Aryl hydrocarbon receptor nuclear translocator-like (ARNTL/BMAL1) is associated with bevacizumab resistance in colorectal cancer via regulation of vascular endothelial growth factor A

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ABSTRACT

Background: The identification of new biomarkers and the development of novel, targetable contexts of vulnerability are of urgent clinical need in drug-resistant metastatic colorectal cancer (mCRC). Aryl-Hydrocarbon-Receptor-Nuclear-Translocator-Like (ARNTL/BMAL1) is a circadian clock-regulated transcription factor promoting expression of genes involved in angiogenesis and tumour progression. We hypothesised that BMAL1 increases expression of the vascular endothelial growth factor A VEGFA gene and, thereby, confers resistance to anti-angiogenic therapy with bevacizumab (Beva), a clinically used antibody for neutralization of VEGFA.

Methods: PCR and immunohistochemistry were employed to assess BMAL1 expression in mice (C57BL/6 J Apcmin/+;BALB/c nu/nu xenografts) and CRC patients under combination chemotherapy with Beva. BMAL1 single nucleotide gene polymorphisms (SNPs) were analysed by DNA-microarray in clinical samples. BMAL1 functions were studied in human CRC cell lines using colorimetric growth, DNA-binding and reporter assays.

Findings: In murine CRCs, high BMAL1 expression correlated with poor preclinical response to Beva treatment. In CRC patients’ tumours (n = 74), high BMAL1 expression was associated with clinical non-response to combination chemotherapy with Beva (p = .0061) and reduced progression-free survival (PFS) (p = .0223, Hazard Ratio (HR) = 1.69). BMAL1 SNPs also correlated with shorter PFS (rs11022780, rs7396943, rs7938307, rs2279287) and overall survival (OS) (rs11022780, p = .014, HR = 1.61). Mechanistically, Nuclear-Receptor-Subfamily-1-Group-D-Member-1 (NR1D1/REVERBA) bound a 672 bp Retinoic-Acid-Receptor-Related-Orphan-Receptor-Alpha-responsive-element (RORE) adjacent to a BMAL1 DNA-binding motif (E-box) in the VEGFA gene promoter, resulting in increased VEGFA synthesis and proliferation of human CRC cell lines.

Abbreviations: Ab, antibody; Apc, adenomatous polyposis coli; ARNTL (BMAL1), Aryl Hydrocarbon Receptor Nuclear Translocator-Like; B2M/B2m, beta-2-microglobulin; Avastin®, Beva human bevacizumab; B2O, mBeva murine bevacizumab; CAV1, caveolin-1; CMS, consensus molecular subtype; CRC, colorectal cancer; DCR, disease control rate (PR + SD); DFS, disease-free survival; E-box, BMAL1/MYC-responsive (DNA) element; ECOG, Eastern Cooperative Oncology Group; FFPE, formalin-fixed paraffin-embedded; G grade, (tumour); GEMM, genetically engineered mouse model; HE, haematoxylin & eosin; HIF1A, Hypoxia-Inducible Factor-1 Alpha; HR, hazard ratio; IHC, immunohistochemistry; KO, knock-out; MVD, microvessel density; N, nodal status (tumour); NC, normal colon (tissue); NR, non-responder; NR1D1 (REVERBA), Nuclear Receptor Subfamily 1 Group D Member 1; NT, non-tumour (normal); OS, overall survival; PCA, principal component analysis; PD, progressive disease; PFS, progression-free survival; PR, partial response; R, responder; RECIST, Response Evaluation Criteria In Solid Tumours; RORA, Retinoic Acid Receptor-Related Orphan Receptor Alpha; RORE, ROR responsive (DNA) element; SD, stable disease; SI, small intestine; SNP, single nucleotide polymorphism; T, size (tumour); TMA, tissue microarray; TU, tumour; UICC, Union internationale contre le cancer (tumour stage); VEGF(R), vascular endothelial growth factor (receptor).

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1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in both men and women and represents a major component of worldwide cancer mortality and morbidity [1]. Current treatment for metastatic CRC (mCRC) includes 5-fluoruracil-based standard of care chemotherapy (e.g. FOLFOX, FOLFIRI) combined with bevacizumab (Beva). Beva is a human monoclonal antibody (Ab) that prevents binding of vascular endothelial growth factor A (VEGFA) to its receptors [1], thus inhibiting angiogenesis. Nevertheless, only a subset of patients responds, and resistance to anti-angiogenic therapy may be intrinsic or adaptive in nature [2]. The underlying molecular mechanisms have yet to be fully revealed. Elucidation of the biology underpinning treatment failure, and the identification of novel predictive markers to identify CRC patients who will benefit from Beva therapy continues to hold clinical relevance [2].

