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NAD Synthesis Pathway Interference is a Viable Therapeutic Strategy for Chondrosarcoma

Elisabeth F.P. Peterse[1], Brendy E.W.M. van den Akker[1], Bertine Niessen[1], Jan Oosting[1], Johnny Suijker[1], Yvonne de Jong[1], Erik H.J. Danen[2], Anne-Marie Cleton-Jansen[1], Judith V.M.G. Bovée[1]

[1]Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands.

[2]Division of Toxicology, Leiden Academic Center for Drug Research, Leiden University, Leiden, the Netherlands.

Running title: NAMPT as a Treatment Target for Chondrosarcoma

Abbreviations list: ACT: Atypical cartilage tumour; α-KG: α-ketogluterate; IC_{50}: Half maximal inhibitory concentration; D-2-HG: D-2-hydroxyglutarate; IDH: isocitrate dehydrogenase; NAD+: nicotinamide adenine dinucleotide; NAMPT: Nicotinamide phosphoribosyltransferase; NAPRT: nicotinic acid phosphoribosyltransferase;

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Corresponding author: Judith V.M.G. Bovée, Postalzone L1-Q, P.O. Box 9600, 2300 RC Leiden, The Netherlands. Tel: +31 71 5266622 e-mail: J.V.M.G.Bovee@lumc.nl

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Abstract

Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT) are rate-limiting enzymes in the nicotinamide adenine dinucleotide (NAD+) synthesis pathway. Chondrosarcoma is a malignant cartilage forming bone tumor, in which mutations altering isocitrate dehydrogenase-1 and -2 (IDH1 and IDH2) activity have been identified as potential driver mutations. Vulnerability for NAD+ depletion has been reported for *IDH1/2* mutant cells. Here, the potency of NAMPT inhibitors as a treatment of chondrosarcoma was explored. Eleven chondrosarcoma cell lines were treated with NAMPT inhibitors, in which the effect on cell viability, colony formation and 3D collagen invasion was assessed. The expression level of NAMPT and NAPRT transcripts in chondrosarcoma cells was determined by qRT-PCR. Methylation of the NAPRT promoter was evaluated using a previously published dataset of genome-wide methylation. In addition, a methylation dataset was used to determine methylation of the NAPRT promoter in twenty *IDH1/2* mutated cartilage tumors. Chondrosarcoma cells showed a dose-dependent decrease in cell viability, 3D collagen invasion and colony formation upon treatment with NAMPT inhibitors, in which nearly half of the cell lines demonstrated absolute IC_{50}s in the low nM range. Increasing IC_{50}s correlated to increasing NAPRT expression levels and decreasing NAPRT promoter methylation. No correlation between *IDH1/2* mutation status and sensitivity for NAMPT inhibitors was observed. Strikingly, higher methylation of the NAPRT promoter was observed in high grade versus low grade chondrosarcomas. In conclusion, this study identified NAMPT as a potential target for treatment of chondrosarcoma.

Implications: Chondrosarcoma patients, especially those of high histological grade with lower expression and hypermethylation of NAPRT, may benefit from inhibition of the NAD synthesis pathway.

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Introduction

Chondrosarcoma represents a heterogeneous group of cartilage forming tumors. It is the second most common primary bone malignancy in humans (1) with different outcomes depending on subtype and histological grade. The far most prevalent subtype (72% of the cases) is conventional central chondrosarcoma, in which the tumor arises centrally in the medulla of the bone. This subtype can be histologically subdivided into atypical cartilaginous tumor (ACT), grade II and grade III chondrosarcomas. ACT (previously known as grade I) accounts for 61% of the cases. The first line treatment is curettage with local adjuvant treatment, resulting in a 5 year survival rate of 83%. Grade II (36%) and grade III (3%) have a worse 5 year survival (combined 53%) due to the higher occurrence of metastases (1-3). These tumors are treated by en bloc resection. Two other subtypes with a worse prognosis are dedifferentiated chondrosarcoma, a highly malignant variant (4), and mesenchymal chondrosarcoma, a rare aggressive subtype in which distant metastasis can be identified even after 20 years (5-7). Chondrosarcoma patients with unresectable disease, due to tumor location, tumor size or extensive metastatic disease, have a 5 year survival of only 2% as the overall efficacy of chemotherapy is limited (8,9).

Gain of function mutations in the *isocitrate dehydrogenase 1* and *-2* (*IDH1* and *-2*) genes have been identified as potential driver mutations of chondrosarcoma because of their high prevalence (38-70% depending on the subtype) (10,11). IDH1 and IDH2 are key enzymes in cell metabolism as they convert isocitrate to α-ketogluterate (α-KG) in respectively the cytoplasm and the mitochondria. The mutant enzyme acquires the activity to convert α-KG to D-2-hydroxyglutarate (D-2-HG). This leads to increased levels of this oncometabolite in cartilage tumors harbouring an *IDH1/2* mutation (12) which competitively inhibits the α-KG dependent enzymes by the high structural similarities (13). Although AGI-5198, a specific small molecule inhibitor of the activity of mutant IDH1, was able to decrease D-2-HG levels in *IDH1* mutant chondrosarcoma cell lines, this did not influence the tumorigenic properties of these cells (14,15) which is in line with findings in *IDH2* mutant leukemia (16,17) and *IDH1*

