Chromosomal instability (CIN) is a hallmark of cancer\(^1\). Yet, many childhood cancers, such as Ewing sarcoma (EwS), feature remarkably ‘silent’ genomes with minimal CIN\(^2\). Here, we show in the EwS model how uncoupling of mitosis and cytokinesis via targeting protein regulator of cytokinesis 1 (PRC1) or its activating polo-like kinase 1 (PLK1) can be employed to induce fatal genomic instability and tumor regression. We find that the EwS-specific oncogenic transcription factor EWSR1-FLI1 hijacks PRC1, which physiologically safeguards controlled cell division, through binding to a proximal enhancer-like GGAA-microsatellite, thereby promoting tumor growth and poor clinical outcome. Via integration of transcriptome-profiling and functional in vitro and in vivo experiments including CRISPR-mediated enhancer editing, we discover that high PRC1 expression creates a therapeutic vulnerability toward PLK1 inhibition that can repress even chemo-resistant EwS cells by triggering mitotic catastrophe. Collectively, our results exemplify how aberrant PRC1 activation by a dominant oncogene can confer malignancy but provide opportunities for targeted therapy, and identify PRC1 expression as an important determinant to predict the efficacy of PLK1 inhibitors being used in clinical trials.
Chromosomal instability (CIN) refers to the ongoing acquisition of genomic alterations comprising circumscribed structural aberrations, deletions of chromosome arms, translocations, and gains/losses of whole chromosomes\(^3\). Through moderate CIN, most cancer types acquire somatic copy number alterations during each cell division creating intra-tumor heterogeneity that enhances the overall ‘fitness’ of the cancer cell population\(^4\). However, excessive CIN causes non-viable karyotypes\(^5\)–\(^8\). While CIN-induction in cancers being intrinsically genomically unstable may have little therapeutic benefit, it may cause massive cell death in cancers with ‘silent’ or nearly diploid genomes, such as pediatric cancers including Ewing sarcoma (EwS)\(^3\)–\(^6\). Indeed, EwS genomes are remarkably silent with diploid genomes, such as pediatric cancers including Ewing sarcoma tissues (Fig. 1a). PRC1 plays pivotal roles in orchestrating cytokinesis, kinesins, and polo-like kinase 1 (PLK1)\(^1\)–\(^10\). Thus, we hypothesized that CIN-induction, e.g., by uncoupling mitosis and cytokinesis\(^11\), may constitute a new therapeutic option for EwS.

**Results and discussion**

To identify a candidate target offering a large therapeutic window, we analyzed curated expression data of 929 normal tissue samples (71 tissue types) and 50 primary EwS tumors\(^12\) for cytokinesis-related genes being highly overexpressed in EwS. As shown in Supplementary Fig. 1, protein regulator of cytokinesis 1 (PRC1) exhibited the greatest fold change among 77 significantly overexpressed cytokinesis-related genes (FDR-adjusted \(P < 0.05\)), being on average \(\sim 8\)-fold higher expressed in EwS than in normal tissues (Fig. 1a). PRC1 plays pivotal roles in orchestrating cytokinesis through bundling of antiparallel microtubules and formation of the spindle midzone via recruitment of regulator and effector proteins, such as centralspindlin, the chromosomal passenger complex, kinesins, and polo-like kinase 1 (PLK1)\(^11\).

Exploration of a cohort of 196 EwS patients\(^13\) uncovered that high PRC1 expression significantly correlated with poor overall survival (\(P = 0.005\)), which was validated in an independent cohort of 144 patients at the protein level (\(P = 0.0037\)) (Fig. 1b, c). Multivariate regression analysis of data from the 96 of these 144 patients with complete clinical annotation revealed that besides metastatic disease at diagnosis, high PRC1 expression was an independent risk factor (HR = 3.1; \(P = 0.04\)) (Supplementary Data 1).

To test for a potential EWSR1-ETS-dependency of PRC1 in EwS, we carried out time-course knockdown experiments in A673 EwS cells harboring a doxycycline (Dox)-inducible shRNA against EWSR1-FLI1 (A673/TR/shEF1). Indeed, knockdown of EWSR1-FLI1 was associated with a striking reduction of PRC1 transcription, which was confirmed in vivo in xenografted A673/TR/shEF1 cells (Fig. 2a, b). Such effect was not observed in xenografts expressing an inducible non-targeting control shRNA (A673/TR/shCtrl) (Fig. 2b).

Since EWSR1-ETS oncoproteins regulate many of their target genes through DNA-binding at enhancer-like GGAA-microsatellites (mSats)\(^14\)–\(^18\), we analyzed available DNase-Seq and ChIP-Seq data from EwS cell lines (A673, SK-N-MC)\(^15\)–\(^19\) for EWSR1-FLI1 and histone marks (H3K4me1, H3K27ac). We found a prominent EWSR1-FLI1 signal located \(-90\) kb telomeric from the PRC1 promoter that mapped to a GGAA-mSat within open chromatin (DNase 1 hypersensitivity site) (Fig. 2c). In both cell lines, silencing of EWSR1-FLI1 was accompanied by a loss of EWSR1-FLI1 signals at this locus, and, more interestingly, with a loss of H3K27 acetylation usually marking active enhancers (Fig. 2c). The EWSR1-FLI1-dependent enhancer activity of this GGAA-mSat was confirmed in reporter assays in A673/TR/shEF1 cells with/without silencing of EWSR1-FLI1 (Fig. 2d).