To this end, we studied transcription factors of the circadian clock machinery which had been previously reported to regulate VEGFA gene expression [3–6] and, thus, may represent suitable candidates for mediating Beva resistance. Synchronised by the solar input to the suprachiasmatic nucleus of the hypothalamus and other brain regions, the “central clock” orchestrates complex signalling networks termed “peripheral clocks” in tissues and organs on an approximate 24 h day/night cycle [7,8]. At the cellular level, these circuits are driven by the master activator basic helix-loop-helix transcription factor “Aryl Hydrocarbon Receptor Nuclear Translocator-Like” (ARNTL/BMAL1, herewith termed BMAL1) and its heterodimer partner “Circadian Locomotor Output Cycles protein Kaput” (CLOCK) [9]. This complex binds to DNA-motifs (E-boxes) in regulatory regions of genes encoding for activating (e.g. Retinoic Acid Receptor-Related Orphan Receptor Alpha, RORA) and repressive (e.g. Nuclear Receptor Subfamily 1 Group D Member 1, NR1D1/REVERBA; PER1/2, CRY1/2) transcription factors inducing negative transcriptional/translational feed-back mechanisms which ultimately generate diurnal oscillations of output genes [7,8]. RORA binds oxysterols, REVERBA nitric oxide, carbon monoxide or Fe(II/III)–heme derived from degraded red blood cells. Both receptors compete for the same site on the DNA, termed ROR responsive element (RORE) [8] and, thereby, regulate target genes, such as BMAL1 and Hypoxia-Inducible Factor-1-Alpha (HIF1A), in a reciprocal mode of fashion. Overall, these properties position circadian clock transcription factors as important sensors of the tissue’s redox and oxygenation status [7,8]. Accordingly, they regulate down-stream genes responsible for the maintenance of cardio-vascular homeostasis, body temperature, metabolism, immune defence and developmental or regenerative processes such as vascular and angiogenesis.

The pivotal role of BMAL1 in orchestrating this hierarchy of regulatory circuits and its cooperation with tissue-specific non-circadian transcription factors [9] provided the rationale to explore its role in resistance to anti-angiogenic therapy: BMAL1 is implicated in several aspects of vessel (dys)function, i.e. coverage of endothelia by pericytes, coagulation / thrombosis, vascular tone / hypertension and atherosclerosis [7,8]. Specifically, BMAL1 is a stimulator of VEGFA production having previously been shown to up-regulate Vegfa promoter activity in mouse xenografts [3]. Conversely, Bmal1 silencing lowered Vegfa mRNA in genetically engineered mouse models (GEMMs) [4,5] and impaired vessel growth in zebrafish [6].

In contrast to the normal body physiology, aberrant expression or arrhythmic activities endow BMAL1 (and other circadian transcription factors) with more specialised functions in disease conditions [7,8]. Clock disruptions, by sleep deprival (e.g. jet lag, shift work, neuro-psychiatric disorders) or gene mutations, are associated with metabolic or inflammatory disturbances and presumably with an enhanced risk for cardiovascular diseases and cancer [10]. Oncogenes (e.g. MYC, RAS) or loss of tumour suppressors (e.g. PTEN) destroy circadian rhythms in cancer cells. For example, mutant KRAS abrogates oscillation cycles of BMAL1 [11], and MYC out-competes BMAL1 at shared E-boxes [12] on target genes. In murine CRC [13] and liver metastases [14], the amplitude and phase of circadian rhythmicity in BMAL1 expression were attenuated, delayed or fully abolished. Thus, a deregulated clock is implicated as a hallmark of cancer [7,8].

In tumour cells, BMAL1 regulates expression of genes/proteins [9] involved in cell proliferation, stemness, senescence or cell survival (P21, CDC25A), endoplasmic reticulum–mediated stress resilience (eIF2α), evasion of apoptosis, e.g. via DNA-damage repair pathways (p53, ATM), cell migration (ROCK2, MTA1), hypoxia (HIF1A) or systemic immune responses (CCL2), supporting a role for BMAL1 as a potential oncogene contributing to chemotherapy resistance or metastasis [7,8]. In patients, high BMAL1 expression has been associated with advanced CRC as well as other cancers [15]. Based on this evidence, we hypothesised that BMAL1 could have a predictive and/or mechanistic involvement in Beva resistance and sought to investigate its role in CRC.

| Research in context |
|--------------------|
| **Evidence before this study** |
| Transcription factors of the circadian clock (such as BMAL1) determine resistance to radiation and chemotherapy in cancer patients. However, their role regarding the efficacy of current targeted therapies such as antibodies against growth factors (like VEGFA) or their receptors remains elusive. |
| **Added value of this study** |
| We identified BMAL1 as a potential marker and functional player in resistance to clinically-in-use VEGFA-neutralizing antibody (bevacizumab) in preclinical, clinical and cellular mechanistic studies of colorectal cancer. |
| **Implications of all the available evidence** |
| Failure to respond to bevacizumab correlated with high expression of BMAL1, a transcription factor which drives persistent synthesis of VEGFA in and secretion from tumour cells. In translational studies, BMAL1 shall be further developed as a predictive biomarker detecting and/or as a druggable target preventing resistance to anti-angiogenic therapy in colorectal cancer patients. |
2. Materials and methods

2.1. Reagents

Chemicals were from Merck (Darmstadt) or Sigma (Taufkirchen), Germany. mBeva (mu_chimeric_B20–4.1) was provided on contract by Roche Diagnostics, Penzberg, Germany (MTA#10082012). Beva (Avastin®) was purchased from the hospital pharmacy [16]. Abs for Western blots were REVERBA (#PA-29865, Thermo Fisher Scientific (FS), Darmstadt, Germany), BMAL1 (sc-65069, BD Bioscience, Heidelberg, Germany), CD31 (#Clone SZ31, Dianova, Hamburg, Germany), BMAL1 (#NB2P–02544, for human, Novus Biologicals, Littleton, CO), BMAL1 (#NB100–2288, for mouse, Novus). siRNA oligonucleotides were from Dharamco (Thermo FS). Human REVERBA (67 kDa, NM_021724, MHS1010–9205700) and BMAL1 (69 kDa, NM_001178.5, MHS5278–202757260) were obtained as full-length cDNA clones in pCMV SPORT6 (Thermo FS). Proximal promoter regions of the human VEFGA (−2 kb/+1) gene were PCR amplified and inserted into pGL3-luc (Promega, Madison, WI). Oligonucleotides are listed in Supplementary Table 1.