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mutant glioma models (15,18). This suggests that while the *IDH1* or *-2* mutations are an early event in tumorigenesis, at later stages other processes involved in chondrosarcoma progression render these cells independent of the mutant IDH1/2 enzymes, which is in line with findings in other *IDH1/2* mutated cancers (15,16). To identify metabolic targets for *IDH1/2* mutated glioma, Tateishi et al. performed a systematic metabolic profiling on glioma cells after short- and long-term mutant IDH1 inhibition (15). This study revealed a vulnerability to nicotinamide adenine dinucleotide (NAD+) depletion in *IDH1/2* mutant cells, demonstrated by an increased sensitivity for nicotinamide phosphoribosyltransferase (NAMPT) inhibitors. This increased sensitivity could be explained by decreased expression levels of nicotinic acid phosphoribosyltransferase (NAPRT) in *IDH1/2* mutated tumors, potentially caused by an increased methylation of the NAPRT promoter (15). NAMPT and NAPRT are rate limiting enzymes of respectively the primary salvage pathway and the Preiss-handler pathway, which are involved in NAD+ synthesis (Figure 1). Tumor cells depend on these pathways for their rapid NAD turnover, as they lack expression of key enzymes in the *de novo* synthesis of NAD from tryptophan (19-21). Therefore, interfering with NAD+ biosynthesis holds great promise as a therapeutic strategy for cancer, which is why we further explored this pathway in chondrosarcoma. We used our large chondrosarcoma cell line panel (n =11) to determine sensitivity to NAMPT inhibitors. In contrary to glioma cell lines, chondrosarcoma cell lines harbouring an endogenous I*DH1* or *IDH2* mutation can grow as a monolayer culture. We determined expression levels of NAMPT and NAPRT, and methylation of the NAPRT promoter in cell lines and in primary tumors. Our results indicate that NAMPT is a promising therapeutic target in chondrosarcoma.

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Materials and Methods

Compounds

NAD+ and the NAMPT inhibitors FK866 (also known as APO866) and GMX1778 (also known as CHS-828) were purchased from Sigma-Aldrich. FK866 and GMX1778 both likely function as substrate mimetics (19). FK866 and GMX1778 were dissolved in DMSO in a concentration of 20 mM and 36.16 mM, respectively, and stored at -20 °C. NAD+ was dissolved in culture medium at a concentration of 10 mM, stored at -20 °C, and used in a concentration of 10 nM, 50 nM and 100 nM. AGI-5198 (Cayman Chemical) was dissolved in DMSO in a concentration of 10 mM, stored at -20 °C and used in a concentration of 1 μM and 10 μM.

Cell culture

The conventional chondrosarcoma cell lines JJ012 (22), SW1353 (ATCC), CH2879 (23), OUMS27 (24), L835 (25) and CH3573 (26), the dedifferentiated chondrosarcoma cell lines L3252B (25), NDCS-1 (27), and L2975 (25), as well as the chondrocyte cell line LBPVA (28) and the cell line HT1080 (ATCC) were cultured in RPMI 1640 (Gibco, Invitrogen) supplemented with 10% (JJ012, SW1353, CH2879, NDCS-1, L2975, HT1080) or 20% (L835, L3252B, OUMS27, CH3573, LBPVA) heat-inactivated Fetal Bovine Serum (FBS) (F7524, Sigma-Aldrich). HT1080 was originally reported to be derived from a fibrosarcoma of bone. As this is a diagnosis of exclusion and this cell line is now known to harbour an *IDH1* mutation, this tumor cell line is most likely derived from a dedifferentiated chondrosarcoma (14). MCS170 (Mesenchymal chondrosarcoma) (29) was cultured in IMDM (Gibco, Invitrogen) with 15% FBS. The lowest passage number possible was thawed (passage number between 11 and 42), the authenticity confirmed by STR profiling with the GenePrint10 (Promega Benelux BV) and tested for mycoplasma using MycoAlert (Lonza, Switzerland) before the start of the experiments. The cells were grown at 37°C with 5% CO_2

in a humidified incubator. Cell lines were never cultured for more than three months, and were tested for mycoplasma every 4 weeks (using RT-PCR).

qRT-PCR

RNA isolation and cDNA synthesis was performed as described previously (30). A standard quantitative reverse transcriptase PCR (qRT-PCR) was performed (31) to determine the expression levels of NAMPT and NAPRT. Primers (Table S1) were designed using primer3 software (http://bioinfo.ut.ee/primer3/). To correct for the amount of cDNA input, gene expression levels were normalized using the expression levels of CYPa, CPSF6 and GPR108 (32,33). Data were normalized using the delta-delta Cq method using Bio-Rad CFX Manager (Bio-Rad).

Proliferation assay

The cell lines were plated in triplicate at a density of 3000 to 20000 cells per well depending on the growth rate. After the cells adhered overnight, the compounds were added in their corresponding concentrations. To determine the effect of AGI-5198 on NAMPT inhibitor sensitivity, cells were pre-treated for 72 hours with 1 μM or 10 μM AGI-5198, after which the cells were counted and the same number of cells were plated for the different pre-treatment conditions. Treatment with AGI-5198 was continued when the NAMPT inhibitors were added. To validate that NAD+ could reverse the effect of the NAMPT inhibitors, NAD+ was added in a concentration of 10 nM, 50 nM or 100 nM at the same time as the NAMPT inhibitors. After 72 hours of incubation, cell viability was measured using the PrestoBlue Cell Viability Reagent (Promega Benelux BV) according to the manufacturer’s instructions. Colorimetric values in the plates were subsequently measured using a Wallac 1420 VICTOR2 (Perkin Elmer). Data were analysed in Graphpad Prism 5.0 (www.graphpad.com). The results shown are the results of three independent experiments.

Absolute IC_{50}s for FK866 and GMX1778 were compared to NAPRT expression levels using Pearson’s correlation (IBM SPSS Statistics 20).

