cell culture plates and incubated for 5 min at room temperature to allow the BME to solidify. Thereafter, the cell culture plates were incubated upside-down for another 25 min at 37°C to allow the material to “sink” to the top of the droplet. Upon completed solidification, BM1* medium (at room temperature (RT) and without BME) was added to the wells so that droplets were very fully submerged in medium.

In the case of bone marrow aspirates as tumor samples (RMS410 and RMS127), sample tubes were spun down (300 g, 5 min, 4°C, slow break) to separate RBCs and nucleated cells (white clot at the bottom of the tube). In the case of RMS410, the normal hematopoietic system was almost entirely superseded by infiltrating tumor cells so that the clot consisted mainly of tumor cells which could directly be plated (in BM1* with BME), which resulted in rapid outgrowth of the model. In the case of RMS127, the percentage of infiltrating tumor cells was estimated by pathology to be low at circa 5–10%. Therefore, initial cultures (plated in BM1* and BME) from the white clot also contained normal nucleated bone marrow cells which, however, were eventually outcompeted by the rapidly growing tumor cells which overtook the culture.

Tumoroid model culturing and propagation

Growing RMS tumoroid models were inspected regularly and showed adequate growth behavior under conventional cell culture conditions (i.e., 37°C, 5% CO₂). All models were regularly tested negative for mycoplasma contamination. Estimated division times ranged from 24 to 72 h for most models. Models were passaged once or twice per week at a confluency of 70 to 80%. For passaging, old culture medium was aspirated and cells were briefly washed with sterile DPBS (Gibco, cat no. 14190144). Cell detachment was performed using TrypLE Express Enzyme (1×, phenol red, Gibco, cat no. 12605010). Depending on the model and the BME percentage, detachment took between 3 and 10 min (higher BME concentrations resulting in longer detachment time). Cells were collected by flushing the well or flask with collection medium and the resulting mixture was spun down (300 g, 5 min, 4°C). Thereafter, the supernatant was removed, and the pellet was resuspended in 1 ml of BM1* and cells were counted using a TC20 Automated Cell Counter (BioRad). Single-cell suspensions were mostly re-seeded at the same density as their parental/previous generation/passage.

Cell culture media

**Base medium (BM)**

To prepare a 500 ml bottle of BM, Glutamax (5 ml, Gibco, cat no. 35050061), Penicillin/Streptomycin (10,000 U/ml, 5 ml, Gibco, cat no. 15140122), and B27 (without vitamin A, 10 ml, Gibco, cat no. 12587010) were added to a full bottle of advanced DMEM/F12 (500 ml, Gibco, cat no. 12634010). BM was stored at 4°C and was used within 2 months.

**Complete culture medium (BM1*)**

To prepare the complete culture medium BM1*, 47.5 ml of the above-mentioned base medium (BM) were taken and pipetted into 50-ml tube. Thereafter, the components below were added (no specific order). BM1* was stored at 4°C and was used within 7–10 days (thereafter, the stability of the growth factors may be compromised).

**Components:**

| Component | Concentration | Reference |
|-----------|---------------|-----------|
| N-acetylcysteine (500 mM) | 125 μl | Sigma, cat no. A9165 |
| MEM nonessential amino acids | 500 μl | Gibco, cat no. 11140035 |
| Sodium pyruvate (100 mM) | 500 μl | Gibco, cat no. 11360070 |
| Heparin (5,000 U/ml) | 5 μl | Sigma, cat no. H3149-10KU |
| hEGF (2 μg/ml) | 500 μl | Peprotech, cat no. AF-100-15 |
| hFGF-basic (40 μg/ml) | 50 μl | Peprotech, cat no. 100-188 |
| hIGF1 (100 μg/ml) | 10 μl | Peprotech, cat no. 100-11 |
| RIK (Y-27632, 100 mM) | 5 μl | AbMole Bioscience, cat no. M1817 |
| A83-01 (5 mM) | 50 μl | Tocris Bioscience, cat no. 2939 |

**Collection medium**

To prepare a 500 ml bottle of collection medium, Glutamax (5 ml, Gibco, cat no. 35050061), Penicillin/Streptomycin (10,000 U/ml, 5 ml, Gibco, cat no. 15140122), and HEPES (1 M, 5 ml, Gibco, cat...
no. 15630049) were added to a full bottle of advanced DMEM/F12 (500 ml, Gibco, cat no. 12634010). Collection medium was stored at 4°C and was used within 2 months.

