**Figure C**

Relative Copy Number (log2)

HF-Eup-1

HF-Eup-2

HF-Eup-3

HF-Eup-4

HF-Eup-5

HF-Eup-6

**Figure D**

Relative Copy Number (log2)

Cell Type, Passage after infection

Vector | HF-Eup-3, P10

EWS-FLI1 | HF-Eup-4, P10
Supplemental Figure S1. Karyotypes of cells employed in this study.
Karyotypes were determined by low-coverage whole genome sequencing.

A, B. Mesenchymal stem (MSC) or progenitor cells (hMPro). hMPro cells from two different donors (a, b) were used in our studies. P: passage after transduction.
C, D. Primary euploid fibroblasts. P: passage after transduction.
E, F. Primary trisomy 8 fibroblasts. The HF-Ts8-3 line was found to be mosaic for trisomy 8 (58% trisomic). P: passage after transduction.
G. Ewing sarcoma cancer cell line MHH-ES1.
Supplemental Figure S2. Characterization of cell cycle progression in EWS-FLI1 expressing cells.

A, Workflow of the analysis of primary mesenchymal progenitor cells and fibroblasts. Black arrow workflow: cells were transduced with control or EWS-FLI1 expressing lentiviruses. Cells

B, Gates used in the analysis (Vector-expressing cells)

| Construct | N   | % GO/G1 | % S  | % S/G2/M | % Mitosis | % Death |
|-----------|-----|---------|------|----------|-----------|---------|
| EWS-FLI1  | 10000 | 19.9    | 43.6 | 35.3     | 0.1       | 10.0    |
| V         | 10000 | 54.8    | 29.7 | 22.8     | 1.3       | 2.1     |

C, Cytokine Levels (top 30)

G, SASP Signature

Supplemental Figure S2. Characterization of cell cycle progression in EWS-FLI1 expressing cells.

A, Workflow of the analysis of primary mesenchymal progenitor cells and fibroblasts. Black arrow workflow: cells were transduced with control or EWS-FLI1 expressing lentiviruses. Cells
harboring the viruses were drug-selected for 2-4 days, which we determined to eliminate uninfected cells. Subsequent manipulations were performed immediately after drug selection. Green arrow workflow: control fibroblasts or fibroblasts carrying additional copies of RAD21 were treated with the indicated genotoxic stress-inducing agents followed by DNA damage analysis. * CPT treatment was only applied for 12 hours. HU: hydroxyurea, CPT: camptothecin, APH: aphidicolin.

B, Cell cycle analysis of unsynchronized EWS-FLI1 expressing hMPro cells: Cells were harvested following 4 days of drug selection after lentiviral infection. DNA content was determined by flow cytometry of DAPI stained cells. Mitotic index was determined by phospho-Histone H3 staining. The histogram on the left shows the DNA content profile of control transduced cells that was used to set the gates for the DNA content analysis. The dot plot on the right shows the phospho-Histone H3 staining in vector transduced control cells that was used to set the gates for the quantification of mitotic cells. These gates were used for all analyses. N: total number of cells assessed.

C, Description of the FUCCI system.

D, Protein quantification for Fig. 1H. Hours indicate the time after release from a serum-starvation induced G1 arrest. Un.: exponentially growing control cells.

E, NBS1 phosphorylation in EWS-FLI1 expressing cells: Euploid human fibroblasts (HF-Eup-3) harboring the indicated lentivirus were released from a serum-starvation induced G1 arrest to determine the phosphorylation state of NBS1. Un: unsynchronized cells; IR: HF-Eup-3 fibroblasts were treated with 2 Gy ionizing irradiation and recovered for 1 hour; no.: cells without radiation treatment.

F, Quantification of β-galactosidase staining in hMPro cells carrying either an empty vector or the EWS-FLI1. Example pictures are shown in Fig. 1L. ***p<0.001, two-tailed t-test, n=3.

G, H, Secreted cytokine levels are higher in hMPro cells carrying the EWS-FLI1 construct after 8 days of EWS-FLI1 expression. Top 30 cytokines in EWS-FLI1-expressing cells are shown; n=2 per bar (G). Secretion of cytokines that are part of the SASP was quantified in (H). N=3, a representative experimental result is shown.