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Cell counting

To confirm that a decrease in cell viability corresponds to an absolute decrease in cell number, the cell lines JJ012, SW1353 and CH2879 were plated in black 96-well μ-Clear Plates (Greiner) in a fully independent experiment. After the cells adhered overnight, the corresponding concentrations GMX1778 were added to the wells in duplicates. After 72 hours of incubation, the PrestoBlue Cell viability Reagent was added as described above. After measuring the Colorimetric values, cells were washed with PBS, fixed in 4% paraformaldehyde for 30 min and stained with Hoechst 33342 (Thermo Fisher Scientific). The cells were counted using Cellomics (Thermo Fisher Scientific) according to manufacturer's instruction. Results were normalized to Mock treated wells.

3D outgrowth/invasion assay

The invasion assay, based on invasion of cells in a 3D extracellular matrix scaffold, was performed as described (34). In short, trypsinized monolayer cultured cells were suspended in PBS containing 2% polyvinylpyrolidone (PVP; Sigma–Aldrich), after which they were printed into 70 μL solidified collagen gels in glass-bottom 96 well plates (Greiner) using injection robotics. Three droplets were injected per well, forming three collagen-embedded tumor spheroids per well. Subsequently, 130 μL medium containing compounds at indicated concentrations was added to each well. To asses outgrowth and invasion of the spheroids, Images were taken one hour post injection and three days post injection using the Motic Motical 3 CMOS 3.0MP Color Digital Camera and the corresponding Motic Images Plus 2.0 ML Software.

Colony formation assay

The colony formation assay was performed according to the "plating before treatment" method as described by Franken et al. (35). After counting, 1000 NDCS-1 cells and 1500 JJ012 cells were seeded per well. The next day, FK866 and GMX1778 were added in the indicated concentrations. The 6 well plates were analyzed at day 10 after staining with

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0,05% crystal violet – 6% glyceraldehyde. Colonies were quantified by manual counting followed by normalization to the negative control. Quantification is done for at least three separate experiments.

Methylation analysis

Methylation of the NAPRT promoter in chondrosarcoma cell lines was determined using a previously described genome-wide methylation dataset (14). In addition, we used a previously conducted but unpublished methylation array of *IDH1/2* mt cartilage tumors, for which genomic DNA of four enchondromas and five ACTs, nine grade II chondrosarcomas and four grade III chondrosarcomas was bisulfite-converted using the EZ DNA Methylation Gold Kit (Zymo Research) and used for microarray-based DNA methylation analysis, performed at ServiceXS using the HumanMethylation450 BeadChip array (Illumina). The bisulfite-converted DNA was processed and hybridized to the arrays according to the manufacturer's instructions. We performed data analysis in R version 3.2.3. 'methylumi' (36) was used to load data from the raw data files and perform data quality checks. One grade II sample was excluded from the analysis based on an inflated average detection p-value. Samples were normalized using the BMIQ procedure from the 'wateRmelon' package (37). We selected probes around the NAPRT gene from base pair position 144658390 until 144668845 on chromosome 8. A heatmap from this region including all samples was generated from the β-values using Gene-E (Broad Institute). We compared the level of methylation of the promoter region of NAPRT (from 144659831 to 144660631 (15)) between low grade (enchondroma and ACT) and high grade (grade II and grade III) tumors. A t-test was performed to compare the groups. In addition, we compared methylation of the CpG island of the NAPRT promoter region to NAPRT mRNA expression in chondrosarcoma cell lines and in a subset (n=13) of these cartilage tumors using the Spearman's correlation (IBM SPSS Statistics 20).

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Results

NAD+ depletion inhibits chondrosarcoma cell viability, invasion and colony formation

To explore whether NAMPT could be a therapeutic target for chondrosarcoma, we treated eleven chondrosarcoma cell lines with FK866 and GMX1778. In contrast to the chondrocyte cell line LBPVA, all chondrosarcoma cell lines showed dose-dependent decreases in cell viability and eight out of eleven chondrosarcoma cell lines showed a more than 75% reduction in cell viability upon treatment with 1 µM NAMPT inhibitor (Figure 2A). Sensitivities for FK866 and GMX1778 were highly comparable within cell lines (Figure 2A). Comparing cell counts with cell viabilities confirmed that a decrease in cell viability was caused by an absolute decrease in cell number (Figure 2B). Cell viability could be rescued by co-incubation with NAD+ in two cell lines tested, demonstrating that the treatment with FK866 and GMX1778 indeed caused on target inhibition (Figure 2C). Interestingly, high concentrations of NAD+ also reduced cell viability in NDCS-1, suggesting that NAD+ levels are tightly regulated in these cells. Absolute IC_{50} values varied between the different chondrosarcoma cell lines: six cell lines had absolute IC_{50} values below 10 nM and three cell lines had absolute IC_{50} values above 100 nM (Table 1). In addition to determining the effect of NAMPT inhibition on cell viability, we studied its effect on JJ012 spheroid outgrowth and invasion using a 3D collagen scaffold model (Figure 2D). Upon treatment with FK866 or GMX1778, a clear decrease in invasive outgrowth was observed. In addition to cell viability and spheroid outgrowth and invasion, these compounds inhibited colony formation of JJ012 and NDCS-1 cells already at a concentration of 0.5 nM (Figure 2E-F). This demonstrates that chondrosarcoma cell lines depend on NAMPT for their NAD+ generation and that NAD+ is essential for their tumorigenic properties.