**Basement membrane extract type 2 (BME)**

To facilitate attachment of cells, culture medium was supplemented with 0.1–0.5% BME (Cultrex Reduced Growth Factor Basement Membrane Extract, type 2, Pathclear, R&D Systems, 3533-005-02). We observed batch-to-batch variations of this product, resulting occasionally in suboptimal attachment of cells when the BME concentration was too low. On average, 0.2–0.3% BME supplementation was sufficient for stably established RMS tumoroid models. However, upon encountering suboptimal cell attachment, BME concentrations were raised to 0.5% in established cultures. During the initial establishment process, higher concentrations of BME (0.3–0.5%) showed increased attachment rates of cells.

**Freezing and storing procedure**

RMS tumor samples as well as established RMS tumoroid cultures were viably frozen as follows: a sufficient number of viable cells (at least 0.5×10^6, mostly 1×10^6) were diluted in 0.5 ml BM1 medium in a cryo tube. Then, an equal volume of freeze-mixture consisting of 80% fetal calf serum (FCS) and 20% DMSO was added dropwise, resulting in final concentrations of 50% BM1 medium, 40% FCS, and 10% DMSO. Samples were frozen slowly using a freezing container in a −80°C freezer. For long-term storage, frozen vials were transferred to liquid nitrogen.

**Thawing procedure**

Cryo-preserved RMS tumoroid cell suspensions were quickly defrosted in a water bath at 37°C and then immediately dissolved in washing medium (to at least 5 ml of total volume to dilute the DMSO). Samples were then spun down (300 g, 5 min, 4°C), supernatant was removed, and the cell pellet was resuspended in 1 ml of BM1 medium. Thereafter, cells were counted using a TC20 Automated Cell Counter (BioRad). Cell viability was on average 20–50% lower compared to viability at the time of freezing. Cells were then plated at a proper density in BM1-supplemented BM1 medium and needed 1–2 weeks to recover before being stable enough for further experiments. Restarting tumoroid cultures from cryo vials was possible for all tumoroid models.

**Early tumor validation by RT-qPCR**

Early during tumoroid establishment (upon first or second passage), a portion of cells was set aside for evaluation of marker gene expression. For this, cells were spun down (500 g, 5 min, 4°C), the supernatant was removed from the pellet, the pellet was dissolved in Trizol (10 min incubation at RT) and was immediately processed or snap-frozen and stored at −80°C until further processing. Upon processing, first the organic and aqueous phases were separated by addition of 20% chloroform, followed by spinning down (maximum centrifugation speed, 15 min, 4°C). The (upper) aqueous phase was further processed using the Direct-zol RNA MiniPrep Kit (Zymo Research) according to the manufacturer’s protocol, including the recommended DNase I treatment. Quality and quantity of isolated RNA were measured using a NanoDrop OneC (Thermo Fisher Scientific). Synthesis of cDNA from isolated RNA as well as a Universal Human Reference RNA that was used as negative control or for normalization (HREF, Stratagen/Agilent # 740000) was performed using an oligo-dT primed SuperScript III Reverse Transcriptase (Promega-based reaction according to the manufacturer’s protocol. RT-qPCR was performed with the obtained cDNAs testing for expression levels of G6PD (forward: 5′-ACGGCAACAGATACAAACAC-3′, reverse: 5′-CGAAGTTCATCTCCCTCC-3′; product size: 86 bp; Amary et al, 2007), DES (forward: 5′-CCGTGCTTCTTTACCTTCTCCT-3′, reverse: 5′-CACCACCTTTCCCTGCTTCAATC-3′; product size: 119 bp), MYOG (forward: 5′-TGCCCAACTGTAGATGTCCTTC-3′, reverse: 5′-CTGCTACAGAGATGTTGGCACT-3′, product size: 81 bp), MYOD1 (forward: 5′-GACGCGGTCTGTAAGCGTAACC-3′, reverse: 5′-ACACCATGCTCAGAGATAA-3′, product size: 148 bp), the PAX-FOXO1 fusion transcript (forward: 5′-GCCGACAGAGCTGCTGGCTAC-3′, reverse: 5′-TAACCTTGTCGTGAGGACAG-3′, product size: 171 bp for PAX3-FOXO1 and 159 bp for PAX7-FOXO1 (Ponce-Castañeda et al, 2014) as well as the PAX3-WTTR1 fusion transcript (forward: 5′-CCACCGAGCATGTTACAG-3′, reverse: 5′-TTCAGGCTTGCTGTCTGATT-5′, product size: 192 bp). Expression levels of DES, MYOG, MYOD1, and PAX-FOXO1 were normalized to G6PD (housekeeping gene) and referenced to the corresponding expression levels in the HREF using the ΔΔCq method (for the fusion transcript only normalization to G6PD as the lack of a fusion expression in HREF did not permit a further reference step).