Statistics and number of experiments are shown in Supplemental Table S2 (multiple tabs).
Supplemental Figure S3. Suppression of the EWS-FLI1 induced growth defects is trisomy 8 specific.

A, Expression of EWS-FLI1 in euploid and trisomy 8 fibroblasts. The indicated cell lines were transfected with empty vector (V) or a vector carrying the EWS-FLI1 fusion (EF) and EWS-FLI1 protein levels were determined by Western blot analysis. GAPDH was used as a loading control. N=2, a representative picture is shown.
**B-D**, Trisomy 9 and 13 do not improve proliferation of *EWS-FLI1* expressing cells. **B**, Karyotypes of trisomy 9 (top) and trisomy 13 (bottom) were determined by low-coverage whole genome sequencing. **C, D**, Proliferation of trisomy 9 (**C**) and trisomy 13 (**D**) cells harboring the indicated lentiviruses was measured after 2-3 days of selection for presence of the lentivirus. Error bars represent S.E.M. of biological replicates. *p<0.05, n.s. not significant, linear regression.

**E, F**, Trisomy 8 enhances repair of replication-induced DNA damage. Analysis of EdU incorporation and γH2AX focus formation in euploid (**E**) and trisomy 8 (**F**) fibroblasts following release from a serum starvation-induced G1 arrest. Error bar: S.E.M. of biological replicates. *p<0.05, two-tailed t-test; n>2. Experiments were done in parallel and in the same way as those described in Fig. 2D and 2E.

**G**, Cyclin D levels were analyzed in the indicated cell lines. Tubulin served as a loading control. Quantifications are shown below. N=2, a representative picture is shown.

**H, I**, Euploid and trisomy 8 fibroblasts were released from a starvation-induced G1 arrest into medium containing aphidicolin (APH, 1μM) for 24 hours (**H**) or camptothcin (CPT, 0.02μM) for 12 hours (**I**) in the presence of EdU (10μM). Drugs were then washed out and the percentage of cells harboring more than 10 γH2AX foci in the EdU positive cells was determined at the indicated times. Error bars represent S.E.M. of biological replicates, n>2. *p<0.05, no significance was detected in **I**, two-tailed nonparametric 2-group Mann-Whitney U test.

**J**, Exponentially growing euploid and trisomy 8 fibroblasts were treated with phleomycin (10μg/mL) for 1 hour. After wash-out the percentage of cells harboring more than 10 γH2AX foci was determined at the indicated times. Error bars represent S.E.M. of biological replicates, n>2. no significance was detected, two-tailed nonparametric 2-group Mann-Whitney U test. Statistics and number of experiments are shown in Supplemental Table S2 (multiple tabs).
Supplemental Figure S4. Chromosome 8 gain is associated with poor prognosis in Ewing sarcoma.

A, Kaplan-Meier survival estimate of patients with tumors with higher chromosome 8 copy number (top 1/3 chr8 gain, red) and patients with disomy 8 tumors (bottom 1/3 no chr 8 gain, black). Patient data used for this analysis are highlighted in yellow in Supplemental Table S3.

B, Cox regression model to estimate death risk associated with chromosome 8 gain.

C, Association of chromosome 8 gain with relapse and metastatic potential in Ewing sarcoma (chi-square).

D, Event-free survival for patients with high and low expression of RAD21, ATAD2, MTBP, E2F5, and MYC.

E, pan-Cancer analysis of whole chromosome gain and chromosome q arm gain.
D, Kaplan-Meier survival estimate of patients with tumors expressing high levels of RAD21, ATAD2, MTBP, E2F5 or MYC (red) and patients expressing low levels of these genes (black). The survival Kaplan-Meier estimates were determined by using the R2 Genomics Analysis and Visualization Platform (http://r2.amc.nl) with a dataset containing 88 patients from (GEO ID: gse17679) (Savola et al. 2011) using the analysis settings described in the Additional Materials and Methods. Event free (top) and overall survival (bottom) are shown.

E, Percentage of cancer types as defined by TCGA (n=38), in which more than 25% of specimens harbor the indicated chromosome gain.
Supplemental Figure S5. Characterization of evolved hMPro cells.

A. Workflow of experimental evolution strategy to identify genes within the 26 ORF-library whose expression increased during continuous culture experiments.