Since GGAA-mSats are typically polymorphic, we tested several cloned fragments (1053 bp including flanking regions) from three

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**Fig. 1 PRC1 is overexpressed in EwS and correlates with poor overall survival.** a Analysis of PRC1 mRNA expression in 50 EwS and 929 normal tissues (comprising 71 normal tissue types). Data are represented as box plots. Horizontal bars indicate median expression levels, boxes the interquartile range, and whiskers the 10th and 90th percentile. Two-sided Mantel-Haenszel test. b Kaplan-Meier survival analysis of 196 EwS patients stratified by thirds of PRC1 mRNA expression (Low, Moderate, High). Two-sided Mantel-Haenszel test. c Left: IHC staining of a TMA comprising 144 EwS tumors for PRC1. Scale bar = 100 \(\mu\)m. Right: Kaplan-Meier analysis of overall survival of 144 EwS patients stratified by their intra-tumoral PRC1 expression levels (Low: IRS < 2; Moderate: IRS 2-5; High: IRS > 5). Two-sided Mantel-Haenszel test.
different EwS cell lines that had different numbers of consecutive GGAA-repeats at this locus (A673, on average 14 repeats [14/14]; EW1, on average 12 repeats [13/11]; TC71, on average 10.5 repeats [11/10]). We noted a strong gain of enhancer activity with increasing numbers of consecutive GGAA-repeats (Fig. 2d), which has been previously described for other GGAA-mSats to contribute to inter-tumor heterogeneity in EwS 17,18. Another genetic variation in the cloned fragments was excluded by whole-genome sequencing and focal Sanger-sequencing. Notably, the average number of GGAA-repeats at this mSat corresponded to the PRC1 expression levels across EwS cell lines at both the mRNA and protein levels (Supplementary Fig. 2b). Consistently, analysis of publicly available proteomics data (https://depmap.org/portal/) comprising 4 EwS cell lines including TC71 and A673 showed that TC71 exhibited the lowest and A673 the highest PRC1 protein levels (Supplementary Fig. 2c).

To confirm the regulatory effect of this GGAA-mSat on PRC1 transcription, we carried out clustered regulatory interspaced...
short palindromic repeats interference (CRISPRi) experiments in two EwS cell lines (A673, RDES). Epigenetic interference with this GGAA-mSat markedly reduced PRC1 expression and proliferation of derivative EwS cells (A673/TRE/dCas9/KRAB, RDES/TRE/dCas9/KRAB) (Fig. 2e). The relationship between PRC1 and this GGAA-mSat was further probed by CRISPR Cas9-initiated homologous deficiency repair (HDR) DNA editing. To avoid knocking out PRC1 itself, which may have led to unphysiologically low or absent PRC1 expression levels, we chose to knockout (KO) its associated enhancer-like GGAA-mSat. Similar to CRISPRi, the genetic knockout (KO) of the PRC1-associated GGAA-mSat was accompanied by significantly lower PRC1 expression levels, proliferation, and sphere-formation in both cell lines (Supplementary Fig. 2d–h). Conversely, replacement of the wildtype mSat comprising 14 consecutive GGAA-repeats by a ‘longer’ haplotype (24 GGAA-repeats) significantly increased PRC1 expression, proliferation, and sphere-formation (Fig. 2f, Supplementary Fig. 2g, h). To elucidate whether PRC1 was directly regulated by EWSR1-FL1, we performed chromosome conformation capture (3C)-PCR assays on A673 cells with either a KO or genetically elongated PRC1-associated GGAA-mSat. These experiments confirmed a physical interaction of both DNA elements, which was abrogated by KO of the PRC1-associated GGAA-mSat (Fig. 2g, Supplementary Fig. 2i). In synopsis, our results indicated that PRC1 is a direct EWSR1-FL1 target whose high but variable expression is controlled by EWSR1-FL1-binding to a polymorphic enhancer-like GGAA-mSat.

To explore the functional role of PRC1 in EwS, we generated three EwS cell lines (RDES, SK-N-MC, TC32) with Dox-inducible shRNAs against PRC1 (shCDS/shUTR) and non-targeting controls (shCtrl). Knockdown efficacy was confirmed by qRT-PCR and western blotting (Supplementary Fig. 3a, b). We first carried out transcription profiling of two of these EwS cell lines (RDES, SK-N-MC) after Dox-induced PRC1 silencing (Supplementary Data 2). Functional gene-set enrichment followed by weighted genic capacity and anchorage-independent growth in vitro and expression, proliferation, and sphere-formation (Fig. 2f, Supplementary Fig. 2g, h). To elucidate whether PRC1 was directly regulated by EWSR1-FL1, we performed chromosome conformation capture (3C)-PCR assays on A673 cells with either a KO or genetically elongated PRC1-associated GGAA-mSat. These experiments confirmed a physical interaction of both DNA elements, which was abrogated by KO of the PRC1-associated GGAA-mSat (Fig. 2g, Supplementary Fig. 2i). In synopsis, our results indicated that PRC1 is a direct EWSR1-FL1 target whose high but variable expression is controlled by EWSR1-FL1-binding to a polymorphic enhancer-like GGAA-mSat.

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Based on PRC1’s essential function in spindle midzone formation21, we reasoned that these phenotypes may be explained by excessive CIN resulting from cytokinesis defects upon PRC1 knockdown. Indeed, cell cycle analysis of synchronized EwS cells showed that PRC1 silencing led to a higher fraction of cells in the G2/M phase over time, which is indicative of a delayed transition through the G2/M-phase that may contribute to the generation of tetraploid cells (Supplementary Fig. 3f). Fluorescence-in-situ-hybridization (FISH) analyses using pan-centromere probes revealed that PRC1 knockdown for only 48 h induced likely non-viable chromosomal abnormalities in ~70% of EwS cells compared to ~10% in controls (Fig. 3g). Moreover, time-lapse live-cell imaging of TC32 and RDES EwS cells with/without PRC1 knockdown demonstrated that PRC1 silencing was associated with a significantly higher number of cells exhibiting mitotic activity without subsequent cytokinesis resulting in chromosome missegregation in both cell lines (P = 0.0022) (Fig. 3h, Supplementary Fig. 3g). These in vitro observations corresponded to a higher degree of nuclear pleomorphism and the presence of so-called, ‘monster’ cells with bizarre, aneuploid, and often multinucleated nuclei, as well as to higher rates of DNA double-strand breaks as indicated by γ-H2AX stains in vivo (Fig. 3i, j). The human origin of monster cells was validated by immunohistochemistry (IHC) using a human-specific anti-mitochondrial antibody (Fig. 3i). In sum, these results indicated that PRC1 is essential for proper cell division in EwS and that disruption of the delicate balance between mitosis and cytokinesis causes non-viable karyotypes in this otherwise genetically silent pediatric cancer.