**Immunohistochemistry (IHC) and H&E stainings**

To perform IHC, tumoroid models were grown as floating 3D spheres. For this, 1–3×10^6 viable cells were put into an ultra-low attachment culture flask (Corning Ultra-Low Attachment 75 cm² U-Flask, Corning, cat no. 3814) in BM1 but without BME supplementation. Establishment of spheres of sufficient size took between 7 and 12 days, depending on the growth characteristics of the respective tumoroid model. Spheres were harvested by carefully transferring the sphere-containing medium from the flask to a 15-ml tube and sedimenting on ice for 10 min. Thereafter, the supernatant was removed, and the sphere-containing pellet was resuspended in cold PBS to wash off any remaining medium. The mixture was again sedimented (see above), and PBS was aspirated. Spheres were now fixed using formalin 10% (v/v), (= 4% w/w) HISTO GRADE, neutralized (pH 7.0 ± 0.2), J.T. Baker, 3933.9020 VWR) for 96 h at 4°C after carefully transferring them to a glass vial. The fixed spheres were then washed twice with PBS and dehydration was performed by adding ethanol solutions with increasing percentages (25% EtOH for 15 min, 50% EtOH for 15 min, 70% EtOH for 15 min—after this step, spheres were stored at 4°C and further processed in batches). Now, spheres were stained with 0.8 g/l Eosin Y dissolved in 96% EtOH (Sigma, E4009) for 30 min and subsequently incubated three times with 100% EtOH for 30 min each. Thereafter, spheres were incubated in n-Butanol (three times 30 min) and melted paraffin (three times) before they were Paraffin-embedded using the HistoCore Arcadia H (Leica Biosystems) following the manufacturer’s protocol in a medium size mold. Hardened paraffin blocks were cut into 4 μm slices using a microtome (HM 355S Automatic...
Microtome, Thermo Scientific). Slides were further processed by deparaffinization (incubation with xylene three times for 3 min, then 100% EtOH two times for 3 min, 95% EtOH two times for 1 min, 70% EtOH once for 1 min, and 50% EtOH once for 1 min) and rehydration (tap water). Thereafter, antigen retrieval was performed by boiling samples for 20 min in citrate buffer (pH 6, for Desmin staining) or Tris-EDTA-Tween buffer (pH 9, for Myogenin and MYOD1). After washing (in TBS-0.025% Triton, twice 5 min) and blocking (1.5 h in TBS-1% BSA), slides were incubated with primary antibody (α-Desmin 1:400, Abcam ab15200 rabbit antibody; α-Myogenin 1:400, Santa Cruz SFD mouse antibody; α-MYOD1 1:200, Cell Marque EP212 rabbit antibody) overnight at 4°C. The next day, slides were washed twice with PBS and incubated with secondary antibodies (Desmin and MYOD1: anti-rabbit-HRP, BioRad 170-6515; Myogenin: anti-mouse HRP, BioRad 170-6516) 1:500 in PBS-1% BSA. Stainings were visualized using Liquid DAB (Hematox 7211) following the manufacturer’s protocol and washed three times with TBS. Counterstaining was performed by incubation with thionine (0.05% for 20 min). After subsequent incubation with 96% EtOH, 100% EtOH and xylene, slides were mounted using Permount mounting medium (Fisher Scientific SP15-100) and visualized using a Leica DMi6 microscope.

H&E stainings were performed manually (steps: xylene three times for 5 min, 100% EtOH twice for 1 min, 95% EtOH twice for 30 s, 70% EtOH for 30 s, washed in demi-water, incubation with hematoxylin (Hematox 7211) for 2:45 min, washed with demi-water, brief incubations with acidic EtOH, washed with demi-water, 95% EtOH for 30 s, eosin incubation for 2:45 min, EtOH 70% for 30 s, EtOH 95% for 30 s, EtOH 100% twice for 30 s, xylene three times for 1 min) or were performed at the in-house pathology department following standard protocols. Visualization was performed as described above.

The quality of stainings was evaluated by an in-house pathologist. Representative images of stained spheres are shown and were compared to H&E as well as IHC stainings obtained in pathology for the RMS tumor sample the tumoroid was derived from (representative images chosen by the pathologist).

Single-cell mRNA sequencing of RMS tumoroid models

Sample processing

For each tumoroid model (RMS127 and RMS444), viably frozen cells were thawed and resuspended in BM* medium. Prior to sorting, 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, #D9542) and DRAQ5 (Thermo Fisher, #65-0880-92) were added to the single-cell suspensions to achieve final concentrations of 1 and 5 μM, respectively. Viable single-cells (DAPI−, DRAQ5−, FSC/SSC) were then sorted into 384-well plates (BioRad, #HSP3801) containing 10 μl of mineral oil (Sigma, #M5310) and 50 nl of barcoded RT primers using a SONY SH800S Cell Sorter. Libraries were prepared according to the SORT-seq protocol (Muraro et al, 2016) and sequenced on the Illumina NextSeq500 (paired-end, 75 bp).

