Efficacy of NEDD8 Pathway Inhibition in Preclinical Models of Poorly Differentiated, Clinically Aggressive Colorectal Cancer

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(Article begins on next page)
NEDD8 pathway inhibition is effective in preclinical models of poorly differentiated, clinically aggressive colorectal cancer.

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Abstract:

Background. The NEDD8 conjugation pathway modulates the ubiquitination, stability and activity of a wide range of intracellular proteins, and its blockade by pevonedistat is emerging as a promising therapeutic approach in various cancer settings. However, systematic characterization of pevonedistat efficacy in specific tumor types and definition of response predictors are still missing.

Methods. We investigated *in vitro* sensitivity to pevonedistat in a large panel of molecularly and functionally annotated colorectal cancer (CRC) cell lines, and exploited gene expression profiling to define a transcriptional predictor of sensitivity.

Results. Out of 122 tested cell lines, 16 (13%) displayed a marked response to pevonedistat, featuring DNA re-replication, proliferative block and apoptosis. Pevonedistat sensitivity did not significantly correlate with microsatellite instability or mutations in *KRAS* or *BRAF*, and was functionally associated with low EGFR pathway activity. A 184-gene transcriptional predictor generated in cell lines was applied to 87 metastatic CRC clinical samples for which patient-derived xenografts (PDX) were available. *In vivo* administration of pevonedistat did not affect growth of any predicted resistant PDX; whereas, it significantly impaired growth of five out of six predicted sensitive models. When applied to gene expression profiles from CRC patients, transcriptional prediction of pevonedistat sensitivity was associated with poor prognosis after surgery and early progression under cetuximab treatment. Histological and immunohistochemical analyses revealed that the pevonedistat sensitivity signature captures transcriptional traits of poor differentiation and high-grade mucinous adenocarcinoma.

Conclusion. These results highlight NEDD8-pathway inhibition by pevonedistat as a potentially effective treatment for poorly differentiated, clinically aggressive CRC.
Introduction

Each year, approximately 700,000 new cases of CRC are diagnosed worldwide, and of these 60% develop metastatic disease [1]. In addition to chemotherapy, inhibition of the epidermal growth factor receptor (EGFR) by antibodies such as cetuximab and panitumumab has been successfully deployed for treatment of CRC [2, 3]. However, EGFR-targeted therapy is ineffective in more than 80% of the cases, due to molecular alterations of downstream signal transducers, like KRAS, BRAF and NRAS [4] [5, 6], and to additional resistance mechanisms [7]. Alternative targeted therapies for CRC have been focused at inhibition of kinases in the same pathway, including HER2, TRK, ALK, IGFR, BRAF and MEK, resulting in a narrow spectrum of activity [8, 9]. As a consequence, there is an unmet need for therapies targeting alternative pathways in CRC. Among them, blockade of protein degradation by proteasome inhibitors has been explored with promising results also in CRC preclinical models [10]. However, the profound, global alteration of protein stability driven by generic proteasome inhibitors limits their therapeutic window and clinical efficacy [11, 12]. As an attractive alternative we considered therapeutic blockade of the NEDD8 conjugation pathway. The NEDD8 gene (neural precursor cell-expressed developmentally downregulated 8) encodes a ubiquitin-like protein that can be covalently conjugated to specific target proteins. Similar to ubiquitin, NEDD8 acts by a conjugation process (‘neddylation”) that involves its sequential transfer to defined substrates through a series of enzymes [13, 14]. The first step of the NEDD8 conjugation process is its activation by the E1 protein complex, composed of the NEDD8 activating Enzyme 1 (NAE1) and UBA3 gene products. Subsequently NEDD8 is transferred to the E2 enzyme UBE2M, to be finally conjugated to specific substrates via a series of E3 ligases [15]. Intriguingly, the best-characterized neddylation targets are cullins 1 to 9, which are regulatable scaffolds of a wide family of E3 ubiquitin-ligases known as cullin-RING ligases (CRLs) [16]. In particular, CRLs are activated by neddylation to ubiquitinate specific sets of substrates involved in many cellular pathways and processes, including cell cycle, transcription, signal
transduction, and development [15]. Moreover, a number of proteins involved in cancer biology, including MDM2, EGFR, HIFα and VHL are directly regulated by neddylation (14). Alterations of the balance between synthesis and degradation of proteins regulated by neddylation can therefore lead to uncontrolled cellular proliferation and evasion of apoptosis, driving the growth of neoplastic cells [17, 18]. For these reasons, inhibition of the NEDD8 conjugation pathway has been recently explored as a candidate therapy for cancer. Pevonedistat (MLN4924), is a first-in class small-molecule selective inhibitor of UBA3, showed remarkable antitumor activity in cell lines from multiple myeloma, and from colon, lung, prostate and breast cancer. These studies also defined the molecular mechanisms involved in the response, which imply DNA rereplication, apoptosis and senescence [19-22]. Pevonedistat has been tested in phase I clinical trials for hematological and solid neoplasms, which confirmed its therapeutic potential although limited to a subset of cases, highlighting the need of biomarkers for patient selection [23-25]. Indeed, the initial characterization studies were performed on a restricted number of preclinical models for each cancer type. This prevented a comprehensive and reliable evaluation of the biomarkers correlating with drug efficacy, which would require large series of samples or preclinical models annotated for drug response and molecular profiles. We recently reported large collections of CRC cell lines and patient-derived xenografts (PDXs) reliably representing the molecular heterogeneity of CRC [7, 9, 26]. Here, we exploited a large panel of cell lines to evaluate the spectrum of activity of pevonedistat and derive a gene expression signature predictive of response. The predictor was then applied to a set of 87 CRC liver metastases (LM-CRC), which were categorized as sensitive or insensitive on the basis of their gene expression profiles. Finally, proof-of-concept validation of the signature was prospectively conducted in vivo using PDXs from predicted sensitive and predicted resistant cases.
Results