2.2. Patient studies

Tissue microarrays (TMAs) of drug-naive CRC patients (n = 48) were purchased from US Biomax (Co483, Rockville, MD). Therapy studies were conducted in accordance with ethical guidelines (Declaration of Helsinki) and approved by the local ethics committees for each participating site. All patients provided written informed consent for the analysis of molecular correlates. Treatment of CRC patients, for the first cohort retrospectively examined (n = 77), was performed as described [17,18]. Surgical specimens [Union internationale contre le cancer (UICC) stage II–IV] were collected prior to any therapy and subjected to histological evaluation according to the Vienna classification [19]. Patients then underwent first-line single or multiple chemotherapy regimens (based on FOLFOX, FOLFIRI) in combination with Beva. Patients were stratified into responders (R) and non-responders (NR) according to progression-free survival (PFS) with a cut-off of 300 days (10 months). PFS was defined as time from start of chemotherapy until the first observed progression. Subgroups were classified by clinical performance following Beva treatment based on restaging examinations according to Response Evaluation Criteria In Solid Tumours (RECIST) [18,20]: Cases were stratified by complete response (CR), partial response (PR), stable disease (SD), progressive disease (PD) or death. Patients who were staged as CR, PR or SD were attributed to the disease control rate (DCR) group (R) vs. PD (NR). In a separate cohort, the impact on the clinical outcome of four single nucleotide polymorphisms (SNPs) in the BMAL1 (ARNTL) gene (Supplementary Table 2) was evaluated in mCRC patients (n = 215) treated with first-line FOLFIRI plus Beva in the randomised phase III TRIBE trial [21,22]. Primary endpoint was PFS, and secondary endpoints were overall survival (OS) and tumour response. PFS was defined as time from randomization until disease progression, death or until last follow up in patients who were alive and remained free of disease progression. OS was defined as time from randomization until death. In both cohorts, patients who had no event observation were censored at the last date of follow up.

2.3. Single nucleotide polymorphism (SNP)

Molecular analyses were performed at the USC / Norris Comprehensive Cancer Center in Los Angeles. The study was conducted adhering to the reporting recommendations for tumour marker prognostic studies [23]. Potentially functional SNPs of interest within the BMAL1 (ARNTL) gene in the randomised phase III TRIBE trial [21,22] were identified according to the following criteria: minor allele frequency > 10% in Caucasians; potential to change gene function in a relevant matter according to public databases (National Institute of Environmental Health Science SNP Function Prediction, snpinfo.niehs.nih.gov, Queen’s University F-SNP, compbio.cs.queensu.ca, NCBI-Pubmed and dbSNP, www.ncbi.nlm.nih.gov as well as Genecards, genecards.org) or tagging SNPs. Linkage disequilibrium among selected SNPs was evaluated by means of SNAP search service (http://archive.broadinstitute.org/mpg/snap/). Genomic DNA from peripheral blood samples was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol and genotyped through the OncoArrayTM platform, a custom array manufactured by Illumina (San Diego, CA), including approximately 530 K SNP markers [24]. Four SNPs in BMAL1 were analysed: rs11022780, rs7396943, rs7938307 and rs2279287 (Supplementary Table 2).

2.4. Animal studies

Animal studies conducted in Germany (35–9185.82/G-176/12/81/G-146/15:81G-188/18) were approved by the government of Baden-Württemberg (Karlsruhe, Germany) and the local institutional board of Heidelberg University. The facilities guaranteed 12 h light on-off cycles at constant room temperature. Animals were injected and sacrificed between 10 a.m. and 2 p.m. to avoid sampling errors due to circadian phases. All animals provided written informed consent for the study and sex and body weight. Tumour-bearing animals were identified by rectal bleeding and Apc min genotype. Mice (n = 11) were injected (i.p., 10 mg/kg, once a week) with mBeva (Roche Diagnostics GmbH, Penzberg) for 4 weeks. Controls comprised tumour-bearing littermates of matched generations (n = 42). Recombinant murine Ab was provided by Roche under material transfer agreement. mBeva (mu_chimeric_B20–4.1 [26]; G6–31 equivalent [27]; [3.08 mg/ml]) neutralises human and mouse VEGFA and has a murine invariant backbone of the Ab chains for applications in immunocompetent mice. Xenograft studies conformed to the 2010/63/EU guidelines and were approved by the Dept. of Health and Children, Dublin, Ireland (B100/3654) and University College Dublin Animal Research Ethics Committee (P12–27). Briefly, BALB/c nu/nu mice (n = 48, Charles River Labs., Wilmington, MA) Cav1−/− (Cav1tm1Mls/J; #004355; Jackson Labs., Bar Harbor, Maine) were randomised (at an age of 4–5 month) according to sex and body weight. Tumour-bearing animals were identified by rectal bleeding and Apc min genotype. Mice (n = 11) were injected (i.p., 10 mg/kg, once a week) with Beva (Roche Diagnostics GmbH, Penzberg) for 4 weeks. Controls comprised tumour-bearing littermates of matched generations (n = 42). Recombinant murine Ab was provided by Roche under material transfer agreement. mBeva (mu_chimeric_B20–4.1 [26]; G6–31 equivalent [27]; [3.08 mg/ml]) neutralises human and mouse VEGFA and has a murine invariant backbone of the Ab chains for applications in immunocompetent mice. Xenograft studies conformed to the 2010/63/EU guidelines and were approved by the Dept. of Health and Children, Dublin, Ireland (B100/3654) and University College Dublin Animal Research Ethics Committee (P12–27). Briefly, BALB/c nu/nu mice (n = 48, Charles River Labs., UK) were subcutaneously implanted with 2 × 106 HCT116 cells in the right flank as previously described [28], and tumours were grown until they reached 200 mm3. Subsequently, animals were randomised into four cohorts (n = 12 per group) and treated with either vehicle [5% (w/v) glucose and PBS] or clinically relevant doses of Beva [10 mg/kg, i.p. once a week] and FOLFOX [Folinic acid: 13.4 mg/kg, 5-FU: 40 mg/kg, Oxaliplatin: 2.4 mg/kg; i.p. once a week 24 h before Beva] either alone or in combination for 4 weeks.