NAPRT methylation correlates with sensitivity for NAMPT inhibitors

We hypothesized that the variation in sensitivity to NAMPT inhibitors between cell lines could be attributed to expression of NAMPT or NAPRT, the rate limiting enzymes of pathways involved in NAD+ synthesis. We performed qRT-PCR analyses in chondrosarcoma cell lines to determine NAMPT and NAPRT expression levels. Expression levels of NAMPT and NAPRT are highly variable between chondrosarcoma cell lines (Figure 3A). NAPRT has the highest expression in CH2879, L3252b, L835, and OUMS27, of which three out of four have IC_{50} values above 30 nM for the NAMPT inhibitors. Plotting IC_{50s} for FK866 and GMX1778 versus NAPRT expression of 10 chondrosarcoma cell lines revealed a correlation between low IC_{50} values and low NAPRT expression levels (p=0.034 and p=0.043, respectively) (Figure 3B). As it has been suggested that low expression of NAPRT is mediated by methylation of the NAPRT promoter (15,38), we assessed the methylation of the NAPRT promoter in 10 chondrosarcoma cell lines (Figure 3C) using a previously published genome wide methylation array dataset (14). The analysis showed that high CpG island methylation was associated with low NAPRT expression levels (Figure 3D) (p=0.029). JJ012, HT1080, SW1353 and NDCS-1 have high β values, demonstrating high methylation of the NAPRT promoter. L835 and L2975 have medium β values, whereas the *IDH* WT cell lines CH2879, CH3573, L3252b and OUMS27 have low β values. This suggested a potential correlation between *IDH*1/2 mutation status and sensitivity for NAMPT inhibitors in chondrosarcoma cell lines. However, (pre)treating JJ012 with AGI-5198, a specific IDH1 mutant inhibitor, did not affect the sensitivity of JJ012 to NAMPT inhibitors (Figure 2E). Furthermore, using previously published datasets and mRNAs of four chondrosarcoma cell lines treated for 10 and 20 passages with AGI-5198 and one *IDH* WT cell line for 10 passages with D-2-HG (14), we demonstrated that the mutant IDH1 enzyme did not influence the methylation of the NAPRT promoter nor NAMPT and NAPRT expression levels (Figure S1A-B).

<u>The NAPRT promoter is hypermethylated in high grade chondrosarcomas</u>

To extend our study to primary tumors, we determined methylation of the NAPRT promoter in an available dataset of twenty *IDH1/2* mutated cartilage tumors. Interestingly, we found significantly higher methylation of the CpG island of the NAPRT promoter in the high grade (grade II and III) chondrosarcomas compared to the low grade (ACT and enchondroma)

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cartilage tumors (p=0.002213) (Figure 4A). To compare methylation to expression in this set of tumors, mRNA of thirteen of these twenty tumors was collected, in which we could not identify a significant correlation between NAPRT CpG island promoter methylation and decreased NAPRT expression levels (p=0.271)(Figure 4B). In addition, we determined NAMPT and NAPRT expression levels in an independent cohort of 32 cartilage tumors, in which also nine *IDH* WT tumors were included (figure 4C-D). Similar to the cell lines, expression of NAMPT and NAPRT was variable in the primary tumors. Expression levels seemed slightly lower in the high grade tumors as compared to low grade, though the difference was not statistically significant (p=0.255, t-test).

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Discussion

The results of this study demonstrate that chondrosarcoma cell lines are vulnerable to NAD+ depletion. Five out of eleven chondrosarcoma cell lines have IC_{50} below 10 nM for the two tested NAMPT inhibitors, suggesting that these cell lines depend on the primary salvage pathway for NAD+ synthesis. Strikingly, the chondrocyte cell line LBPVA was unaffected by NAMPT inhibition, further demonstrating the therapeutic potential of NAMPT inhibitors for chondrosarcoma patients.

To identify a possible biomarker for distinguishing sensitivity to NAMPT inhibition, we assessed methylation and expression levels of NAPRT, the rate limiting enzyme of the Preiss-Handler pathway for NAD+ synthesis. Comparing IC_{50} values to NAPRT expression levels demonstrated that low NAPRT expression is significantly correlated to increased sensitivity for NAMPT inhibitors. Therefore, NAPRT expression partly explains the difference in NAMPT inhibitor sensitivity between the different cell lines. Strikingly, we observed higher methylation of the CpG island of the NAPRT promoter in high grade chondrosarcomas versus low grade cartilage tumors, suggesting that NAMPT can be a promising target especially in these clinically challenging patients.

We did not observe a correlation between the presence of a mutant IDH1/2 enzyme and sensitivity for NAMPT inhibitors in chondrosarcoma. Furthermore, inhibition of the IDH1 mutant enzyme by AGI-5198 did not influence sensitivity for NAMPT inhibitors nor methylation of the NAPRT promoter. This is in line with previous studies from our group, where we demonstrated that *IDH1/2* mutations do not affect immunohistochemical levels of 5-hmC, 5mC and trimethylation of H3K4, -9, and -27 (39), and prolonged inhibition of the IDH1 mutant enzyme does not affect global gene expression, CpG island methylation nor histone H3K4, -9, and -27 trimethylation in chondrosarcoma cell lines (14). Our observations are in contrast to the conclusion from Tateishi. et al, who suggest that mutant IDH1 downregulates NAPRT expression, making *IDH1* mutant cell lines more sensitive to NAMPT inhibitors (15). However, only two chondrosarcoma cell lines were included in that study,

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which both harbour an *IDH1 or IDH2* mutation and are sensitive to NAMPT inhibition. No co-treatment with a mutant IDH1 inhibitor and a NAMPT inhibitor was performed and NAPRT expression was not assessed in primary tumors; the link between mutant *IDH1* and NAPRT expression was demonstrated by the introduction of an *IDH1* mutation in *IDH* WT glioma cells. Therefore, experimental differences and a different tumor type may explain the discrepancy between experimental results.