Analysis of single-cell mRNA-sequencing data

Sequencing reads were demultiplexed, mapped to the Genome Reference Consortium human build 38 genome, and transcript counts were generated using a custom implementation of the zUMIs pipeline (Parekh et al, 2018). Count tables from each plate were read into R (v4.1.0), merged and metadata fields were compiled. The merged count and metadata tables were used to initialize a Seurat (v4.0.3) object (Hao et al, 2021), and cells with < 500 expressed genes, < 800 or > 50,000 unique transcripts, a percentage of mitochondrial transcripts < 50%, a percentage of hemoglobin genes > 1% or a ratio of intergenic to genic transcripts > 2 were excluded from further analysis. The filtered object was then log-normalized, using a scaling factor of 10,000, and the top 2,000 most variable genes in the dataset were defined by calling the FindVariableFeatures Seurat function (default parameters). These genes were scaled, centered and used as input for running principal component analysis (PCA). The top five principal components were then used to project the data in two-dimensions using t-distributed stochastic neighbor embedding (t-SNE). Single-cell CNV profiles were constructed using the InferCNV R package (v1.8.0; Tickle et al, 2019). In addition to the default parameters, an average expression threshold per gene of 0.3 and a standard deviation filter of 2 was used to denoise the results. A dataset of cord blood mononuclear cells (CBMCs) generated in-house was used as the ‘normal’ reference sample.

RNA and DNA isolation from tumor and derived tumoroid samples for whole-genome sequencing (WGS) and bulk mRNA sequencing (RNA-seq)

Tumoroid models

To isolate RNA and DNA for WGS and RNA-seq, tumoroid cells were collected as a pellet, snap-frozen, and stored at −80°C. Isolation of RNA and DNA from the same pellet was performed using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen # 80224) according to the manufacturer’s protocol. Lysis in RLT buffer was followed by homogenization using a QiaShredder column as described. The flow-through fraction in RLT buffer was used for the Allprep DNA and RNA isolations. Quality and quantity of isolated RNA and DNA were measured by using the NanoDrop OneC (Thermo Fisher Scientific), Bioanalyzer 2100 (Agilent), Qubit Fluorometer (Thermo Fisher Scientific), and marker-checks using RT-qPCR (as above).

Tumor samples and germline control samples (EDTA blood)

Resected tissue and/or biopsies were processed within 10 min after removal from the patient. DNA and RNA were isolated from the same piece of fresh frozen tissue using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen # 80224) using the QIAcube Connect (Qiagen). Reference DNA was isolated from peripheral white blood cells (EDTA blood) using the same method.

Whole-genome sequencing (WGS) of tumor and tumoroid samples

Sequencing

One hundred and fifty nanograms of total DNA were used for library preparation using the KAPA HyperPlus kit (Roche), according to manufacturer’s instructions. Libraries from tumor and normal tissue were pooled in a 3:1 ratio, with a total of 7 tumor/normal pairs per S4 sequencing kit. Libraries were sequenced using 2 × 150 cycles on a NovaSeq 6000 (Illumina).
**Preprocessing, alignment, and annotation**

The WGS sequencing data were processed as per the GATK 4.0 best practices workflow for variant calling, using a wdl and Cromwell-based workflow. Reads were aligned to GRCh38 using bwamem (v0.7.13), and quality control (QC) was performed using FastQC (v0.11.5) and picardTools (v2.20.1). Somatic variants were identified using MuTect2 from GATK v4.1 and annotated using Vep (v92). Likewise, copy-number alterations (CNAs) were identified using GATK v4.1.

**Identification of nonsynonymous single-nucleotide variants (SNVs)**

Raw VCF files from germline, tumor, and tumoroid samples were processed with vcf-tools (v0.1.13; Danecek et al., 2011). Only variants that met the criteria (passed all quality filters in addition to PHRED quality score > 100 (250 for indels) and maximum read depth of 10) were kept for further analyses. Indels were also filtered out if the minimum mapping quality (MQ) was below 60. Bcftools (https://github.com/samtools/bcftools) was used to remove all common snps (VAF ≥ 1%) present in dbSNP (https://www.ncbi.nlm.nih.gov/snp/, v151 downloaded in August 2020). Filtered files were then loaded into R (v4.0.2) and processed with the package VariantAnnotation (Obenchain et al., 2014) and packages from the tidyverse. Variants in tumor and tumoroid models also present in the germline sample were removed from further analyses; remaining variants were filtered for VAFs > 0.3 and those tagged as missense variant, stop gained, stop lost, start lost, in-frame insertion, out-of-frame deletion, and frameshift variant were selected as nonsynonymous somatic mutations. Figures were generated with ggplot2 (v.3.3.2).