Effects of NEDD8-pathway inhibition by pevonedistat on CRC cell lines.

From a collection of 151 CRC cell lines [9], a panel of 122 genetically unique models were tested for in vitro sensitivity to pevonedistat at concentrations ranging from 1nM to 10μM. Overall, 16 cell lines (13%) displayed marked sensitivity (Fig. 1A; table S1; see Methods). A subset of lines with different sensitivity and genetic background was selected to better characterize the in vitro response. As indicated by the activation of caspase 3/7, pevonedistat selectively induced apoptotic cell death in sensitive cells, regardless of the presence of mutations in BRAF or KRAS (Fig. 1B). All responsive cells also displayed a dramatic increase in the fraction of cells with DNA content > 4N (Fig. 1C), indicative of profound cell cycle alterations leading to rereplication, a known effect of NEDD8 pathway inhibition [20]. To date, only one CRC cell line (HCT-116) has been tested for response to pevonedistat in vivo, in mouse xenografts [19]. To further evaluate the in vivo activity of this compound on CRC models, the most sensitive cell line in the panel (HuTu-80) was xenotransplanted in nude mice, which were then treated with pevonedistat. As shown in fig. S1, drug treatment induced tumor stabilization, similarly to what had previously been reported in HCT116 xenografts [19]. Immunohistochemistry (IHC) on HuTu-80 explanted tumors highlighted pevonedistat-induced reduction of proliferating cells and accumulation of the known CRL substrate p21 (fig. S1), indicating in vivo drug target engagement.

Molecular correlates of response to pevonedistat in CRC cell lines

Cell lines stratified by the following molecular features were assessed for differential pevonedistat sensitivity in vitro (Fig. 2A): (i) MSI status; (ii) BRAF or RAS mutation; (iii) cetuximab sensitivity in KRAS/BRAF wild type cells; (iv) assignment to one of the three major transcriptional subtypes: Inflammatory/Goblet, TA/Enterocyte and Stem/Serrated/Mesenchymal [27]. While MSI status, BRAF
or RAS mutations, and cetuximab sensitivity were not significantly associated with pevonedistat sensitivity or resistance, the TA/enterocyte transcriptional subtype, known to be enriched in cetuximab-sensitive cells [9], displayed significant pevonedistat resistance (t test, P < 0.005; table S2).