Phase I clinical trials to test the safety of NAMPT inhibition have been performed with FK866, GMX1778 and its prodrug GMX1777 (19). However, further evaluation was discontinued due to dose-limiting toxicities (19). To increase the therapeutic index of NAMPT inhibitors, co-administration with nicotinic acid (NA) has been proposed (19). NA can be used to synthesise NAD+ in NAPRT-proficient cells, thereby decreasing the toxicity without interfering with its efficacy in the treatment of NAPRT-deficient tumors. Indeed, it was shown that the effect of NAMPT inhibitors on tumorigenic properties of HT1080 and SW1353 chondrosarcoma cell lines and HT1080 xenografts was not affected by co-administration of NA (40), suggesting that this could be a suitable approach to decrease dose-limiting toxicities in chondrosarcoma patients.

Collectively, this study demonstrates that NAMPT inhibitors hold potential therapeutic promise for chondrosarcoma patients, especially for those with high histological grade as these, due to hypermethylation of the NAPRT promoter, are dependent on the primary salvage pathway for their NAD+ synthesis.

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Table 1: Absolute IC_{50} values for the NAMPT inhibitors FK866 and GMX1778 of the chondrosarcoma cell lines.

Cell Line	**IC_{50} FK866 (nM)**	**IC_{50} GMX1778 (nM)**
JJ012	1.54	1.09
HT1080	3.04	5.02
L835	497.15	358.18
SW1353	28.08	29.11
L2975	7.17	9.44
CH2879	7.51	8.39
NDCS-1	2.49	4.09
CH3573	5.67	30.68
L3252b	>1000	>1000
MCS170	>1000	>1000
OUMS27	50.70	32.94

Figure Legends

Figure 1: Schematic representation of nicotinamide adenine dinucleotide (NAD)+ biosynthesis. Nicotinamide phosphoribosyltransferase (NAMPT) and nicotinic acid phosphoribosyltransferase (NAPRT) are the rate limiting enzymes of the primary salvage pathway and the Preiss-Handler pathway, respectively. NAD+ is recycled in the primary salvage pathway. NM=nicotinamide, NMN=nicotinamide mononucleotide, NA= nicotinic acid (vitamin B3), NAMN = nicotinic acid mononucleotide.

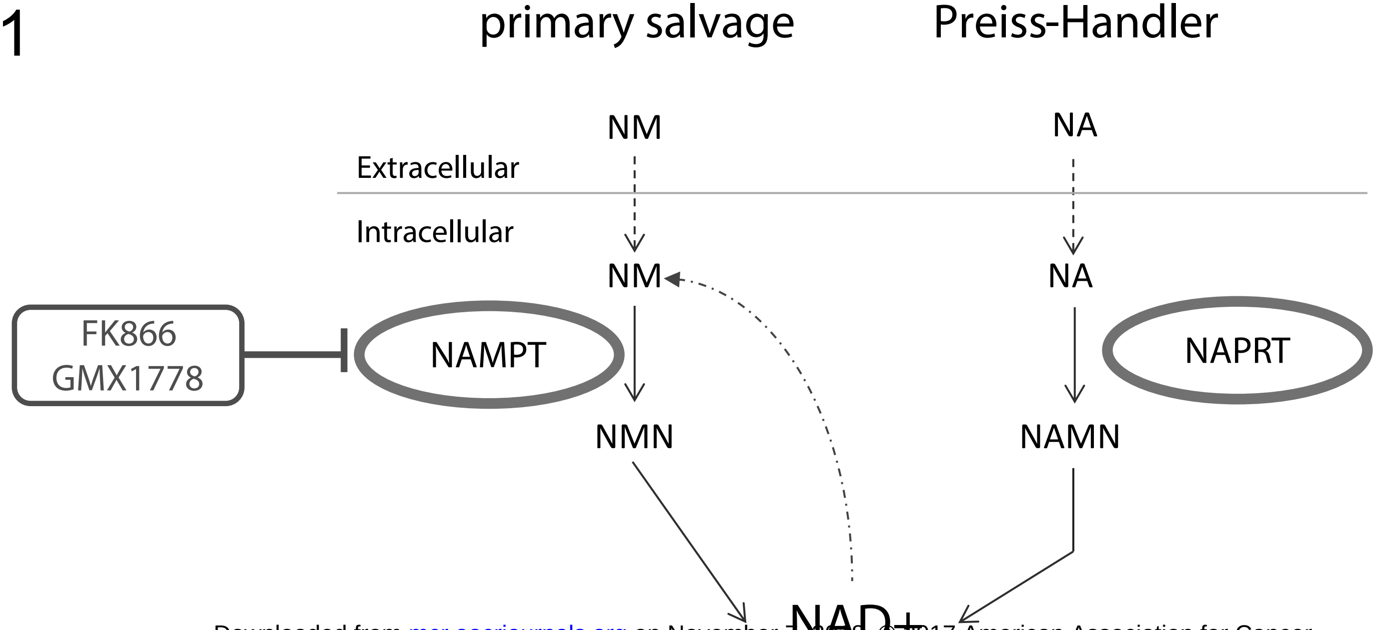
Figure 2: Chondrosarcoma cell lines are sensitive for NAD+ depletion. A: Eleven chondrosarcoma cell lines and a chondrocyte cell line were treated for 72 hours with two NAMPT inhibitors: FK866 and GMX1778, after which cell viability was assessed by a PrestoBlue assay. All cell lines showed a dose-dependent decrease in cell viability except the LBPVA control and in 8/11 cell lines this reduction was more than 75%. B: Cell count results (using Cellomics) were very similar to cell viability results (using PrestoBlue). C: JJ012 (*IDH1* mt) and NDCS-1 (*IDH* WT) were co-treated with NAMPT inhibitors and 10 µM, 50 µM or 100 µM NAD+. NAD+ abolished the effect of the NAMPT inhibitors, demonstrating that the effect of NAMPT inhibitors is caused by an NAD+ depletion. D: 3D outgrowth and invasion of JJ012 cells in a collagen scaffold-embedded spheroid model. JJ012 spheroid outgrowth and invasion was inhibited by treating the cells with NAMPT inhibitors. 130µl medium containing 10 nM compounds was added on top of the 70µl collagen gel. E: Representative wells from JJ012 and NDCS-1 colony formation during treatment with FK866 and GMX1778. F: Quantification of at least three different colony formation assays.