**Signature analysis**

Somatic signature analysis was performed using the R programming language (v3.6.3) and the R packages MutationalPatterns (v3.2.0; Manders et al., 2022) and VariantAnnotation (v1.32.0; Obenchain et al., 2014; Blokzijl et al., 2018). Somatic variants were filtered on both the variant allele fraction (VAF) and depth (DP). For both the control and the tumor/tumoroid samples we used DP ≥ 20. For the tumor/tumoroid samples we used VAF > 0.3, while in control samples the VAF had to be 0. Samples with 50 or less mutations would have been excluded, but this was not the case for any samples in our cohort. A mutation matrix was generated that shows how often each of the 96 types of base substitutions occurred in each sample. A variational Bayesian non-negative matrix factorization (NMF) was performed on this matrix to extract four de novo mutational signatures for base substitutions. The cosine similarities were then calculated between these de novo signatures and a set of signatures consisting of both the COSMIC signatures (v3.2, GRCh38) and the SIGNAL exposure signatures (Kucab et al., 2019; Alexandrov et al., 2020). The four signatures most similar to the de novo signatures (SB51, SB55, SBS18, and temozolomide [TMZ]) were then used for signature refitting. Signature refitting was performed using the fit_to_signatures_strict function using the best_subset method with a max_delta of 0.004. Our approach of first performing de novo signature extraction followed by refitting is similar to the approach suggested by Maura et al. (2019).

To determine the similarity between the samples, the cosine similarities of their base substitution profiles were calculated. This resulted in a cosine similarity matrix that was used to calculate the distance between samples, which was then used for hierarchical clustering.

**Evaluation of clonal heterogeneity using WGS data**

To evaluate clonal heterogeneity in our RMS tumoroid models, two approaches were used based on the WGS data from each tumor/tumoroid pair. First, we compared the VAFs of somatic mutations in both tumor and tumoroid models by selecting variants from coding regions with a VAF of at least 0.1 in either tumor or tumoroid model. To ensure the VAFs were representative of the ratio of cell populations in the culture, we removed all SNVs from noncopy number neutral regions or tagged as sites with loss of heterozygosity (LOH) as detected by the GATK pipeline. VAFs of SNVs matching those criteria were plotted in a correlogram using R.

Second, we investigated the alternate allele (B-allele) fractions as they can indicate the presence of clonal and/or subclonal populations based on estimated allelic ratios. To this end, we extracted the median alternate allele fraction from the modeled segments from each sample; to ensure comparability across region segments, a consensus region bed file was generated prior to generating the density plots. Frequencies were then plotted per tumor/tumoroid pair as density plots using R.

For both analyses, RMS127 was excluded due the low tumor cell percentage of the tumor sample.

**Bulk mRNA (RNA-seq) of tumor and tumoroid samples:**

**transcriptional profile and gene fusions**

**Sequencing**

Three hundred nanograms of total RNA were used for library preparation using the KAPA RNA HyperPrep kit with RiboErase (Roche), according to manufacturer’s instructions. The protocol was optimized to achieve an insert size of ~300–400 nt.

RNA libraries were pooled with a total of 25 samples per S1 kit or 60 samples per S2 kit. Libraries were sequenced using 2 × 150 cycles on a NovaSeq 6000 (Illumina).

**Preprocessing: alignment, annotation, and detection of gene fusions**

The RNA sequencing data were processed as per the GATK 4.0 best practices workflow for variant calling, using a wdl and Cromwell-based workflow (https://gatk.broadinstitute.org/hc/en-us/sections/360007226651-Best-Practices-Workflows). This included performing quality control with Fastqc (v0.11.5) to calculate the number of sequencing reads and the insert size (Andrews S., 2010. FastQC: a quality control tool for high throughput sequence data, http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Picard (v2.20.1) for RNA metrics output and MarkDuplicates ("Picard Tools." Broad Institute. http://broadinstitute.github.io/picard/). The raw sequencing reads were aligned using Star (v2.7.0) to GRCh38 and gencode version 31. Gene fusion detection was performed using Star fusion (v1.6.0; Haas et al., 2019). Finally, expression counts were determined at exon and gene level using Subread Counts (Liao et al., 2019).

**Processing of transcriptome data**

Raw count tables were loaded into R (v4.0.2) and processed with the packages from the tidyverse. Count matrices were transformed
into log2-scale transcripts per million (log2 TPM) tables and genes annotated with the same gene symbol merged.

Comparison of transcriptional profiles across samples (kidney and rhabdomyosarcomas) was performed via correlation performed in R using the base package.