Based on these results, we hypothesized that targeting cytokinesis may offer a new therapeutic strategy for EwS. Since—to the best of our knowledge—there are no direct inhibitors against PRC1 available, we explored whether targeting its major upstream interacting partner PLK1 could serve as an alternative target. Indeed, PLK1 phosphorylates and binds to PRC1, which enables the interaction of the PLK1-PRC1 complex that is critical for its translocation to the central spindle to initiate cytokinesis11,21,22. In accordance, both genes were highly significantly co-expressed in patient EwS tumors (n = 196, r Pearson = 0.58, P = 2.2E-16) (Supplementary Fig. 4a), and combining both markers yielded a highly significant association with worse overall survival (P = 0.0013) (Supplementary Fig. 4b, c). Thus, we treated our EwS models with two small-molecule ATP-competitive PLK1 inhibitors (BI2536, BI67277 [alias Volasertib]) that are currently used in clinical trials (Supplementary Data 3). Both inhibitors showed strong anti-proliferative effects on EwS cells at lower nanomolar levels in vitro (Fig. 4a, b). Even more strikingly, direct and indirect downregulation of PRC1 either by RNA interference or genetic KO of the PRC1-associated GGAA-mSat dramatically diminished the sensitivity of EwS cells toward both PLK1 inhibitors (Fig. 4a, b). Conversely, upregulation of PRC1 via CRISPR-mediated elongation of the PRC1-associated GGAA-mSat significantly increased their sensitivity toward both inhibitors (Fig. 4b). Importantly, it should be noted that the percentage of viable cells at the time of the beginning of PLK1-treatment was ~90–95% and that the percentage of apoptotic cells was equal in the control and treatment groups (Supplementary Fig. 4d, e). Similar to long-term suppression of PRC1, long-term treatment of EwS cells with both inhibitors strongly reduced clonogenic and anchorage-independent growth, and significantly induced apoptosis (Fig. 4c, d, Supplementary Fig. 4f). Together, these findings suggested that EwS cells with high PRC1 expression are very sensitive to PLK1 inhibition and that this sensitivity can be almost abolished by the suppression of PRC1.

To confirm the PRC1-dependency of PLK1 inhibition in vivo, we used our CRISPR Cas9-initiated HDR edited A673 EwS cells and xenografted them in NOD/scid/gamma (NSG) mice. Once tumors were palpable, mice were treated with BI2536 (40 mg/kg) or BI67277 (30 mg/kg) once per week via tail vein injection at clinically achievable dosages (Supplementary Data 4). Treatment of mice xenografted with highly PRC1 expressing EwS cells led to strong inhibition of tumor growth and even tumor regression after only three cycles of treatment without overt adverse effects, such as weight loss (Fig. 4e, f, Supplementary Fig. 4g, h). In addition, both PLK1-inhibitors led to a significant increase in the number of aneuploid cells and, monster’ cells (Supplementary Fig. 4i). Strikingly, this effect could be abrogated by genetic KO of
Fig. 3 PRC1 safeguards genome stability in EwS cells. a Transcriptomic network analysis after PRC1 knockdown. b Cell viability 72 h after PRC1 silencing. Data are displayed as individual dots. Horizontal bars represent means and whiskers SEM, n = 6 biologically independent experiments; two-sided Mann-Whitney test. c Analysis of apoptosis (summary of both shPRC1). Data are displayed as individual dots. Horizontal bars represent means and whiskers SEM, n = 6 biologically independent experiments; two-sided Mann-Whitney test. d Sphere-formation after PRC1 knockdown. Data are displayed as individual dots. Horizontal bars represent means and whiskers SEM, n = 9 biologically independent experiments; two-sided Mann-Whitney test. Representative images; scale bar = 50 µm. e The volume of xenografts with/without PRC1 silencing. Data are mean and SEM, n indicates the number of animals per condition: n = 5 shCtrl and n = 6 shCDS/shUTR in both cell lines. Two-sided Mann-Whitney test. f Representative micrographs and quantifications of PRC1 (IRS), Ki-67, and cleaved caspase-3 (CC3). Data are displayed as individual dots. Horizontal bars represent median IRS of PRC1 or means and whiskers SEM for positivity, respectively; the number of animals per condition is as in e; two-sided Mann-Whitney test; scale bar = 100 µm. g Representative images (scale bar = 1 µm) and quantification of aberrant metaphases. Horizontal bars represent means and whiskers SEM, n = 6 biologically independent experiments. Parentheses indicate a total number of analyzed mitoses (summary of both shPRC1). Two-sided Chi-squared test. h (Left) Time-lapse images of TC32 cells with/without PRC1 knockdown. F-actin (red), DNA (blue). Arrows = merged nuclei. Scale bar = 10 µm. (Right) Quantification of cytokinesis failure (summary of both shPRC1). Data are displayed as individual dots. Horizontal bars represent means and whiskers SEM; n = 6 biologically independent experiments. i Representative micrographs of xenografts (H&E, human mitochondria (mt)), and quantification of monster’ cells per low-power field (LPF). Data are displayed as individual dots. Horizontal bars represent means and whiskers SEM, the number of animals per condition is as in e; two-sided Mann-Whitney test; scale bar = 20 µm. j Representative micrographs for γ-H2AX, and quantification per HPF. Data are displayed as individual dots. Horizontal bars represent means and whiskers SEM, the number of animals per condition is as in e; two-sided Mann-Whitney test; scale bar = 100 µm.
the PRC1-associated enhancer-like GGAA-mSat (Supplementary Fig. 4h, i). Remarkably, mice with total tumor regression did not show any sign of tumor recurrence up to 25 days after the last cycle as confirmed by necropsy and IHC (Supplementary Fig. 4j, k). In contrast, xenografts from A673 EwS cells with KO of the PRC1-associated GGAA-mSat exhibited delayed tumor growth and only progressive disease during treatment despite a 4th injection was administered (Fig. 4f, Supplementary Fig. 4h, i). Together, these findings suggested that genomically silent pediatric cancers, such as EwS, may be very sensitive to PLK1 inhibitors. Fig. 4 High PRC1 expression levels prime EwS cells for PLK1 inhibition. a Heatmaps depicting the average percentage of growth inhibition in EwS cells with/without PRC1 silencing after 72 h of PLK1 inhibition; n = 6 biologically independent experiments. b Analysis of IC50 in CRISPR Cas9-edited A673 cells after 72 h of PLK1 inhibition. Data are displayed as individual dots. Horizontal bars represent means and whisks SEM, n = 6 biologically independent experiments. c Sphere-formation of EwS cells treated with BI2536 or BI6727. Data are displayed as individual dots. Horizontal bars represent means and whisks SEM, n = 9 biologically independent experiments. e Schematic of PLK1 inhibitor treatment in vivo. d Analysis of apoptosis of EwS cells with/without PLK1 inhibition for 72 h. Data are displayed as individual dots. Horizontal bars represent means and whisks SEM, n = 6 biologically independent experiments. e Excess over Bliss analysis of RDES cells with/without PRC1 knockdown treated with combinations of Vincristine (VCR) and the PLK1 inhibitors in vitro (red indicates synergy). Summary level (mean) data from n = 6 biologically independent experiments. f Analysis of tumor volume of mice treated with either vehicle, VCR (1 mg/kg/d, i.p.) alone, BI2536 or BI6727 for 72 h. Data are displayed as individual dots. Horizontal bars represent means and whisks SEM, n = 6 biologically independent experiments. j Dose-response curves of two Doxorubicin (Doxo)-resistant EwS cell lines (A673, TC71) treated with Doxo combined with BI2536 or BI6727 for 72 h. The reverse index (RI) is given. Dots represent means and whisks SEM, n = 6 biologically independent experiments. k Schematic illustrating key findings of this study. Two-sided Mann-Whitney test in all panels.
inhibition in case of high PRC1 expression. In support of this notion, analysis of matched in vivo gene expression and drug response data from pediatric tumor types (including EwS)\textsuperscript{24} with relatively silent genomes revealed that good responses to BI6727 (Volasertib) were observed exclusively among PRC1 high expressing xenografts (defined by median expression; \(P = 0.0325\), Fisher’s exact test)—an effect not observed for PLK1 and MKI67 (Supplementary Data 5). However, since this dataset contained xenografts from only 4 different EwS cell lines, we extended our analyses using publicly available drug-response and gene expression data from the DepMap project, comprising 11 EwS cell lines (https://depmap.org). In this dataset, we observed a relatively strong negative correlation (\(r_{\text{Pearson}} = -0.54\)) of PRC1 mRNA levels with lower cell viability upon Volasertib treatment (\(P = 0.04\)) (Supplementary Data 6). This correlation even remained significant when focusing on the 9 of 11 EwS cell lines that exhibited a confirmed EWSR1-FLI1 fusion (\(r_{\text{Pearson}} = -0.72\), \(P = 0.02\)) (Supplementary Fig. 4i). Similar to our observations made in the in vitro dataset (Supplementary Data 5)\textsuperscript{25}, such correlations were not observed for PLK1 nor MKI67 irrespective of the EWSR1-FLI1 status (PLK1: \(r_{\text{Pearson}} = -0.19/0.4\), \(P = 0.29/0.14\); MKI67: \(r_{\text{Pearson}} = 0.04/-0.09\), \(P = 0.46/0.41\)) (Supplementary Data 6).