B. Evolution of co-infected hMPro (2nd trial)

C. RNAseq at days 0, 12, 36

Deliver 26 gene ORFs with EWS-FLI1 or vector by lentiviral transduction
Continuous culturing
RNAseq at days 0, 12, 36

GAPDH
MYC
MYC+
EWS
EWS
hMPro

K

hMPro cells

EWS-FL1+ 26 ORFs
EWS-FL1+ Vector

Day 0 Day 12 Day 32 Day 0 Day 12 Day 32

MTRI 0.7 2.0 0.7 1.1 0.7 1.0
ATAD2 0.6 0.4 2.0 0.6 1.5 2.3
E2F3 1.2 0.9 2.5 1.7 1.3 1.0
DDAT1 1.2 1.6 1.6 1.1 0.8 0.9
PUPF0 1.5 1.1 1.4 0.8 0.9 0.9
PMPG1 1.0 0.9 1.3 0.8 1.0 1.0
LYHDS 0.7 0.9 1.3 0.8 1.0 1.1
MTDH 1.5 1.1 1.2 1.1 1.6 1.6
TSTA3 1.1 1.0 1.1 0.9 0.9 0.9
EFSH 1.1 1.3 1.1 1.2 1.4 1.3
Pfx 1.2 1.4 1.1 1.1 1.2 1.0
RAG1 1.0 0.8 1.1 0.9 1.3 1.4
AZN1 1.2 1.3 1.1 1.5 0.8 1.9
BOP1 1.2 0.8 1.0 0.6 0.9 1.0
ARMG1 0.9 1.4 1.0 1.2 1.1 0.8
SHMRP 1.3 0.5 1.0 0.6 1.1 0.7
EFSH 1.2 1.0 1.0 0.8 1.2 1.0

hMPro with 26-ORF +EWS-FLI1
B, Repetition of the experiment shown in Fig. 5A. Primary hMPro cells were infected at passage 7 (P7). Proliferation of cells was measured 2-3 days after transduction of cells with the indicated lentiviral constructs. Error bars represent S.E.M. of biological duplicates. ****p<0.0001, linear regression.

C, Fold change expression of members of the 26-ORF library was determined in relation to cells that were not transduced with the library. Genes whose expression increased over time by 2 fold or more are highlighted in red. NA: not analyzed because the transcript was not detected in control cells.

D-F, Cells size analysis of hMPro cells harboring the indicated lentivirus constructs after 0 (D), 12 (E) and 36 (F) days of culturing. Cell size was determined using a Coulter counter (Beckman Coulter). hMPro cells transduced with a control vector were not measured on day 36, because very few cells had remained on the plate by then.

G, Appearance of cells harboring the indicated lentivirus constructs.

H, hMPro cells from the evolution experiment shown in Fig. 5A were cultured for 26 days. Cells were then incubated in the presence of 10 µM EdU for 5 hours. The percentage of EdU positive cells harboring more than 10 γH2AX foci was determined. n>50 cells/cell type.

I, Focus formation of cells harboring the indicated lentivirus constructs. Cells transduced with the 26-ORF library and the EWS-FLI1 fusion appear to grow more than other cells but did not form clear foci.

J, Immunocompromised mice were injected subcutaneously with either the TC32 Ewing sarcoma cell line (left flank) or with hMPro cells transduced with the EWS-FLI1 fusion and the 26 ORF library (right flank). Mice were sacrificed and imaged 44 days after injection.

K, hMPro cells harboring the EWS-FLI1 fusion were transduced with a lentivirus containing MYC. MYC protein levels were measured after 10 days of growth. GAPDH was used as a loading control. N=2, a representative picture is shown. Statistics and number of experiments are shown in Supplemental Table S2 (multiple tabs).
Supplemental Figure S6. Effects of RAD21 and MYC overexpression.

A, RAD21 protein levels in euploid fibroblasts (HF-Eup-3) expressing RAD21 under the control of the doxycycline-inducible TET-ON promoter. Quantification of the blot is shown in (C). N=2, a representative picture is shown.