Growth curve experiments

Growth behavior in 3D sphere cultures prior to drug screenings (see below) was tested via performing growth curve experiments. Tumoroid cells were plated at different densities (typically between 250 and 4,000 viable cells per well) in 384-well round bottom ultralow attachment spheroid microplates (Corning, cat no. 3830). To facilitate 3D sphere formation, cells were grown in BM1 medium without BME and after dispensing them into the plates, plates were spun (350 g, 5 min, slow break) to concentrate cells in the center of the wells. Plates were incubated at standard conditions and cells were granted a recovery period of 48 h. Thereafter, readouts were performed using CellTiter-Glo 3D Cell Viability Assay (CTG3D, Promega, cat no. G9683) according to the manufacturer’s protocol at three time points: immediately (T0), 48 or 72 h (T48 or T72), and 120 h (T120). Measured luminescence (via FLUOstar Omega, BMG Labtech) was averaged per readout per cell density and the corresponding background signal from medium was subtracted. Obtained measurements from T48/T72 to T120 were normalized to T0 to calculate the relative growth over the period of 120 h. For the following drug screening experiments (see below), a cell density was chosen that showed logarithmic growth behavior in growth curve experiments. Defined starting number of cells facilitated the establishment of a sphere and were optimized for intrinsic growth factor levels and space depletion in the well over the course of the experiment. Growth curve experiments were performed once per model but with at least ten technical replicates per number of cells plated.

Drug screenings

For drug screenings, tumoroid models were processed according to the same protocol as for the growth curve experiments, with a number of cells seeded that was determined in those experiments (see above). Drugs, dissolved in DMSA or water, were added 48 h after seeding of the tumoroid spheres fully automated via a robotics system: Up until 2019, this was facilitated via a Caliper Sciclone-Robotic Liquid Handler using a dilution of the drugs in medium and after dispensing them into the plates, plates were pipetted. From 2020 onward, screenings were performed at the high-throughput screening (HTS) facility of the Princess Máxima Center with a Biomek i7 liquid handler, using the acoustic liquid handler Echo550 for direct drug transfers. Before the screening, the 384-well working plates containing the dissolved drugs are shaken (30 min, RT) and centrifuged (1 min, 350 g). Tumoroid spheres were treated with a tenfold dilution series of the drugs on the library plate (0.1 nM to 10 μM). Positive control samples were treated with DMSO, negative control samples with staurosorpine (final concentration of 10 μM). Readouts were performed using CellTiter-Glo 3D Cell Viability Assay (CTG3D, Promega, cat no. G9683) according to the manufacturer’s protocol at T0 (before addition of drugs–control) and at T120 (120 h after addition of drugs–readout). Dose-response was estimated per drug and concentration in relation to the DMSO-treated cells (set to 100%) and empty controls (set to 0%). Quality of the screenings was approved after assessment of the cell growth (absorbance signal of T120 over T0), the negative, positive, and empty controls and, if applicable, the amount of variability between the duplicates.

Depending on the available number of cells at drug screening, a subset of models (RMS007, RMS109, RMS110, RMS000ECC, RMS000ETY, RMS000FLY, RMS000HQ, RMS000HWO, RMS000HWQ) could be screened in technical duplicates while for the other models (RMS006, RMS012, RMS013, RMS102, RMS108, RMS127, RMS335, RMS410, RMS444, RMS000CPU) only screening without technical duplicates was feasible. To test reproducibility, we analyzed the correlation between the technical duplicates in the tumoroid models for which those were available (Appendix Fig S1). As we observed a very high correlation there (R = 0.91), indicating a high reproducibility, we deemed it appropriate to combine the analysis of samples tested in duplicate and those not tested in duplicate given the scope of the assay was to assess whether we could obtain biologically meaningful results (any “hit” should have been further validated). RMS tumoroid models RMS000ETY and RMS000HWO were excluded from the analysis as they did not show an increase of CTG3D signal between T0 and T120 (Table EV1), indicating that these two models did not grow sufficiently under the screening conditions (while they displayed a minor increase in signal during the growth curve experiments).

Further data analysis was performed using R (v3.6.3). Area Under the Curve (AUC) values were calculated for every drug per tumoroid model and replicate (in the case of the tumoroid models with technical replicates) using the auc function of the MESS package (v0.5.6). In the case of RMS tumoroid models with technical duplicates, the two resulting AUC values per drug were correlated (using the cor function of base R) to calculate the correlation between replicates and plotted using the plot function of base R (see above). For the further downstream analysis, these replicate AUC values were averaged, resulting in a matrix with one AUC value per drug per RMS tumoroid model. On this matrix, unsupervised clustering was performed using the get_dist function from the factoextra package (v1.0.7) using the arguments “pearson” for RMS tumoroid models and “euclidean” for drugs to measure dissimilarity with further clustering using the hclust function from base R using the “average” argument for linkage. Data were visualized using the heatmap.2 function from the gplots package (v3.0.3) using dendrogram generation from the established clustering. Principal component analysis (PCA) was performed using the prcomp function from base R with exclusion of the RMS tumoroid model RMS000FLV due to its outlier behavior. PCAs were visualized using the fviz_pca_ind and fviz_pca_var functions from the above-mentioned factoextra package.