In the clinical setting, Vincristine (VCR) and Doxorubicin (Doxo) are highly active chemotherapeutics employed in first-line treatment and relapsed EwS\textsuperscript{10}. Hence, we assessed the potential synergistic effects of PLK1 inhibition with both drugs. As shown in Fig. 4g, the microtubule-destabilizing drug VCR showed highly synergistic effects (positive Bliss score) at nanomolar concentrations in RDES cells, which was strongly diminished upon PRC1 knockdown. This PRC1-dependent effect was confirmed in TC32 EwS cells (Supplementary Fig. 5a) and corresponded to prior observations of the synergy of BI6727 with VCR in this cell line\textsuperscript{24}. This combination therapy also significantly reduced the necessary IC50 of PLK1 inhibitors (Supplementary Fig. 5b), which may help to mitigate their potential adverse effects. It should be noted that almost identical effects on the PRC1-dependent PLK1 efficacy were observed in TC32 and RDES cells (Fig. 4g, Supplementary Fig. 5a), which differ in their STAG2 and TP53 mutation status (TC32: STAG2-mut/TP53-wt; RDES: STAG2-wt/TP53-mut)\textsuperscript{7,8}, suggesting that both mutations have likely no impact on the efficacy of PLK1 inhibition in EwS cells, although it has been reported that PLK1 can phosphorylate STAG1\textsuperscript{25,26}. Along the same lines, there was no statistically significant overlap between STAG2-mutated or TP53-mutated and PRC1 highly expressing patient EwS tumors (Supplementary Data 7). Similar to the in vitro findings, we noted a strong synergistic effect of BI6727 and VCR in vivo, even when applying a 5-fold reduced dose of BI6727 (now 5 mg/kg) as inferred from our in vitro synergy assays (Supplementary Fig. 5c). In fact, while VCR or BI6727 (at the reduced dose) as single agents only delayed tumor growth, a combination of both drugs led to tumor regression in all mice without adverse effects, such as weight loss (Fig. 4h, Supplementary Fig. 5d, e). However, such a synergistic effect was not observed for the intercalating drug Doxo across both cell lines and PLK1 inhibitors regardless of the PRC1 levels (Supplementary Fig. 6a). Yet, it is noteworthy that both PLK1 inhibitors were still effective at nanomolar concentrations in EwS cell lines being highly resistant to Doxo (Supplementary Fig. 6b), and that they could partially restore their Doxo-sensitivity (Fig. 4i, j).