Figure 3: NAPRT expression correlates to sensitivity for NAMPT inhibitors and to methylation of the CpG island of the NAPRT promoter. A: NAMPT and NAPRT are heterogeneously expressed in the different chondrosarcoma cell lines. Expression levels were determined using qRT-PCR analyses. B: log IC_{50s} for the NAMPT inhibitors FK866 and GMX1778 significantly correlated to NAPRT expression levels. C: NAPRT methylation in a

previously published methylation array of ten chondrosarcoma cell lines. β-values are visualized using Gene-E (broad institute), where red represent high methylation of the NAPRT promoter and green represents low methylation of the NAPRT promoter. D: NAPRT expression significantly correlates to methylation of the CpG island of the NAPRT promoter in chondrosarcoma cell lines. E: Pre-treating JJ012 cells for 72 hours with AGI-5198, followed by a 72 hours combined treatment of AGI-5198 and the corresponding NAMPT inhibitor did not influence the dose-dependent decrease in cell viability. Cell viability was determined by a PrestoBlue assay.

Figure 4: A: The CpG island of the NAPRT promoter is hypermethylated in high grade compared to low grade *IDH1/2* mutant cartilage tumors. β-values are visualized using Gene-E (broad institute), where red represent high methylation of the NAPRT promoter and green represents low methylation of the NAPRT promoter. mRNA was available of cell lines marked with a *. B: Methylation of the CpG island of the NAPRT promoter weakly correlated to NAPRT expression in cartilage tumors *. C: qRT-PCR analyses of NAMPT expression in an independent cohort of 32 cartilage tumors. D: NAPRT expression in a cohort of 32 cartilage tumors shows a non-significant trend for higher expression in low grade versus high grade cartilage tumors. Expression was normalised to cartilage. EC: enchondroma, ACT: Atypical Cartilaginous tumor, CS2: Chondrosarcoma grade II, CS3: Chondrosarcoma grade III.

1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Preiss-Handler
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NA
Extracellular
Intracellular
NM
NA
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GMX1778
NAMPT
NAPRT
NMN
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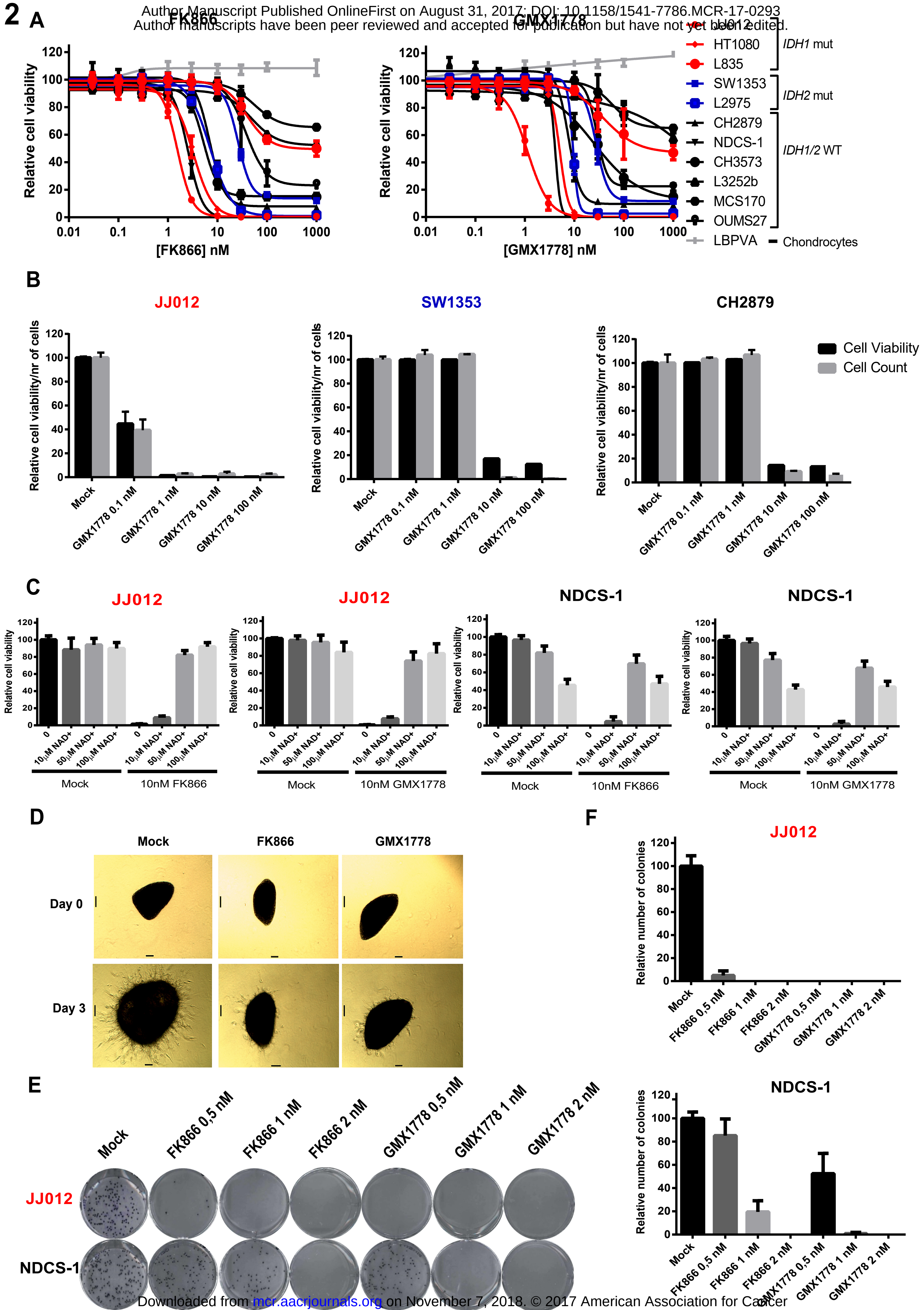
NAD+