We then exploited gene expression profiles to assess whether pevonedistat sensitivity was associated to specific transcriptional traits. To this aim, genes were ranked for differential expression between the 16 most sensitive and the 44 most resistant lines (Fig. 2B; Table S2; see Methods) and tested for preferential up- or down-regulation of gene functional signatures, according to the Gene Set Enrichment Analysis [28] (GSEA; Fig. 2C; table S3). GSEA highlighted upregulation of epithelial mesenchymal transition (EMT), hypoxia and TNF-alpha/NFKB pathways in pevonedistat-sensitive models. Interestingly, transcriptional signatures of EGFR/KRAS pathway activation and signaling dependence were instead upregulated in the pevonedistat-resistant group, in line with the above-described lower response to pevonedistat of TA/enterocyte subtype cells. Overall, this analysis confirmed that sensitivity to pevonedistat is associated with specific biological features, which are captured by transcriptional profiles.

A transcriptional predictor of pevonedistat sensitivity was thus built by selecting the genes with statistically significant differential expression between the most sensitive and most resistant cell lines (see Methods), which resulted in 184 gene transcripts sharply distinguishing sensitive from resistant cells (Figure 2D; table S4). A “pevonedistat sensitivity score” (PSS) was calculated for each cell line by subtracting the averaged expression values of genes upregulated in resistant cells from the average expression of genes upregulated in sensitive cells (table S5).

**Preclinical validation of the pevonedistat response transcriptional predictor**

To provide independent, preclinical in vivo validation of pevonedistat activity and of its transcriptional predictor in CRC, we applied the 184-gene predictor to an expression dataset generated from 87 LM-
CRC samples, for which PDX derivatives were available (see Methods). Of the 184 genes identified in cell lines, 163 were also detected in the LM-CRC dataset. This enabled derivation of a PSS whereby PDX samples were ranked by predicted sensitivity (Fig. 3A; table S5). For validation purposes, six PDX models were selected from samples with high PSS and five from samples with low PSS. KRAS mutants, BRAF mutants and wild-type cases were represented in both groups, with the notable exception that no BRAF-mutant case had a low score.

For each model, at least six xenografts were treated with pevonedistat - and a corresponding cohort with vehicle - for 4 weeks. As shown in Fig. 3B, no low-PSS model displayed significant growth inhibition. Conversely, five out of the six models with high score were growth-inhibited by 80% or more. In four cases, growth curve analysis (Fig. 3C; fig. S2) evidenced prolonged tumor stabilization by pevonedistat. As observed in cell line xenografts, highly sensitive PDXs displayed pevonedistat-induced Ki67 downregulation and accumulation of the CRLs substrates p21 and p27 (Fig. 4). Altogether, these results confirm that a sizeable fraction of CRCs respond to NEDD8 pathway inhibition by pevonedistat in vivo. Importantly, the transcriptional predictor of response generated in cell lines efficiently identified responsive LM-CRC cases, which included also KRAS or BRAF mutant tumors.

**Association of pevonedistat response prediction with clinical aggressiveness and poor differentiation of CRC.**

To assess whether CRC cases predicted to respond to pevonedistat might have distinctive clinical features, the 184-gene predictor was applied to gene expression profiles from 290 CRC samples annotated for disease-free survival (DFS) after surgery [29] (GEO ID: GSE14333; table S5). As shown in Fig. 5A, cases predicted to be sensitive to pevonedistat (top 15% highest PSS) had significantly lower DFS (log rank chi square, P < 0.003). The predictor was then applied to LM-CRC samples
annotated for clinical response to cetuximab [30] (GEO ID: GSE5851; table S5). In agreement with the results obtained in vitro, predicted sensitivity to pevonedistat was associated to significantly lower progression-free survival (PFS) in patients treated with cetuximab monotherapy (t test, P < 0.002; Fig. 5B).

We then evaluated possible relationships between the pevonedistat sensitivity signature and established morphological markers currently in use for CRC stratification. We noticed that many known markers of CRC differentiation were downregulated in sensitive cell lines (fig. S3), suggesting that a positive prediction of response could be associated with poor differentiation. To verify this hypothesis, a transcriptional colon tissue-specific score that we previously calculated for all CRC cell lines [9] was compared with the PSS, and found to be strongly anticorrelated (Fig. 5C; Pearson correlation = -0.88). Indeed, most of the colon differentiation genes were massively downmodulated in pevonedistat-sensitive lines, as shown by GSEA analysis (Fig. 5D). Accordingly, pevonedistat-sensitive cell lines had a significantly lower colon score than pevonedistat-resistant cells (Fig. 5E; t test, P < 0.0001).