2.5. Cell culture

Simian Virus 40 (SV40) large T-antigen transformed human embryonic kidney (HEK293T) and human colon adenocarcinoma (SW480, HCT116) cell lines (all purchased from the American Type Culture Collection, Rockville, MD) were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) (#41965) supplemented with 10% (v/v) fetal calf serum (#SV30160), 20 mM glutamine and penicillin / streptomycin (1000 U/ml; all from Thermo FS), herewith termed “complete” medium, as recommended by the distributors. CRC cell lines were chosen based on their mutational status for the KRAS gene and Wnt-pathway components, the major oncogenic drivers of the largest consensus molecular subgroups (CMS2/3) of human CRC [29]. Cell lines were routinely tested (every 3 months) using PlasmoTest™ (#17C01-MM, Mycoplasma Detection Kit, InViVoGen, Toulouse, France). Cell cultivation did not exceed 25 consecutive passages after thawing from liquid N2. Transfection procedures were done as described by the manufacturer of TurboFect® reagent (Thermo FS). Colorimetric cell viability assay based on...
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was conducted according to the manufacturer’s protocol (Roche Diagnostics GmbH, Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) was done as recommended by the distributor (DVE00, R&D Systems, Minneapolis, MN) measuring VEGFA in supernatants of monolayer cell cultures. Conditioned medium (CM) was prepared by growing cells for 72 h to subconfluence in 6-cm dishes using complete DMEM, followed by collection of supernatants and storage at −80 °C. Starvation medium (SM) was composed of basal DMEM without any supplements. Clinically relevant mBeva (1–10 μg/ml) [30] and nontoxic hemin (≤ 30 μM) [31] concentrations were applied for in vitro assays.

2.6. Western blotting

The methods including preparation of cell and tissue protein lysates were done as described previously [32].

2.7. Reverse transcription PCR (RT-PCR) and quantitative PCR (qPCR)

Methods were performed on total RNA as published [25]. PCR primers are listed in Supplementary Table 1.

2.8. Electrophoretic mobility shift assay (EMSA)

Biotin-labelled oligonucleotides (Supplementary Table 1) were annealed and served as a binding probe for nuclear extracts as described before [33] and following the guidelines of the manufacturer (LightShift Chemiluminescent EMSA Kit, Thermo FS).

2.9. Chromatin-immunoprecipitation (ChiP)

ChiP was conducted as published previously [34] followed by genomic qPCR using primers as indicated in Supplementary Table 1.

2.10. Immunohistochemistry (IHC)

Ab and haematoxylin & eosin (HE) stainings were performed on formalin-fixed paraffin-embedded (FFPE) samples as described [35]. IHC was done manually on murine samples or using Dako Autostainer Plus (Agilent, Santa Clara, CA) on human specimens. Abs were diluted IHC was done manually on murine samples or using Dako Autostainer Plus (Agilent, Santa Clara, CA). Automatised calculation of CD31+ microvessel density (MVD) and vessel size followed an established protocol [36]. Staining frequency and intensity in epithelial (tumour (TU); non-tumour/normal (NT)) and stroma (lamina propria) cells were analysed observer-blinded [35]. Scores were: 0+ = negative (0–25%), 1+ = weak (25–50%), 2+ = moderate (50–75%), 3+ = strong (75–100% positive nuclei compared to total nuclei per field). Images from bright field microscopy (DM BE Quantimet 600 HR, Camera: DC500, Software: IMSO, Leica Microsystems, Wetzlar, Germany) were quantitatively assigned using the software Image J (https://imagej.nih.gov/ij/) (n > 50 nuclei per field, n = 5 fields per image).

2.11. cDNAmicroarray

As published before [25], cRNAs from CRC and adjacent normal colon tissue of Apc min/+ Cav1 +/- mice were hybridised to GeneChip® Mouse Exon 1.0 ST arrays (Affymetrix, Wycombe, UK), followed by Gene Set Enrichment Analysis (GSEA). CEL files were submitted to the NCBI GEO data set repository with the accession number GSE124838 and ID 200124838 under the following link: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124838.