CRISPR/Cas9 knockout of TP53 and functional evaluation

RMS012 tumoroid cells were kept under standard conditions (BM1* supplemented with 0.1% BME) prior to the experiment. Upon reaching 70% confluency, tumoroid cells were passaged as usual and seeded with high density into wells of a 24-well plate. After 24 h of recovery, transfection was performed: Nucleic acid–Lipofectamine 2000 complexes were prepared according to the standard Lipofectamine 2000 protocol (Invitrogen). Four μl of Lipofectamine 2000
reagent in 50 µl Opti-MEM medium (Gibco) and 1.5 µg of DNA (pSpCas9(BB)-2A-GFP control or sgRNA TP53 plasmid in 50 µl Opti-
MEM medium) were mixed, incubated for 5 min, and added to the
cells. Plasmids were kindly shared by Jarno Drost (PMC, NL). For
the plasmid sequence refer to Drost et al (2015). Twenty-four hours
after transfection, transfection efficiency was evaluated using fluo-
rescence microscopy, detecting GFP positive cells. Forty-eight hours
after transfection, selection with nutlin-3 (10 µM) was started. Four
days after the start of selection, first nutlin-3 resistant colonies could
be detected in TP53 knockout plasmid transfected cells whereas
cells transfected with control plasmids died due to nutlin-3 expo-
sure. Putative knockout cells were further expanded.

Knockout was confirmed using Western Blotting and Sanger
sequencing of genomic DNA: For Western Blotting, snap-frozen
tumoroid cell pellets were lysed in Phosphatase-substituted RIPA
buffer and run on a 10% precast gel for P53 detection (BioRad).
Protein levels of P53 (1:1,000, Santa Cruz DO-1 P53 antibody) were
detected while Histone 3 (1:2,000, Abcam ab1791 Pan-H3) served as
loading control. For visualization, secondary antibodies (goat anti-
rabbit, BioRad 1706515/goat anti-mouse BioRad 1706516) conju-
gated with horseradish peroxidase (HRP) were used together with
ECL substrate (Perkin Elmer) on an imaginer (BioRad ChemiDoc).

Protein levels of P53 (1:1,000, Santa Cruz DO-1 P53 antibody) were
detected while Histone 3 (1:2,000, Abcam ab1791 Pan-H3) served as
loading control. For visualization, secondary antibodies (goat anti-
rabbit, BioRad 1706515/goat anti-mouse BioRad 1706516) conju-
gated with horseradish peroxidase (HRP) were used together with
ECL substrate (Perkin Elmer) on an imaginer (BioRad ChemiDoc).

The experiment was performed three times. Further data analysis
was performed using Phusion High-Fidelity DNA Polymerase (New
England BioLabs) using the following primers: forward 5′-CCCATT
TACAGTCCCCCTTG-3′, reverse 5′-CAGGAAGCCAGGCGTAAAAG-3′.
PCR products were cleaned up and concentrated using a DNA
Clean-up and Concentration Kit (Zymo Research) according to the
manufacturer’s protocol. Gel electrophoresis indicated the presence
of a specific product which was sent for Sanger sequencing using
the following primers: forward 5′-TTGTTACATGAGACCCAGG-3′,
reverse 5′-GAAGTCTCATGGAAGCCAGG-3′. Obtained sequences
were aligned and inspected using the Benchling browser tool
(https://www.benchling.com/). Furthermore, sequencing data was
submitted for TIDE (Tracking of Indels by Decomposition, http://
tide.nki.nl) analysis to infer the composition of Indels in the knock-
out population (Brinkman et al, 2014).

For detection of DNA double strand breaks, induction of γH2AX
was measured using Western Blotting. Fusion-negative embryonal
rhabdomyosarcoma cell line RD (kindly shared by Jan Molenaar,
PMC, NL) served as a control (either untreated or treated with 1, 5,
or 10 µM of staurosporine (Sigma) for 24 h). RD cells (ATCC identi-
fier CCL-136, species human) were cultured under conventional
conditions as detailed by the American Type Culture Collection
( ATCC) with regular testing for mycoplasma contamination. For
Western Blotting, snap-frozen tumoroid (RMS012 TP53 KO) or
tumor (RD) cell pellets were lysed in Phosphatase-substituted RIPA
buffer and run on a 15% self-cast gel for γH2AX (p.S139) detection.
Protein levels of γH2AX (p.S139, 1:2,000, Abcam ab26350 antibody)
detected while GAPDH (1:1,000, Abcam ab9485 antibody) served as
loading control. Visualization was performed as described
above for the confirmation of the knockout. As above, Western Blot-
ting analysis was performed twice, and a representative blot is
shown.