B, C, RAD21 mRNA (B) and protein (C) levels in euploid fibroblasts (HF-Eup-3) carrying RAD21 under the doxycycline-inducible TET-ON promoter grown in medium containing different

D, E, Doublings of HF-Eup-3, RAD21 or Vector, respectively, in the presence of increasing concentrations of doxycycline. N=2, n.s., *p<0.05, **p<0.01.

F, Images of EWS-FLI1+Vector and EWS-FLI1+RAD21 fibroblasts.

G, β-galactosidase staining of EWS-FLI1+Vector and EWS-FLI1+RAD21 fibroblasts.

H, I, Western blot images of RAD21, MYC, and H3 proteins in fibroblasts treated with EWS-FLI1+Vector, EWS-FLI1+RAD21, EWS-FLI1+MYC, and EWS-FLI1+RAD21+MYC vectors. Doubling times are shown for each treatment group.

J, Percentages of cells with γH2AX foci in fibroblasts treated with EWS-FLI1+Vector, EWS-FLI1+RAD21, EWS-FLI1+MYC, and EWS-FLI1+RAD21+MYC vectors.
amounts of doxycycline. Numbers on top of each bar indicate the fold increase over the no doxycycline control (0µg/mL).

D, E, Growth kinetics of euploid fibroblasts (HF-Eup-3, 4) carrying the TET-ON- RAD21 fusion or vector control grown in medium containing the indicated amounts of doxycycline. Error bars represent S.E.M. of biological duplicates. *p<0.05, **p<0.01, n.s. not significant, linear regression.

F, G, β–galactosidase staining (F) and quantification (G) after 8-day expression of EWS-FLI1 in hMPro cells. Scale bar: 100µm. Error bars: S.E.M. of biological replicates (n=3).

H, Expression levels of RAD21 and MYC. H3: loading control. Numbers underneath the blot indicate degree of downregulation relative to the vector transduced cells. N=2, a representative picture is shown.

I, Proliferation of hMPro cells harboring the indicated lentiviral constructs was measured after 5 days of transduction. Error bars: S.E.M. of biological duplicates. ***p<0.001, linear regression.

J, DNA damage analysis in hMPro cells carrying the indicated constructs. Cells were sampled at day 10 of the proliferation analysis shown in (I). Cells were then incubated in the presence of 10µM EdU for 5 hours. At least 3 fields with over 50 EdU positive cells were quantified. ^ p<0.01, two-tailed t-test compared to Vector control. No statistically significant difference was detected between cells carrying the EWS-FLI1+RAD21 constructs and the EWS-FLI1+RAD21+MYC constructs. *p<0.05, one-way ANOVA test.

Statistics and number of experiments are shown in Supplemental Table S2 (multiple tabs).
Supplemental Figure S7. Deletion of one copy of RAD21 in trisomy 8 fibroblasts.
A. RAD21 exon 4 of clone 1 and clone 2 in Fig. 6A-E was PCR amplified using primers P1 and P2 and sequenced using next generation deep sequencing. This analysis showed that about 30% of reads harbored a GA insertion in clone 1 or a deletion of the bases CGA in clone 2.

B. An example of the T7EI analysis to determine the efficiency of RAD21 knock out: RAD21 was PCR amplified from the indicated cell lines, denatured and re-annealed. Degree of mismatch in the re-annealed DNA molecules was assessed by T7 endonuclease I (T7EI) cleavage. An AATI Fragment Analyzer was used to visualize and quantify T7EI-cut mis-match products as shown.

C. Karyotypes of clone 1 and 2 in (a), before transduction with the EWS-FLI1 fusion or the vector control as determined by low-coverage whole-genome sequencing.

D. Exponential growing cells were pulse-labeled with EdU for 1 hour and DNA content was determined by DAPI staining. Flow cytometry was used to analyze cell cycle profiles. Number inside each gate represents the percentage of EdU positive population; n=number of cells analyzed.

E. Examples of single colonies formed by MHH-ES1 cells with two copies of RAD21 (crRAD21) and three copies of RAD21 (crCtrl). Scale bar: 500µm.

F. Three independent failed CRISPR-RAD21 MHH-ES1 clones (carrying the crRAD21 construct but not being target-edited) show similar proliferation as the MHH-ES1 cells carrying three copies (crCtrl: control). Error bars represent S.E.M. of biological duplicates.