Conversely, cetuximab-sensitive models were characterized by a significantly higher colon score (Fig. 5F; t test, P < 0.0001). Overall, these results indicate that pevonedistat sensitivity and its transcriptional predictor are associated to poor differentiation in CRC cell lines.

Of the four known CRC differentiation markers with the highest differential value between pevonedistat-sensitive and resistant cells (CDH17, KRT20, CDX1 and CDX2), cadherin-17 mRNA proved to be the most anticorrelated with the PSS in the 87 liver metastases dataset (Pearson correlation = -0.68). We therefore selected a representative set of 15 CRC liver metastasis samples with variable PSS and stained them for CDH17 protein expression by immunohistochemistry (IHC). Protein levels of CDH17 were well correlated with the respective mRNA levels (fig. S4) and, most importantly, strongly anticorrelated with the PSS (Fig. 5G; Pearson correlation = -0.84). CDH17 protein expression was also evaluated in the 11 PDX models selected for in vivo tests, and again found to be inversely correlated
with response to pevonedistat (Pearson correlation = -0.83; Fig. 5H). These data confirm CDH17 detection by IHC as a potentially valuable negative predictor of pevonedistat sensitivity in CRC.

To evaluate possible positive morphological predictors of pevonedistat sensitivity, hematoxylin/eosin-stained sections of CRC liver metastases with variable PSS values were independently analyzed by a pathologist. Interestingly, all mucinous adenocarcinoma samples invariably had high PSS values, while the great majority of non-mucinous and mixed cases had low or negative PSS (Fig. 6A). The mucinous histotype was strongly associated with low CDH17 expression (Fig. 6B). The same pattern was observed in the 11 PDX models selected for in vivo tests, where the mucinous histotype was invariably detected in all pevonedistat-responsive cases, but not in the five resistant cases with low PSS (Fig. 6C-D; fig. S5. The only case with mucinous histotype and high PSS that did not respond to pevonedistat, M023, displayed instead high CDH17 expression (fig. S6).

**Discussion**

In this work, we exploited a large tissue-specific collection of CRC cell lines to assess pevonedistat efficacy; we defined in this cohort a transcriptional predictor of response; and, finally, we applied the predictor to a powerful preclinical platform, composed of fresh tumor samples matched with PDX derivatives, that reliably mirror disease response and its correlation with predictive biomarkers [9, 26]. The 122 CRC cell lines selected for pevonedistat testing covered the whole spectrum of CRC molecular and transcriptional subtypes known to date [9]. The fraction of responsive cell lines (13%) was even higher than that of cetuximab-sensitive models (9%). The main biological responses elicited by pevonedistat in sensitive CRC lines were re-replication, G2 arrest and apoptosis, in agreement with previous findings in solid tumors and hematological malignancies [19, 20]. Of relevance, the observed functional anticorrelation with EGFR pathway activity/dependence and the lack of significant
association with KRAS or BRAF mutation render this therapeutic approach potentially eligible also for cetuximab-resistant and ‘RAS’ pathway-mutant CRC.

Gene expression profiling of CRC cell lines at the baseline provided further information about functional features associated with pevonedistat sensitivity/resistance. In particular, pevonedistat-sensitive cells displayed transcriptional traits of EMT, possibly driven by hypoxia and TNFα signaling [31-33], a phenotype associated to poor prognosis of CRC [34, 35]. Conversely, resistant cell lines displayed marked traits of EGFR/KRAS pathway activity and associated sensitivity to EGFR blockade [36, 37]. These results confirmed that global transcriptome analysis could reliably capture functional traits associated with pevonedistat sensitivity or resistance. Indeed, the genes of the pevonedistat sensitivity signature, obtained by supervised analysis, displayed highly concordant expression across cell lines (Fig. 2 D), indicative of coherent transcriptional modules. This signature was therefore applied as a response predictor in an independent dataset of 87 LM-CRCs. Although identified in in vitro cultured CRC cell lines, genes of the predictor displayed coherent expression also in LM-CRC samples, which allowed their stratification by a +/- 2.5-fold range of PSS. PDXs derived from high-PSS selected LM-CRCs were markedly sensitive to pevonedistat in five out of six cases, while none of the predicted resistant PDXs responded to pevonedistat treatment. This indicates that the transcriptional signature can efficiently predict in vivo sensitivity. Notably, in all xenografts tested, pevonedistat did not display detectable side effects.