Collectively, our results demonstrate that the EWSR1-FLI1-mediated high PRC1 expression sensitizes EwS cells for PLK1 inhibition, and renders PRC1 as a promising predictive biomarker for therapies evoking cytokinesis defects and mitotic catastrophe (Fig. 4k). We anticipate that CIN-directed therapies have the potential to profoundly affect clinical outcomes, especially in cancers with largely silent genotypes, such as EwS. Still, careful biomarker-guided selection of patients is required to identify those patients who may benefit in particular from therapeutic modulation of CIN. In this regard, PLK4 inhibition was recently recognized as a therapeutic treatment option specifically for TRIM37-amplified neuroblastoma and breast cancer via causing centromere-dysfunction\textsuperscript{27,28}. Notably, our data may also shed new light on why previous preclinical testing of PLK1 inhibition in non-preselected EwS models may have yielded heterogeneous results on its efficacy\textsuperscript{23}. In fact, especially the TC71 cell line used in this screen exhibited a relatively low response toward BI6727 and the lowest PRC1 expression levels among all four cell lines tested in vivo\textsuperscript{23} (Supplementary Data 5). These findings correspond to the rather low PRC1 expression levels and short PRC1-associated GGA-A-mSat of the TC71 cell line as demonstrated in this study (Supplementary Fig. 2b). Yet, it should be noted that the interaction of PLK1 and PRC1 is complex: While PRC1 phosphorylation by PLK1 is required for the formation of the PRC1-PLK1 protein complex and its translocation to the spindle midzone, it has been reported that PLK1 can also negatively regulate PRC1 to prevent premature midzone formation before cytokinesis\textsuperscript{29}. In turn, microtubules can stimulate PRC1 phosphorylation by PLK1, creating a potential negative feedback loop controlling PRC1 activity\textsuperscript{29}. These facts imply that PRC1 highly expressing cells may have adapted by increasing PLK1 to dampen the effects of high PRC1, which would be in agreement with our finding that PRC1 and PLK1 are significantly co-expressed in patient EwS tumors (Supplementary Fig. 4a). Accordingly, it is tempting to speculate that when PLK1 is inhibited, the PRC1 function may become deregulated and toxic to the cell. Although this is subject to future research, it is conceivable that the PRC1-related mechanism identified in our EwS model may be translatable to other cancers for which immunohistochemical detection of high PRC1 levels could serve as a broadly available, and inexpensive predictive biomarker.

**Methods**

**The provenience of cell lines and cell culture conditions.** Human cell lines were provided by the following repositories and/or sources: A673, HEK293T, and RDES cells were purchased from ATCC. SK-N-MC was provided by the German Collection of Microorganism and Cell Cultures (DSMZ). TC71 and TC32 cells were kindly provided by the Children’s Oncology Group (COG). A673/TR4/dE1F1 cells were described previously\textsuperscript{30}. Ew1 cells were kindly provided by O. Delattre (Institut Curie Research Center, Paris, France). All cell lines were cultured in RPMI-1640 medium with stable glutamine (Biocrom) supplemented with 10% tetracycline-free fetal calf serum (FCS; Sigma-Aldrich) and 100 U/ml penicillin and 100 µg/ml streptomycin (Merck) at 37°C with 5% CO2, in a humidified atmosphere. Cell lines were routinely tested for mycoplasma contamination by nested PCR, and cell line identity was regularly verified by STR-profiling. Doxorubicin-resistant (Doxo-res) EwS cells were established through continuous culture with serially increasing Doxo concentrations starting at ~10 nM corresponding to predetermined IC50 values. After successful adaptation to the given concentrations indicated by re-growth, cells were cultured with serially ascending Doxo concentrations by multiplying the IC50 values by factor 1.1–2.0. Doxo-res variants were maintained with ~200 nM Doxo.

**RNA extraction, reverse transcription, and quantitative real-time PCR (qRT-PCR).** Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel). One microgram of total RNA was reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR reactions (final volume 20 µl) were performed using SYBR Green Mastermix (Applied Biosystems) mixed with diluted cDNA (1:10) and 0.5 µM forward and reverse primer on a BioRad CFX Connect instrument and analyzed using BioRad CFX Manager.
Cloning of GGAA-mSats and luciferase reporter assays. To assess the average enhancer activity of both alleles of the PRC1-associated GGAA-mSat in a given cell, 1033 bp fragments (hg19 coordinates: chr15:91,623,953–91,625,005) including PRC1-associated GGAA-mSats (hg19 coordinates: chr15:91,624,412–91,624,459) (Supplementary Fig. 2a) from three EwS cell lines (A673, EW1, TC71) were PCR-cloned upstream of the SV40 minimal promoter into the pGL3-Fluc vector (Promega, #E1761). Primer sequences are given in Supplementary Data 8. The presence of additional variants devoid of the GGAA-mSat was ruled out by whole genome sequencing of the parental cell lines and Sanger-sequencing of the cloned fragments. Each DNA fragment (2 x 50 ng per reaction) representing 2 alleles of the mSat-containing pGL3-Fluc vectors of a given cell line in equal mass and with the Renilla pGL3-Bluc vectors (Promega) (ratio 100:1) in a six-well plate with 2 ml of growth medium. Transfection medium was replaced by medium with/without Doxycycline (Dox) (1 µg/ml, Sigma-Aldrich) 4 h after transfection. After 72 h the cells were lysed and assayed with a dual luciferase assay system (Bethold). Firefly luciferase activity was normalized to Renilla luciferase activity.

CRISPR interference. The pLKO.1-puro U6 sgRNA BfuAI large stuffer lentiviral backbone for expression of the sgRNAs and the pHAGE TRE dCas9-KRAB lentiviral plasmid21 encoding dCas9-KRAB were obtained from Addgene (#52628 and #50917). Candidate sgRNAs were identified by searching for G(N5-G) motifs 100 bases upstream/downstream of the PRC1-associated GGAA-mSat that correspond to the target sites of U6 Pol III transcription and the spCas9 PAM recognition element (NGG) (Supplementary Data 8)32. Relevant off-target matches were determined and ruled out by CRISPR-Cas9 guide RNA design checker (IDT). sgRNAs were then cloned individually into BfuAI sites at the pLKO.1-puro U6 sgRNA BfuAI large stuffer plasmid and verified by Sanger-sequencing. Lentiviral expression plasmids were constructed by transfection of the lentiviral expression plasmid along with pPAX2 (Addgene #12260) and pMD2.G (Addgene #12259) packaging plasmids. 48 h post-transfection, the lentivirus-containing medium was harvested and concentrated using the Lenti-X concentrator solution (Clontech). The A673 and RDES dCas9-KRAB stable EwS cell lines were generated by infecting the cell lines with pHAGE TRE dCas9-KRAB lentivirus in combination with G418 selection (800 µg/ml). A673 and RDES cells stably expressing dCas9-KRAB were transduced with pLKO.1-puro sgRNA lentiviral particles and treated with puromycin (1 µg/ml) and G418 (400 µg/ml) to select and maintain stable cell lines. After selection, cells were left untreated or induced with Dox at 1 µg/ml, respective of the CRISPR expression construct. Gene expression in the parental lentiviral RNP complex was analyzed by qRT-PCR and cell viability was assessed by standardized cell counting (CellTiter-Blue assay, Promega). For long-term experiments, human EwS cell lines RDES, SK-N-MC, and TC32 were transduced with lentiviral pLKO-TET-ON all-in-one vector system (Plasmid #21915, Addgene) containing a puromycin-resistance cassette for Dox-inducible expression of shRNAs against PRC1 (shPRC1) or a non-targeting control shRNA (shCtrl). Dox-inducible vectors were generated according to a publicly available protocol34 using In-Fusion HD Cloning Kit (Clontech) containing as insert a non-targeting shRNA (shCtrl) or PRC1 (shPRC1) (Supplementary Data 8). shRNAs were amplified in Stellar Competent Cells (Clontech) and integrated shRNA was verified by Sanger-sequencing (sequencing primer: 5'-GGCGAGGAATATCACCATTATGGCTTACAGA-3'). Lentiviral particles were generated in HEK293T cells. Virus-containing supernatant was collected to infect the human EwS cell lines. Successfully infected cells were selected with 2 µg/ml puromycin (InVivoGen). The shRNA expression for PRC1 knockdown in EwS cells was achieved by adding 1 µg/ml Dox every 48 h to the medium. Generated cell lines were designated RDES/TR/shCtrl, RDES/TR/shCDS, RDES/TR/shUTR, SK-N-MC/TR/shCtrl, SK-N-MC/TR/shCDS, SK-N-MC/TR/shUTR, TC32/TR/shCtrl, TC32/TR/shCDS and TC32/TR/shUTR.