2.12. Software and statistics

Results are means ± S.E. from at least 3 subjects or mice per genotype and treatment group or independent cell passages. Data were normalised to house keeper genes or proteins as indicated in legends to figures and calculated as % or fold vs. control. Matched tumour (TU) and normal/non-tumour (NT) intestinal tissues from the same patient or mouse were assessed. Graphpad Prism (version 4.0; GraphPad Software, San Diego, CA) and SAS (version 9.3; SAS Institute, Cary, NC) were used to analyse the data. All tests were two-sided. P-values *p < .05 were considered significant. Transcription factor binding sites were identified with AliBaba2.1 (gene-regulation.com/pub/programs/allibaba2/) based on TRANEXAC 4.0.

For SNP-analysis, Kaplan-Meier curves and log-rank test were used in univariable analysis; events/total, Hazard Ratio (HR), median and Wald P-value were based on multivariable Cox proportional hazards regression models adjusting for patient characteristics including gender, age, Eastern Cooperative Oncology Group (ECOG) performance status, primary tumour site, primary tumour resected, liver limited disease, adjuvant chemotherapy, BRAF status, RAS status, and top three principle components from the principal component analysis (PCA) for European ancestry SNPs [23,24]. The associations between SNPs and tumour response were evaluated using chi-square test. Exploratory subgroup analyses by gender, primary tumour location, and RAS mutational status were also performed. Interaction test for SNPs and gender, tumour location and RAS status was conducted using the same multivariable Cox proportional hazard models. All P-values were from two-sided Wald tests at a 0.05 significance level. All tests were calculated by using the SAS statistical package version 9.4.

3. Results

3.1. mBeva reduces CRC growth in GEMMs

To explore mBeva efficacy in vivo, we employed a genetically engineered mouse model (GEMM) that develops macroscopic vascularised CRC driven by co-deficiency of adenomatous polyposis coli (Apc) and caveolin-1 (Cav1) as previously described [25]. Gene set enrichment analysis of cDNA microarrays (GSE124838) revealed a pro-invasive signature in CRCs from Apc min/+ Cav1 +/- mice [25]. Likewise, iron-regulated genes were up-regulated in tumour (TU) vs. non-tumour/normal (NT) colon tissue by ~2 to 60-fold (Supplementary Table 3, Supplementary Fig. 1). Minor changes were recorded for clock-related genes (e.g. Clock, Revverb e.a.), prompting us to assess whether those transcription factors play a role in CRC in vivo.

To this end, tumour-bearing Apc min/+ Cav1 +/- mice (n = 11) were treated (i.p. 10 mg/kg once a week) with mBeva for 4 weeks. The control cohort comprised tumour-bearing littermates of matched generations (n = 42). Mice were sacrificed, and serum and organs collected for cryopreservation or formalin-fixed paraffin embedding (FFPE). Macroscopic (Fig. 1a) and microscopic (Fig. 1b) histopathological analyses of murine tissues were performed to determine the response to mBeva treatment (Supplementary Tables 4–5). mBeva decreased incidence and multiplicity of CRC: 30 of 42 (71.4%) controls bore colon tumours vs. 4 of 11 (36.4%) treated mice (*p = .0411, Fisher Exact test) (Fig. 1c), and 11 of 42 (26.2%) controls had >1 colon tumour vs. 0 of 11 (0%) treated animals (Cochrane Armitage trend test, “p = .0157” (Fig. 1d). In sum, 27 of 42 (64.3%) controls had high-grade tumours vs. 1 of 11 (9.1%) treated mice (“p = .0015, Fisher Exact test) (Fig. 1e), and 21 of 42 (50%) tumours displayed dilated glands (“cysts”), ulcerations with hemorrhage and other signs for cell death compared with 1 of 11 (9.1%) under therapy (“p = .0172, Fisher Exact test) (Fig. 1f).

In addition, tumour size was reduced from [9.7 ± 12 mm2] in controls to [5.8 ± 2.8 mm2] in mBeva-treated mice [30.8 ± 14.1 mm2] in control mice [30.8 ± 14.1 mm2]. Results are means ± S.E. from at least 3 subjects or mice per genotype and treatment group or independent cell passages. Data were normalised to house keeper genes or proteins as indicated in legends to figures and calculated as % or fold vs. control. Matched tumour (TU) and normal/non-tumour (NT) intestinal tissues from the same patient or mouse were assessed. Graphpad Prism (version 4.0; GraphPad Software, San Diego, CA) and SAS (version 9.3; SAS Institute, Cary, NC) were used to analyse the data. All tests were two-sided. P-values *p < .05 were considered significant. Transcription factor binding sites were identified with AliBaba2.1 (gene-regulation.com/pub/programs/allibaba2/) based on TRANEXAC 4.0.