Tumor response achieved in sensitive PDX models was growth stabilization, rather than regression. Of note, and in line with the tumor stabilization observed in phase I clinical trials [24], these results do not reflect the extensive cell death and profound growth inhibition observed for sensitive cell lines in vitro. The same discrepancy was seen when xenografts from the most sensitive of the 122 cell lines were treated with pevonedistat, which only induced tumor stabilization (fig. S1). This suggests that the lack of tumor regression in vivo could be due to suboptimal drug delivery/pharmacodynamics and/or
microenvironmental protective factors. However, given the uniqueness of the pathway affected, it is likely pevonedistat will have additive or synergistic effects when combined with other treatments. Indeed, preclinical evidence of improved efficacy by combination with pevonedistat has already been obtained for apoptosis inducers like TRAIL and TNF-α [38, 39], DNA-damaging agents [40-44] and radiation therapy [45, 46]. Some of these combinations are currently being evaluated in clinical trials on unselected cases (NCT01862328). Future exploration of similar combinations in CRC will greatly benefit from the possibility of selecting patients by the predictor described here.

From the clinical point of view, it should be noted that the gene expression-based predictor of pevonedistat response captured a subset of cases with distinctive prognostic, pharmacological and morphofunctional features: (i) human CRC samples with high PSS had a significantly worse prognosis; (ii) observed or predicted pevonedistat sensitivity was inversely associated to cetuximab sensitivity, from cell lines to patients, and occurred also in KRAS- and BRAF-mutant cases, intrinsically resistant to EGFR blockade; (iii) low mRNA and protein levels of the differentiation marker CDH17 [47], encoding for a cell-adhesion molecule expressed in intestinal epithelium, were strongly correlated to pevonedistat sensitivity in vitro and in vivo. Therefore, positive CDH17 IHC staining could be exploited as a negative predictor of pevonedistat response; conversely, a positive predictor could be the high grade, mucinous phenotype, which accounts for about 10% of CRCs and is characterized by adverse prognosis and poor response to chemotherapy and targeted agents [48-50]. Recently, low-grade mucinous CRCs have been distinguished from high-grade cases by their differentiated morphology [51]. Notably, all the five PDX models displaying sensitivity to pevonedistat were poorly differentiated (CDH17-negative) and mucinous, therefore belonging to the high-grade mucinous subgroup.

In summary, this study shows that NEDD8 pathway inhibition by pevonedistat may be a promising therapeutic strategy for poorly differentiated, clinically aggressive mucinous CRC, not eligible for alternative tailored treatments.
Materials and Methods

Cell lines and drugs

All cell lines were maintained in their original culturing conditions according with supplier guidelines, as reported elsewhere [9]. Cell lines of the HROC series [52-54] were obtained from Michael Linnebacher. For in vitro experiments pevonedistat (MLN4924) was obtained from Active Biochem (CAT#: A-1139). For in vivo studies the compound was provided by Millennium Pharmaceuticals, Inc. (Cambridge, MA). For additional details on cell cultures and assays see Supplementary Methods.