Gene expression microarray analysis and functional gene-set enrichment analyses. To identify gene involved in cytokeratins in EwS, we used curated knowledge of EwS gene expression data (GSE66914) were downloaded from the GEO, processed as previously described19, and displayed in the UCSC genome browser. The following samples were used in this study: ENCODE_SKNMC hg19_DNAAssH_rep1 GSM1517546, GSM1517555, and GSM1517555. Transfection medium was replaced by medium with/without Firetriviral plasmid31 encoding dCas9-KRAB were obtained from Addgene (#52628 and #50917). Candidate sgRNAs were identified by searching for additional variants devoid of the GGAA-mSat using the publicly available gene expression data generated on Affymetrix HG-U133Plus2.0 (GSE9907) and the medium from transfected cells was replaced by a fresh medium without HDR enhancer after 24 h. Genomic DNA isolation and detection of editing efficacy at a bulk level were performed 48 h after transfection by assessment of the fluorescence intensity and by PCR amplification and gel electrophoresis. Finally, single-cell cloning was carried out to select successfully edited clones, which were verified by Sanger-sequencing (Supplementary Fig. 2d).
and statistical significance levels were calculated with limma (R package, v.3.44.3)37. Then, the resulting P-values were adjusted for multiple testing based on false discovery rate correction. Finally, up- and downregulated genes with significant fold changes (FCs) (FDR-adjusted P < 0.05) were analyzed for Gene Ontology (GO)-term enrichment using clusterProfiler (R package, v.3.16.1)38. Annotiation of GO-terms was done using the org.Hs.eg.db (R package, v.3.11.4).

To assess the impact of PRC1 on gene expression and on alternative splicing in Ews, a microarray analysis was performed. To this end, 5 × 10⁵ cells were seeded in T25 flasks and treated with Dox for 60 h (Dox-refreshment after 48 h). Thereafter, total RNA was extracted with the ReliaPrep mRNA Cell and Tissue MiniPrep System (Promega) and transcriptome profiled at IMGM laboratories (Martinsried, Germany). The data was analyzed using the R language and samples analyzed with the Bioconductor BioMart software. Gene Ontology (RIN) > 9 were hybridized to Human Affymetrix Clariom D microarrays. Data were quantile normalized with Transcriptome Analysis Console (v4.0; Thermo Fisher Scientific) using the SST-RMA algorithm. Annotation of the data was performed using the Affymetrix library for Clariom D Array (version 2, human) on the gene level. DEGs with consistent and significant FCs across EWSR1 and cell lines were identified as follows: First, the normalized gene expression signal was log2 transformed. To avoid false discovery artifacts due to the detection of only minimally expressed genes, we excluded all genes with a lower or just minimally higher gene expression signal than that observed for ERG (mean log2 expression signal of 6.29), which is known to be not expressed in EWSR1-FLI1 positive EWS cell lines8. Accordingly, only genes with mean log2 expression signals (w/o Dox condition) of at least 6.5 were further analyzed. The FCs of the shCtrl samples and both specific shRNAs were calculated for each cell line separately. Then, the FCs observed in the respective shCtrl samples were subtracted from those seen in the shPRC1 samples, which yielded the FCs for each specific shRNA in each cell line. Then these FCs were averaged to obtain the mean FC per gene across shRNAs and cell lines. To identify enriched gene sets, genes were ranked by their expression FC between the groups Dox (−/−) and a pre-ranked gene set-enrichment analysis (GSEA) was done using Igsa (R package, v.1.14.3)49 to find the biological processes GO definitions from MSigDB (v7.0, c2-gsea-all)96. To test for GSEA results against a candidate GO term, GO enrichment was filtered for significance (adjusted P < 0.01; Normalized enrichment score > 1.75) and used to create a symmetric GO adjacency matrix based on the jaccard distance between GO terms and respective gene lists. GO clusters were identified using dynamicTreeCut (R package, v.1.6.3.1), and the GO network was visualized in Cytoscape (v3.8.0)41.

**Western blotting.** EwS cells with Dox-inducible shRNAs were treated with Dox (1 µg/ml for 72 h). To test for relative PRC1 protein expression levels across EwS cell lines, EwS cells were cultured in standard culture conditions until reaching 70% confluence. Whole cellular protein was extracted using RIPA buffer containing 1 mM Na₂VO₄ and protease inhibitor cocktail (Sigma-Aldrich). Proteins were separated on a 10% gel and blotted on PVDF membranes. Membranes were incubated with rabbit polyclonal anti-PRC1 antibody (1:1000, 15617-1-AP, Proteintech) or mouse monoclonal anti-GAPDH (1:800, sc-32233, Santa Cruz). Then, membranes were incubated with horseradish peroxidase (HRP) coupled anti-rabbit IgG (H + L) (1:5000, R3436HPR, OriGene) or anti-mouse IgG (H + L) (1:3000, W022R, Promega). Proteins were detected using chemiluminescence HRP substrate (Millipore-Merck).