For SNP-analysis, Kaplan-Meier curves and log-rank test were used in univariable analysis; events/total, Hazard Ratio (HR), median and Wald P-value were based on multivariable Cox proportional hazards regression models adjusting for patient characteristics including gender, age, Eastern Cooperative Oncology Group (ECOG) performance status, primary tumour site, primary tumour resected, liver limited disease, adjuvant chemotherapy, BRAF status, RAS status, and top three principle components from the principal component analysis (PCA) for European ancestry SNPs [23,24]. The associations between SNPs and tumour response were evaluated using chi-square test. Exploratory subgroup analyses by gender, primary tumour location, and RAS mutational status were also performed. Interaction test for SNPs and gender, tumour location and RAS status was conducted using the same multivariable Cox proportional hazard models. All P-values were from two-sided Wald tests at a 0.05 significance level. All tests were calculated by using the SAS statistical package version 9.4.
Fig. 1. mBeva inhibits CRC growth in GEMMs. (a) Macroscopic representative image of (2 adjacent) vascularised distal CRCs in a resected colon from Apc<sup>min/−</sup> Cav1<sup>+/−</sup> mice. Original magnification 10×. tu = tumour, p = proximal, r = rectum. Scale bar = 5 mm. (b) Microscopic representative images of HE-stained FFPE colon tissue sections from control and mBeva-treated Apc<sup>min/−</sup> Cav1<sup>+/−</sup> mice. nc = normal colon, te = tumour epithelial region, ts = tumour stroma region, c = cyst/dilated gland. Original magnification 50×. Scale bar = 250 μm. (c) mBeva reduces incidence of CRCs. Apc<sup>min/−</sup> Cav1<sup>+/−</sup> mice were treated with mBeva (i.p., 10 mg/kg, once per week) for 4 weeks. Data are absolute case numbers (mice with or without tumours) (*p = .0411 mBeva vs. control, Fisher Exact test). (d) mBeva reduces multiplicity of CRCs. Data are presented as in panel c (number of tumours per mouse) (*p = .0157 mBeva vs. control, Cochran Armitage trend test). # = no cases recorded. (e) mBeva reduces colon tumour grade (G), i.e. improves histomorphology from “high-grade/dedifferentiated” to “low-grade/well-differentiated”. Data are absolute numbers [cases] from n ≥ 11 animals per group (*p = .0015 mBeva vs. control, Fisher Exact test). (f) mBeva reduces colon tumour ulcers/cysts. Data are absolute numbers [cases] from n ≥ 11 animals per group (*p = .0172 mBeva vs. control, Fisher Exact test). (g–h) mBeva has no effect on microvessel density (MVD). Immunohistochemistry (IHC) detecting CD31 in Apc<sup>min/−</sup> Cav1<sup>+/−</sup> CRC tissue sections: (g) Representative images. Original magnifications 100×, 200×. (h) Quantitative analysis of MVD by vessel size. Data are means [weighted counts of CD31+ vessels per mm<sup>2</sup>] ± S.E. from n ≥ 6 animals per group (large vessels: p = .0582 mBeva vs. control; t-test procedure). Detailed statistics for all preclinical parameters is presented in Supplementary Tables 4-6.
15.8 n = 25] (*p = .0123, Wilcoxon two-sample test). Treated animals had less sub-epithelial lymphoid follicles (excluding Peyer’s Patches) [0.3 ± 0.6 n = 11] in the intestine than controls [2.7 ± 3.5 n = 34] (*p = .0017, Wilcoxon two-sample test) and fewer adenomas in the small intestine (SI) (Supplementary Fig. 2).

To assess the ability of mBeva to attenuate neoangiogenesis, i.e. tumour vessel growth, immunohistochemistry (IHC) was conducted on FFPE-tissue sections of Apc<sup>min/+</sup> CRCs. We employed CD31 (Platelet/Endothelial Cell Adhesion Molecule-1, PECAM1) as a surrogate marker for in situ detection of microvessel density (MVD) [37]. However, morphometric quantification of MVD based on CD31+ staining positivity failed to uncover differences between mBeva-treated and untreated tumour-bearing mice (Fig. 1g-h). There was a trend towards reduction of large vessels following treatment (counts per mm²: 12.4 ± 7.9 mBeva n = 6 vs. 19.8 ± 6.9 control n = 12, p = .0582, t-test procedure) (Supplementary Table 6). Thus, neutralization of VEGFA in vivo prevented CRC growth in mice without a major impact on neoangiogenesis.

3.2. BMAL1 is associated with murine CRCs that were resistant to mBeva therapy

To gain further understanding as to why mBeva fails to reduce MVD in vivo, RT-qPCRs on total RNA extracted from intestinal organs were performed. We focussed on BMAL1 based on its recognised role in cancer therapy resistance [7,8]. Arntl (herein termed BMAL1) mRNA was up-regulated in small intestinal tissue from untreated Apc<sup>min/+</sup> (MIN) compared with untreated WT-type animals (MIN 2.0 ± 0.5 vs. WT 0.7 ± 0.1-fold, *p = .0043, Mann Whitney test, n = 6 per group) (Fig. 2a). Similar results were obtained for VEGFα, and a trend was stated for Cd31. Notably, mBeva did not attenuate BMAL1 mRNA (p = .0576, Kruskal Wallis test, n = 9 per group) in colon tissue from mBeva-treated vs. untreated tumour-bearing Apc<sup>min/+</sup> mice (Fig. 2b). Likewise, VEGFα and Cd31 remained elevated. These findings indicated that VEGFα neutralization despite attenuation of tumour growth fails to reduce vessel formation in vivo, presumably by up-regulation of alternate pathways, such as BMAL1.

To measure BMAL1 protein, IHC was performed on CRC tissue from treated tumour-bearing Apc<sup>min/+</sup> mice (Fig. 2c). Responder (R) vs. non-responder (NR) mice were defined by 2D tumour areas (NR > 6 mm² > R) and the proliferation marker Ki67. Notably, high BMAL1 staining was associated with preclinical non-response of individual mice to mBeva. Accordingly, BMAL1 protein expression was decreased in CRCs of R compared with NR animals (p = .0821 Fisher Exact test; *p = .0447 t-test), and R mice were characterised by smaller tumour size and lower Ki67+ (*p = .0695 Fisher Exact test; *p = .0170 t-test) proliferation index (n = 4 per group, R vs. NR). Collectively, these data proposed that failure of mice to respond to mBeva correlates with high BMAL1 expression.