2
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FK866
GMX1778
Relative cell viability
[FK866] nM
[GMX1778] nM
JJ012
HT1080
L835
IDH1 mut
SW1353
L2975
IDH2 mut
CH2879
NDCS-1
CH3573
L3252b
MCS170
OUMS27
IDH1/2 WT
LBPVA
Chondrocytes
B
JJ012
SW1353
CH2879
Relative cell viability/nr of cells
Cell Viability
Cell Count
Mock
GMX1778 0.1 nM
GMX1778 1 nM
GMX1778 10 nM
GMX1778 100 nM
C
JJ012
NDCS-1
Relative cell viability
10nM FK866
10nM GMX1778
D
Mock
FK866
GMX1778
Day 0
Day 3
E
JJ012
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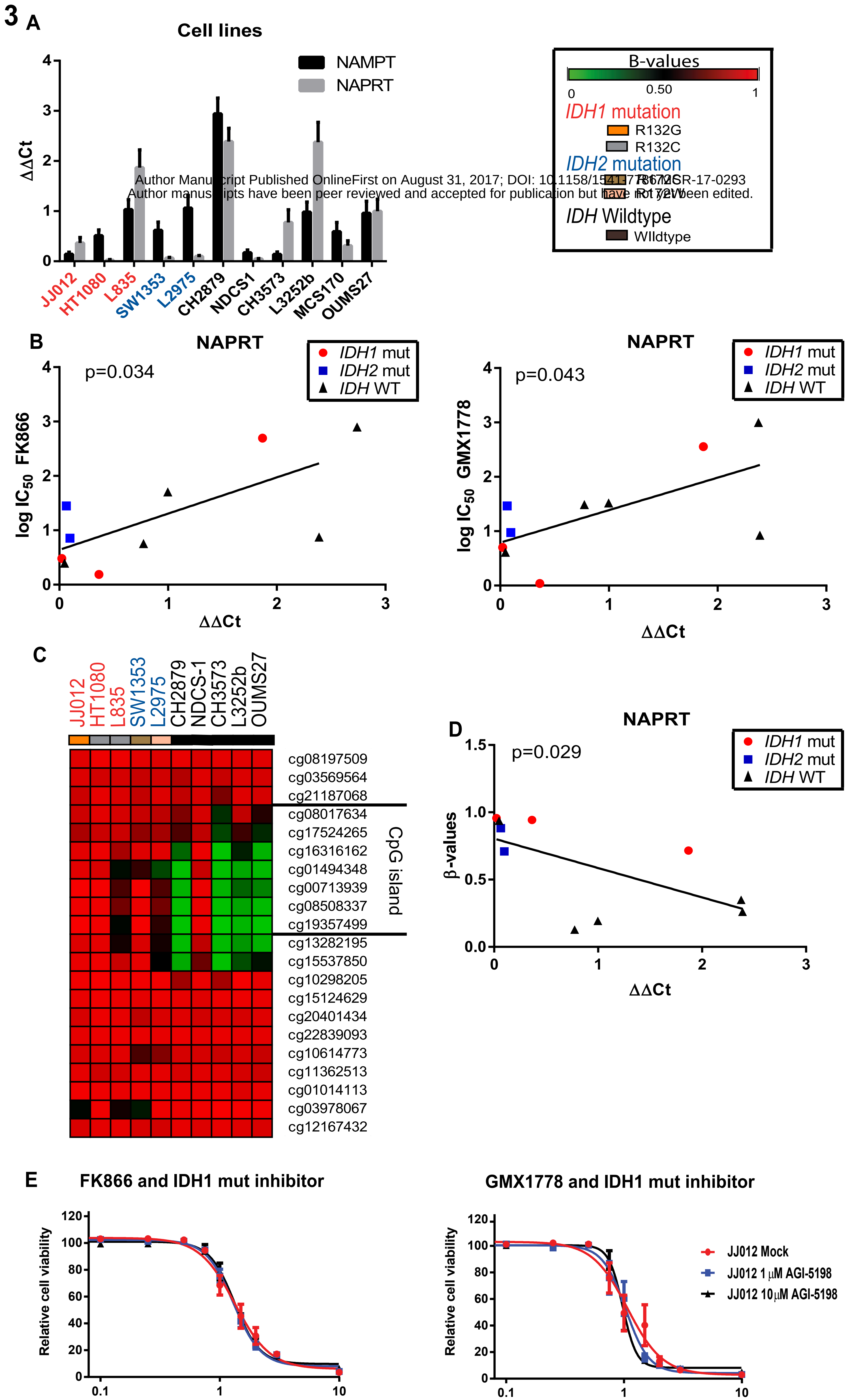
GMX1778 2 nM
F
Relative number of colonies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Cell lines
NAMPT
NAPRT
ΔΔCt
JJ012
HT1080
L835
SW1353
L2975
CH2879
NDCS1
CH3573
L3252b
MCS170
OUMS27
B-values
0
0.50
1
IDH1 mutation
R132G
R132C
IDH2 mutation
R172S
R172W
IDH Wildtype
Wildtype
B
NAPRT
p=0.034
IDH1 mut
IDH2 mut
IDH WT
log IC50 FK866
ΔΔCt
NAPRT
p=0.043
log IC50 GMX1778
ΔΔCt
C
JJ012
HT1080
L835