**Proliferation assays.** For proliferation assays, 2 × 10⁵ EwS cells harboring Dox-inducible shRNAs were seeded in wells of 6-Well plates and treated with 1 µg/ml Dox for 72 h (Dox-downduration after 48 h). Cell viability was determined by counting cells including their supernatant in standardized hemocytometers (C-Chip, Biochrom) via the Trypan-Blue (Sigma-Aldrich) exclusion method.

**Fluorescence-in-situ-hybridization (FISH).** After shRNA-induced PRC1 knockdown for 48 h, EwS cells with ~70% confluency were incubated for 6 h with 0.2 mg/ml colcemid (Demeosem Solution D1925 Sigma 10 µg/ml in HBSS). Meta-phase phase cells were harvested by trypsinizing, treated with a pre-warmed hypotonic solution (75 mM KCl) for 30 min at 37 °C, and fixed in fresh, ice-cold 3:1 methanol/acetic acid solution for 3 × 15 min. The fixed cells were kept overnight at 4 °C. Frozen slides were brought to RT on a heating plate. 20 µl of fixed cell suspension was dropped onto precoated glass slides, air-dried, and two times for 5 min in 1× phosphate-buffered saline (PBS), the slides were post-fixed in 30% (v:v) ethanol at each time point post releasing (24 h, 48 h, and 72 h), treated with 100 µg/ml RNase (ThermoFisher), and stained with 50 µg/ml propidium iodide (PI) (Sigma-Aldrich). For analysis of apoptosis, RDES, SK-N-MC, and TC32 cells harboring Dox-inducible shRNA against PRC1 and respective controls were seeded at 8 × 10⁵ cells per 10 cm dish with/without the addition of Dox (1 µg/ml) for 72 h. Dox was renewed 48 h after seeding. RDES and TC32 EwS cells were seeded at 8 × 10⁵ cells per 10 cm dish with/without the addition of PLK1 inhibitor BI2536 (5 nM) or BI6727 (20 nM) or DMSO. For analysis of apoptosis, RDES and TC32 cells harboring Dox-inducible shRNA against PRC1 and respective controls were seeded at 8 × 10⁵ cells per 10 cm dish with/without the addition of Dox (1 µg/ml) and analyzed at different time points after shRNA-mediated knockdown (24 h and 48 h). The CRISPRCas9-initiated HDR edited A673 EwS cells and A673 wt cells were seeded at 1 × 10⁶ cells per 10 cm dish and analyzed 24 h after of apoptosis by fluorescence microscope (Zeiss Axioskop 2 Plus, Germany). Samples were assayed by BD Accuri C6 Cytometer (BD Biosciences). An example of a gating strategy was given in Supplementary Fig. 7.

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karyotypic changes were classified and quantified according to the International Standard of Cytogenetic Nomenclature (ISCN)\(^{[6]}\) and as previously described\(^{[6]}\): aneuploidy (chromosome loss/gain), non-congressed, multinodal, and ‘monster’ cells.

**Time-lapse live-cell imaging.** TC32 and RDES EwS cells harboring a Dox-inducible shRNA against PRCI or a non-targeting control shRNA were injected in a 1:1 mix of cells suspended in HBSS formulated with Calcium and Magnesium (ThermoFisher) in the right amount of NGS medium. Time-lapse images were acquired every second day with a caliper and tumor volume was calculated by the formula \(V = \frac{4}{3}\pi r^3\). When the tumors reached an average volume of \(100\) mm\(^3\), mice were sacrificed by cervical dislocation. Then, xenograft tumors were extracted and fixed in 4%-formalin and parafﬁn-embedded (FFPE) for (immuno)histology. Deep Red Acting Tracking Stains (A57245, Life Technologies) in 250 μl Live Cell Imaging Solution (A14291D1, Invitrogen). The staining solution was then carefully removed and the tumors were subsequently counterstained with Hoechst 33342 Ready Flow Reagent (RFP01, Invitrogen) for 15 min in the dark at 37 °C. After exposure, cells were washed 3x with Live Cell Imaging Solution at 37 °C and then imaged and analyzed in 300 μl prewarmed FluoroBrite® DMEM (A1896701, Life Technologies) supplemented with 10% FCS, 4 mM GlutaMax (Invitrogen), and 20 mM HEPES (Invitrogen). The environment throughout imaging was controlled at 37 °C, 5% CO\(_2\), and 90% humidity. Time-lapse images were acquired with an inverted Zeiss AxioObserver Z1 microscope equipped with an AxioCam MRm grayscale CCD camera and controlled by ZEN pro software (Zeiss). Brightfield, Cy5 (AHF F36-523), and DAPI (Zeiss Filter Set 49, 488049-9901-000) were captured using x40 oil, 1.4 numerical aperture (NA) objective combined with 2 × 2 binning modes. Fluorescence and bright-ﬁled images were acquired using a 20× lens. The objective was z-stacked in 10 planes, 2 μm intervals for 2 x binning modes. Tumor and BrDU-positive cell positions were determined as follows: Ulcerated tumors, loss of body surface adherence. At the experimental endpoint, cell growth inhibition was assessed using a Resazurin assay. CompuSyn (ComboSyn, Inc.) was used for determining the DRI of each PLK1 inhibitor at the IC50 level. After the start of the treatment using a Resazurin assay. The excess over Blass was calculated using synergyﬁnder (R package v.2.2.4)\(^{[49]}\). For assessment of the dose reduction index (DRI), 5000 cells/well of Dox-inducible shRNA expressing RDES and TC32 EwS cells were seeded in triplicate wells of 96-well plates. Cells were pre-treated for 48 h with Dox to induce the PRC1 knockdown before BI6727 or BI2536 was added (10 nM Doxorubicin (Doxo), 4 nM Vincristine (VCR), and/or 0.1 nM H2AX raised in rabbit (1:8000, ab81299, Abcam), or as previously described. The product of these two grades de

**Human samples and ethics approval.** Human FFPE tissue samples were retrieved from the archives of the Institute of Pathology of the LMU Munich (Germany) and the Gerhard-Domagk Institute of Pathology of the University of Münster (Germany) with approval of the institutional review boards. All patients gave informed consent. Tissue-microarrays (TMAs) were stained and analyzed with the approval of the ethics committee of the LMU Munich (approval no. 550-16 UE).