Gene expression data analysis

Gene expression data from the 122 CRC cell lines were extracted from the Gene Expression Omnibus (GEO, accession: GSE59857; Table S2), normalized and preprocessed (expression filtering, removal of redundant probes, log₂ transformation, log₂ratio vs. average) as previously described [9]. To construct a transcriptional predictor of response to pevonedistat, log₂ signal values in the 16 sensitive and 44 resistant cells were compared by t-test (P <0.001 and > 1.7 fold-change (absolute log2 ratio). False discovery rate (FDR) analysis was performed by Monte Carlo simulation considering 2,000 random sample permutations. The fraction of false positive hits was very low (1.56 %). The expression of the signature genes was visualized by Gedas software [55]. Gene expression data from 87 CRC-LM samples for which PDX derivatives were extracted from a 185 CRC-LM GEO dataset (Isella et al., manuscript submitted; GSE73255). Expression filtering was performed selecting probes detected in at least one sample. Among redundant probes, the probe with the highest average value was selected for further analysis. For survival analysis, gene expression profiles from 290 primary CRC surgical specimens were downloaded from GEO (GSE14333) [29]. For progression-free survival analysis, gene expression data of metastatic CRC samples were downloaded from GEO (GSE5851) and 61 LM-CRC were exploited for further analysis [30]. Additional details are provided in Supplementary Methods.
**Human tumor samples and animal studies**

All patients provided informed consent and samples were procured and the study was conducted under the approval of the Review Boards of the institution. Clinical and pathologic data were entered and maintained in our prospective database. Female NOD-SCID mice (Charles River Laboratories) were used in all in vivo studies. All animal procedures were approved by the Ethical Commission of the Institute for Cancer Research and Treatment and by the Italian Ministry of Health. Further details on the experimental procedures are provided in Supplementary Methods.

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G. Picco contributed study design, data analysis, performed *in vitro* and *in vivo* experiments and manuscript writing. C. Petti performed *in vivo* and *in vitro* experiments. K. Grillone and T. Rossi performed *in vitro* experiments. G. Migliardi contributed to *in vivo* experiments. C. Isella contributed to bioinformatics analyses. F. Sassi and A. Sapino performed and interpreted histochemical and morphological analyses. L. Trusolino, A. Bardelli, F. Di Nicolantonio and A. Bertotti contributed study design and manuscript writing. E. Medico contributed study design, data analysis, bioinformatics, manuscript writing and project oversight. **Competing interests:** The authors declare no competing financial interests.
**Figures legends**

**Fig. 1. Sensitivity of CRC cell lines to Pevonedistat in vitro.** (A) The indicated cell lines (n=122) were treated with increasing concentrations of Pevonedistat for 4 days and cell viability was assessed by measuring ATP content. Bars represent relative cell viability compared to vehicle after 96 hours of treatment (average between viability at 0.5 and viability at 1 µM pevonedistat). Cells lines with viability < 40% were considered sensitive (n=16; 13%). Red (sensitive) and blue (resistant) squares at the bottom of the bars highlight representative cell lines selected for further characterization of the response. (B) Histograms representing the relative caspase 3-7 activity of pevonedistat-sensitive (red) and resistant (blue) cell lines after 48h of exposure to the drug at incremental concentration, as indicated. Caspase activity was normalized versus viability values (CellTiter-Glo), obtained from experiments performed in parallel ± SD (n = 3) (C) DNA content evaluation by flow cytometry in CRC cell lines. Black histograms: vehicle-treated cells. Colored histograms: cells treated for 24h with 1µM pevonedistat. Only sensitive cell lines display a remarkable increase of re-replicating cells (>4N) when treated with pevonedistat (red histograms).

**Fig. 2. Molecular prediction of pevonedistat sensitivity in CRC cell lines.** (A) Dot plots displaying sensitivity to Pevonedistat (average viability at 0.5 and 1 µM) of 122 CRC cell lines stratified by MSI status, KRAS/BRAF mutational status, cetuximab sensitivity (only in KRAS and BRAF wild-type cases) and consensus transcriptional subtypes, as indicated. (B) Dose-response to Pevonedistat of 16 highly sensitive and 44 highly resistant CRC cell lines (red and blue curves, respectively). (C) Gene sets associated to cellular functions/pathways upregulated in pevonedistat-sensitive (red text) or resistant (blue text) CRC cell lines. The radar plot scale indicates the GSEA normalized enrichment.
score (NES). (D) Heatmap representing the expression of the 184 signature genes (rows) in pevonedistat-sensitive and resistant cell lines, ordered from left to right by decreasing sensitivity score.