SW1353
L2975
CH2879
NDCS-1
CH3573
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CpG island
D
NAPRT
p=0.029
β-values
ΔΔCt
E
FK866 and IDH1 mut inhibitor
Relative cell viability
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JJ012 Mock
JJ012 1 μM AGI-5198
JJ012 10μM AGI-5198
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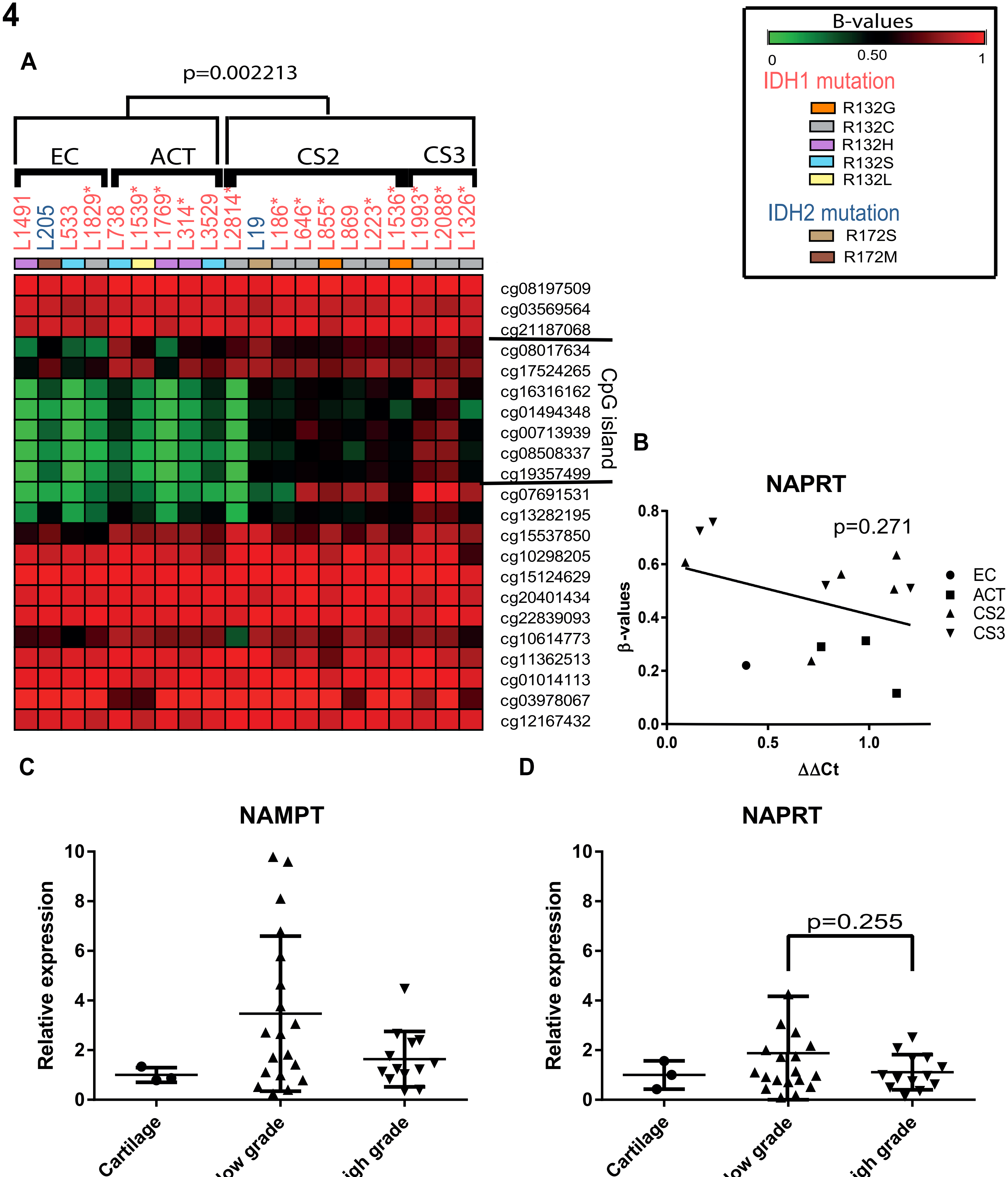
4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CpG island
B-values
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IDH1 mutation
R132G
R132C
R132H
R132S
R132L
IDH2 mutation
R172S
R172M
B
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p=0.271
β-values
ΔΔCt
EC
ACT
CS2
CS3
C
NAMPT
Relative expression
Cartilage
low grade
high grade
D
NAPRT
p=0.255
Relative expression
Cartilage
low grade
high grade

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NAD Synthesis Pathway Interference is a Viable Therapeutic Strategy for Chondrosarcoma

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