**Immunohistochemistry (IHC) and immunoreactivity scoring.** For IHC, 4-μm FFPE sections were cut and antigen retrieval was carried out by heat treatment with Target Retrieval Solution Citrate pH 9.0 (32689, Agilent Technologies). Slides were incubated with the primary antibodies in rabbit polyclonal anti-PRC1 antibody raised in rabbit (1:1000, Dako) or for 120 min at RT followed by the monoclonal secondary horseradish peroxidase (HRP)-coupled horse-anti-rabbit antibody (ImmPRESS Reagent Kit, MP-7401, Vector Laboratories) or for 120 min at RT followed by Dako REAL® Detection kit, Alkaline Phosphatase/Red Rabbit/Mouse (K005, Dako). AEC-Plus (K3461, Agilent Technologies) or substrate RED (K005, Dako) was used as chromogen in the corresponding detection system. Samples were counterstained with hematoxylin (H-3401, Vector Laboratories and T865.1 Roth). For cleaved caspase-3 (CC3) staining, antigen retrieval was carried out by heat treatment with Target Retrieval Solution Citrate pH 9.0. Immunoreactivity quantification was assessed using a computerized image analysis system (Cell Signaling). For 60 min at RT followed by ImmPRESS Reagent Kit (MP-7401, Vector Laboratories) or for 120 min at RT followed by Dako REAL® Detection kit, Alkaline Phosphatase/Red Rabbit/Mouse (K005, Dako). AEC-Plus (K3461, Agilent Technologies) or substrate RED (K005, Dako) was used as chromogen in the corresponding detection system. The percentage of mitotic cells that exited mitosis as a single cell was reported as those that had cytokinesis.

**In vivo experiments.** NOD/Seid/gamma (NSG) mice have been housed in individually ventilated cages (IVC) under specific pathogen-free (SPF) conditions with strict dark/light cycles (darkness from 6 p.m. to 6 a.m.), an ambient temperature of 21–23 °C and a humidity of 45–65%. 5 x 10^6 EwS cells harboring a shRNA against PRC1 or a non-targeting control shRNA were injected in a 1:1 mix of cells suspended in HBSS formulated with Calcium and Magnesium (ThermoFisher) in the right amount of NGS medium. The environment throughout imaging was controlled at 37 °C, 5% CO\(_2\), and 90% humidity. Time-lapse images were acquired with an inverted Zeiss AxioObserver Z1 microscope equipped with an AxioCam MRm grayscale CCD camera and controlled by ZEN pro software (Zeiss). Brightfield, Cy5 (AHF F36-523), and DAPI (Zeiss Filter Set 49, 488049-9901-000) were captured using x40 oil, 1.4 numerical aperture (NA) objective combined with 2 × 2 binning modes. Fluorescence and bright-ﬁled images were acquired using a 20× lens. The objective was z-stacked in 10 planes, 2 μm intervals for 2 x binning modes. Tumor and BrDU-positive cell positions were determined as follows: Ulcerated tumors, loss of body surface adherence. At the experimental endpoint, cell growth inhibition was assessed using a Resazurin assay. CompuSyn (ComboSyn, Inc.) was used for determining the DRI of each PLK1 inhibitor at the IC50 level. After the start of the treatment using a Resazurin assay. The excess over Blass was calculated using synergyﬁnder (R package v.2.2.4)\(^{[49]}\). For assessment of the dose reduction index (DRI), 5000 cells/well of Dox-inducible shRNA expressing RDES and TC32 EwS cells were seeded in triplicate wells of 96-well plates. Cells were pre-treated for 48 h with Dox to induce the PRC1 knockdown before BI6727 or BI2536 was added.
in vitro efficacy of Doxo depending on the co-treatment with PLK1 inhibitor BI2536 or BI6727 was assessed by a Resazurin assay. To this end, cells were treated for 72 h with different concentrations of Doxo (0–2000 nM) and co-treated with the non-toxic dosage of PLK1 inhibitor BI2536 or BI6727 (10 nM) as defined above. The predicted ED50 concentrations were calculated using CompuSyn (ComBioSyn, Inc.). For both cell lines, the reverse index (RI) was calculated by dividing the ED50 of Doxo alone by the ED50 of the combination treatment (Doxo plus 10 nM of PLK1 inhibitor).

Statistical analyses. If not otherwise specified, statistical data analysis was performed using PRISM 8 (GraphPad Software Inc., CA, USA) on the raw data. If not otherwise specified in the figure legends comparison of two groups in functional in vitro experiments was carried out using a two-sided Mann-Whitney test. Contingency tables of FISH counting were analyzed by the Chi-square or Fisher’s exact test as appropriate. Pearson correlation analysis was done and visualized using the R package ggpubr (v0.4.0). If not otherwise specified in the figure legends, data are presented as box-dot plots with horizontal bars representing means and whiskers representing the standard error of the mean (SEM). The sample size for all in vitro experiments was chosen empirically. For in vivo experiments, the sample size was predetermined using power calculations with \( \beta = 0.8 \) and \( \alpha = 0.05 \) based on preliminary data and in compliance with the 3R system (replacement, reduction, refinement). In retrospective Kaplan-Meier analyses of overall survival, curves were calculated from all individuals. Statistical differences between the groups were assessed by a Mantel-Haenszel test. The sample sizes of the patient cohorts were not predetermined. Retrospective analysis of survival probabilities was done on an exploratory basis. Cox multivariate statistical analyses were carried out in IBM SPSS Statistics software (version 23.0; IBM Corp). A hazard ratio (HR) > 1 indicated that patients were at risk of a worse prognosis. Potential associations of the

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\text{PI} = \sum_{i} \text{gene score}
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were available51. To develop a prognostic index (PI), EwS expression of 11 genes and immunohistochemical analyses identify SOX2 as major risk factor for overall survival and relapse in Ewing sarcoma patients. *Ebiomedicine* 47, 156–162 (2019).

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Author contributions

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

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Competing interests

The authors declare no competing interests.

Additional information

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