3.3. Beva treatment correlates with BMAL1 protein expression in mouse CRC xenografts

To interrogate the association of BMAL1 expression with human VEGFA Ab (Beva) treatment in an independent animal model, CRC tissues from HCT116 xenografts were analysed. Tumour-bearing mice (n = 12 per group) were subjected to a 4-weeks treatment (i.p.) with vehicle (5% glucose w/v), Beva (10 mg/kg), FOLFOX (40 mg/kg 5-FU; 3.4 mg/kg oxaliplatin; 13.4 mg/kg folic acid) or FOLFOX followed by Beva 24 h later (as detailed in Materials & Methods). After 4 weeks, mice treated with FOLFOX in combination with Beva had tumours significantly smaller (*p < .001 FOLFOX + Beva vs. vehicle control, t-test) than mice with tumours treated with either monotherapy arms or vehicle (Fig. 2d). At the end of 4 weeks, mice were euthanised, and tumours were harvested. FFPE-tissue sections were stained with BMAL1 Ab by IHC (Fig. 2e). Histopathologic evaluation of positivity scores demonstrated that Beva not only failed to lower BMAL1 protein in xenografts but rather increased its expression (75 ± 5% Beva vs. 40 ± 7% vehicle control, n = 4 per group, *p = .0286, Mann Whitney test). BMAL1 protein was also detected in murine and patient (pos. control) tissue lysates subjected to Western blotting (Fig. 2f). Thus, resistance to mBeva was associated with high BMAL1 expression, both in GEMM and xenograft CRC mouse models.

3.4. Clinical non-response to Beva correlates with BMAL1 protein expression in CRC

To determine BMAL1 protein expression in CRC patients, IHC studies were first performed on tissue microarrays (TMAs) of tumour (TU, n = 40) and non-tumour/normal colon (NT, n = 8) specimens from drug-naive cases. BMAL1 staining (Supplementary Fig. 3) was reduced (by ~50% TU vs. NT) in tumour compared with normal colon tissue in both epithelial and stromal (lamina propria) cells. BMAL1 positivity decreased with increasing tumour aggressiveness [19], such as cellular de-differentiation (grade, G), growth into submucosal layers (size, T) and invasion of local lymph nodes (nodal status, N) (*p < .05 vs. NT, Kruskal Wallis test). Still, a major proportion of patients (33 of 40 / 83%) retained BMAL1 staining in tumour cells, which justified subsequent studies regarding its possible connection to therapy response.

Therefore, to assess whether BMAL1 could serve as a marker of Beva resistance in humans, a retrospective series of CRC tissue specimens (TU, n = 77) was analysed by IHC (Supplementary Tables 7–10) [17,18]. There was a significant association between low BMAL1 staining (scores 0/1) in tumour cells and stable disease (SD) in patients undergoing Beva therapy (Cochrane Armitage trend test *p = .0061; Fisher Exact test *p = .0130; n = 44) (Fig. 3a). Accordingly, high BMAL1 staining (scores 2/3) in the tumour correlated with progressive disease (PD) (Fisher Exact test p = .0087; n = 44) (Fig. 3b). Consistently, patients who had high BMAL1 staining in their tumour tissue were less likely to belong to the disease control rate group (DCR: PR/SD) responding to Beva (R vs. NR, Cochane Armitage trend test p = .0021; n = 63) (Fig. 3c).

Analysis of progression-free survival (PFS) data demonstrated that high BMAL1 protein expression in tumour cells correlated with poor clinical outcome [R (PR/SD) vs. NR (PD/Death); scores 0–2 vs. 3: Fisher Exact test *p = .0413; n = 77] (Fig. 3d). Data for individual patient cases from restaging examinations according to Response Evaluation Criteria In Solid Tumours (RECIST) [20] are shown in Supplementary Fig. 4. High BMAL1 positivity in tumour tissue was also associated with the likelihood to show a progress (PD) in the first restaging according to RECIST (Supplementary Fig. 5).

Overall, high BMAL1 protein expression in tumours was associated with reduced PFS (Fig. 3e). Kaplan–Meier patient survival curves by PFS in months (n = 74 cases) were plotted according to the first observed progress (PD) according to RECIST vs. BMAL1 staining scores: [0–2 vs. 3: p = .1667 log rank test, HR = 1.601 (95% CI 0.79–3.84); *p = .0399 Wilcoxon test]. Additional pair-wise comparisons are presented in [Supplementary Fig. 6]: [2 vs. 3: p = .1617 log rank test, HR = 1.687 (95% CI 0.79–4.18); *p = .0223 Wilcoxon test]. Collectively, these results indicate that a high BMAL1 expression in tumours correlates with a poor clinical outcome following Beva treatment.

Further analysis of an independent cohort of CRC patients using cBioPortal of Cancer Genomics™ [38] [Colonorectal Adenocarcinoma, TCGA, Provisional, n = 633] (Supplementary Table 11, Supplementary Fig. 7) underscored that alterations in the BMAL1 (ARNTL) gene, mainly amplifications, mutations and changes in mRNA transcription, confer poor prognosis. However, due to limited case numbers, significance was not reached.