To assess differential response to prexasertib (Med-
chemExpress), RMS012 tumoroid models (TP53 wildtype and TP53
knockout) were processed according to the same protocol as for
the growth curve experiments (see above), with 500 cells seeded
per well. Prexasertib (dilution series from 200 to 0.78125 nM) was
added 48 h after seeding of the tumoroid spheres. Control samples
were treated with DMSO. Readouts were performed using CellTiter-Glo 3D Cell Viability Assay (CTG3D, Promega, cat no.
G9683) according to the manufacturer’s protocol at T72 (72 h after
addition of drugs). Dose-response was estimated per model and
concentration in relation to the DMSO-treated cells (set to 100%).
The experiment was performed three times. Further data analysis
was performed using R (v3.6.3). A sigmoidal fit for the dose-
response curve was calculated per replicate for both the knockout
and wild-type sample with a three-parameter log-logistics function
using the drc package (v3.6.3; Ritz et al, 2015). The statistical sig-
nificance of the differences in fitted IC50 values between knockout
and wildtype were obtained using a two-sided e-test. For visualiza-
tion purposes, a three-parameter sigmoidal fit per model (not per
replicate) was used.

Image postprocessing and figure preparation

Microscopy images from RMS tumoroid IHC and H&E stainings as
well as images from Western Blotting were postprocessed according
to good scientific practice with Adobe Photoshop 2021 and Fiji
(v2.0.0-rc-69/1.52; Schindelin et al, 2012). Images from original
RMS tumors (H&E and IHC) were not processed. Figures were pre-
pared using Adobe Illustrator 2021.
Data availability

Bulk sequencing data (i.e., whole-genome sequencing and bulk mRNA sequencing) have been made openly available at the European Genome-Phenome Archive (EGA) with the identifiers EGAD00001008466 (“WGS soft tissue sarcoma tumoroid biobank”), EGAD00001008467 (“hTERT-Ras primary pediatric kidney tumor controls for the soft tissue sarcoma tumoroid biobank”), and EGAD00001008709 (“RNA-Seq soft tissue sarcoma tumoroid biobank”). Single-cell mRNA sequencing data of the RMS tumoroid models RMS127 and RMS444 as well as the CBMC controls are similarly available at EGA with the identifier EGAD00001009002 (“sc-RNA-Seq soft tissue sarcoma tumoroid biobank”). Code for bulk sequencing data analysis is made openly available at https://github.com/teresouza/rms2018-009.

Expanded View for this article is available online.

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Author contributions

Michael T Meister: Conceptualization; supervision; investigation; visualization; methodology; writing — original draft; writing — review and editing. Marian JA Groot Koerkamp: Data curation; investigation; methodology. Terezinha de Souza: Data curation; investigation; visualization. Willemijn B Breunis: Investigation; methodology. Ewa Frazer-Mendelewskua: Investigation. Mariel Brok: Investigation. Jeff DeMartino: Investigation; visualization. Freek Manders: Investigation; visualization. Camilla Calandrini: Investigation. Hinri HD Kerstens: Data curation. Alex Janse: Data curation. M Emmy M Dolman: Resources. Selma Eising: Resources. Karin PS Langenberg: Resources. Marc van Tuil: Investigation. Rutger KG Knops: Resources. Sheila Tervisschavanschueltina: Resources. Laura S Hiemcke-jiwava: Resources. Uta Flucke: Resources. Johannes HM Merks: Resources. Max M van Noesel: Resources. Bastiaan Bj Tops: Resources. Jayne Y Hehir-Kwa: Resources. Patrick Kemmeren: Resources. Jan J Molenaar: Resources. Marc van de Wetering: Resources. Ruben van Boxtel: Resources. Janno Drost: Resources; writing — review and editing. Frank CP Holstege: Conceptualization; supervision; funding acquisition; writing — original draft.

In addition to the CRediT author contributions listed above, the contributions in detail are:

- Conceptualization: MTM and FH; Methodology: MTM, MJACK and WBB.
- Investigation: MTM, MJACK, TS, WBB, EF-M, MB, JDe, CC, FM, and MT.
- Resources: MEMD, SE, KPSL, RRCK, STS, LSH-J, UF, JHMM, MMN, BBJT, JYH-K, PK, JJM, MIW, RB and JDr; Data curation: MJACK, TS, AJ and HHDK; Writing—Original Draft: MTM and FCPH; Writing—Review and Editing: MTM and JDr; Visualization: MTM, TS, JDe, and FM; Funding Acquisition: FCPH; Supervision: MTM and FCPH.

Disclosure and competing interests statement

The authors declare that they have no conflict of interest.

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