**Fig. 3. Pevonedistat inhibits in vivo growth of CRC PDXs predicted responsive.** (A) Waterfall plot displaying 88 CRC liver metastases ordered by decreasing pevonedistat sensitivity score (PSS, y-axis), from left to right. Red and blue bars highlight cases with high and low PSS, selected for validation in PDX models. The heatmap reports expression (log2 ratio vs average) of signature genes in the same 88 samples. (B) Bar graph reporting growth inhibition of PDXs after treatment for 28 days with Pevonedistat vs vehicle-treated counterparts. Red bars, high PSS cases; blue bars, low PSS cases. (C) PDX growth curves of the cases displaying the highest growth inhibition among the predicted sensitive (left panels, red curves) and among the predicted resistant (right panels, blue curves). Black curves, vehicle-treated.

**Fig. 4. Pevonedistat response in vivo is associated with reduced ki67 and increased p21 and p27 protein levels.** Immunohistochemical staining and morphometric quantitation of ki67 (A), p21 (B) and p27 (C) of two representative PDX tumors derived from a sensitive (M085, red bars) and a resistant (M065, blue bars) case at the end of treatment with vehicle (black bar) or pevonedistat (colored bar), as indicated. Results are means ± SD of 10 fields (20×) for each experimental point. Bar graphs are flanked by micrographs of representative fields. Scale bar, 50 μm.

**Fig. 5. Pevonedistat response prediction is associated with clinical aggressiveness and poor differentiation of CRC.** (A) Kaplan-Meier analysis of disease-free survival after surgery of primary CRC cases with top 15% highest PSS score (red line) vs all remaining cases (blue line). (B) Kaplan-
Meier analysis of progression-free survival of liver metastatic cases with top 15% highest PSS score (red line) after treatment with cetuximab monotherapy. (C) Dot plot displaying the correlation between the PSS (y-axis) and the tissue-specific “colon” score (x-axis) in 122 CRC cell lines. (D) GSEA enrichment analysis of colon differentiation genes in pevonedistat-sensitive vs resistant cell lines. (E-F) Dot plots displaying the correlation between the tissue-specific “colon” score and viability upon pevonedistat (E) and cetuximab (F) treatment. Pevonedistat-sensitive lines are marked in red (left panel), cetuximab sensitive lines are marked in blue (right panel). (G) Correlation between PSS and CDH17 protein expression detected by IHC in 16 liver metastatic CRC samples. (H) Correlation between growth inhibition measured in 11 PDXs tested in vivo and CDH17 protein expression of the same PDXs evaluated by IHC.

Fig. 6. Pevonedistat sensitivity is associated with high-grade, mucinous CRC. (A) Dot plot reporting on the x-axis the PSS values for CRC liver metastases grouped by their mucinous aspect: mucinous, mixed, non-mucinous. (B) Bar plot showing, on the y-axis, the fraction of mucinous (red), mixed (grey) and non-mucinous (blue) cases grouped by their CDH17 staining signal, from 1 (absent) to 4 (high). (C-D) Micrographs of representative PDXs responsive or resistant to pevonedistat (left and right panels, respectively), stained by haematoxylin and eosin. Scale bar 50 μm.
References

Figure 1
Cell viability (%)

Resistant (n=44)
Sensitive (n=16)

Gene Sets
Up in Sensitive Cells

Gene Sets
Up in Resistant Cells

TNFA signaling via NFKB
Hypoxia
UP in MSI CRC
DOWN by EGFR
EMT
UP by KRAS
UP in MSS CRC
UP in Gefitinib sensitive

Pevonedistat-sensitive/resistant Log₂ Ratio
Growth inhibition (%)

Tumor volume (mm$^3$)

Figure 3
Figure 4

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<td>Pevo</td>
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<td><img src="image" alt="Image" /></td>
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</tr>
</tbody>
</table>

p < 0.001

C

<table>
<thead>
<tr>
<th></th>
<th>M085</th>
<th></th>
<th>M065</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>P27-positive nuclei (% area)</td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
<td><img src="image" alt="Graph" /></td>
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</tr>
</tbody>
</table>

p < 0.001
A

Mucinous

Mixed

Non-muc

-1.0  -0.4  0.2  0.8  1.4

PSS

B

Fraction of samples

100%

75%

50%

25%

0%

1  2  3  4

CDH17 staining

C

pevonedistat-sensitive

M023

M039

M015

D

pevonedistat-resistant

M059

M064

M065