3.5. Clinical non-response to Beva correlates with BMAL1 SNPs in CRC

Finally, the impact on the clinical outcome of four single nucleotide polymorphisms (SNPs) in the BMAL1 (ARNTL) gene was evaluated in
Fig. 2. Bmal1 detects murine CRCs resistant to mBeva therapy. (a) Bmal1/Arntl mRNA is up-regulated in tumour-bearing mice. Total RNA was extracted from frozen small intestinal (SI) tissue of wild-type (WT) and Apc min/+ (MIN) mice (n = 6 per group). CT-values from RT-qPCRs normalised to beta-2-microglobulin (B2m) are calculated as -fold ± S.E. (*p < .05, Mann Whitney test). (b) mBeva fails to reduce Bmal1 mRNA (p = .0576, Kruskal Wallis test) in tumour-bearing mice. Total RNA was extracted from colon tissue of untreated and mBeva-treated Apc min−/− mice (n = 9 per group). RT-qPCRs were analysed as in panel a. (c) Non-response to mBeva correlates with high Ki67 (p = .0095 Fisher Exact test; *p = .0170 t-test) and Bmal1 (p = .0621 Fisher Exact test; *p = .0447 t-test) protein expression in Apc min−/− CRCs. IHC staining positivity scores are from absolute animal numbers [cases] (n = 4 per group, NR vs. R). NR = non-responder; R = responder; # = no case recorded. Quantitative analyses (left) and representative images (right, original magnification 100×). (d) Beva and FOLFOX in combination inhibit tumour growth in BALB/c nu/nu mouse xenograft CRCs compared to monotherapy. Animals were injected (s.c.) with HCT116 cells, and, after 37 days, treated once per week (i.p) with vehicle (CON.), Beva (10 mg/kg) or FOLFOX (40 mg/kg 5-FU, 3.4 mg/kg oxaliplatin, 13.4 mg/kg folinic acid) or in combination (Beva given 24 h later than FOLFOX) for 4 weeks. Tumour volumes [in mm3] are means ± S.E. (n = 12 per group, *p < .001 FOLFOX + Beva vs. vehicle control, t-test). (e) Beva fails to reduce Bmal1 protein in mouse xenografts. IHC staining frequency from CRC xenografts (in panel d) is calculated in % ± S.E. (n = 4 per group, *p = .0286 Beva vs. vehicle control, Mann Whitney test). Quantitative analyses (top) and representative images (bottom, original magnifications 25×, 100×). (f) Detection of Bmal1 protein in tissue lysates from murine or human normal colon (NC) and CRC (TU). Representative Western blots.
mCRC patients (TU, n = 215) treated with first-line FOLFIRI plus Beva in the randomised phase III TRIBE trial [21,22]. Baseline patient and tumour characteristics are listed in Supplementary Table 12. Three of the SNPs were in introns, while rs2279287 located to the upstream (promoter) regulatory region of the gene (Supplementary Table 2). Among the evaluated SNPs, rs11022780 showed a significant association with overall survival (OS) (Fig. 4a). Patients with any C allele had a shorter median OS than those harboring T/T allele (25.0 vs. 32.9 months) both in univariate (HR = 1.63, 95% CI 1.14–2.32, *p = .006) and multivariable (HR = 1.61, 95% CI 1.10–2.32, *p = .014) analysis. This effect was significant in the subgroup analysis for tumour location (Fig. 4a) and RAS mutational status (Supplementary Fig. 8a). Notably, rs2279287, rs7938307 and rs7396943 SNPs revealed significant associations with a shorter PFS in subgroups by gender, tumour location or RAS mutational status (Fig. 4b, Supplementary Fig. 8b–c). Results from interaction tests for SNPs and gender, tumour location and RAS status are summarised in Supplementary Table 13. The association of SNPs with outcome in the overall patient cohort is presented in Supplementary Table 14, while significant results of subgroup analyses are reported in Supplementary Table 15.

In summary, BMAL1 (ARNTL) gene alteration(s) and high protein expression correlated with unfavourable clinical outcomes in CRC patients treated with Beva. Overall, BMAL1 seems to act as a “bad guy” conferring resistance to Beva, both in mice and humans.
Fig. 4. Clinical non-response to Beva correlates with BMAL1 SNPs in human CRC. SNPs in the BMAL1 (ARNTL) gene correlate with poor prognosis and clinical response in CRC patients of the phase III TRIBE cohort [21,22]. Significant results of adjusted Kaplan-Meier plots by months are shown. Events/total, HR, median and Wald P-value were based on the multivariable Cox proportional hazards regression model as detailed in Materials & Methods. Complete statistics is presented in Supplementary Tables 12–15. (a) SNP rs11022780 is associated with shortened OS in the total population and upon subgroup analysis for tumour location. (b) SNPs rs2279287 and rs7938307 are associated with shortened PFS upon subgroup analysis for gender and tumour location.
3.6. REVERBA binds to a RORE-like element in the human VEGFA promoter

To decipher the molecular mechanism, underlying the observed in vivo resistance to Beva in presence of BMAL1, two human CRC cell lines (SW480, HCT116) with activating mutations in Wnt/Ras signalling pathway components (such as in APC and KRAS) were chosen as in vitro models for the APC min-/+ GEMM and the majority of human CRCs (CMS2/3) [29].

BMAL1 is an activator of REVERBA [7,8] and VEGFA [6,39] gene transcription. We therefore hypothesised that the two transcription factors may be mechanistically connected in mediating Beva resistance. To this end, we first performed an in silico search using AiliBaba2.1 for RORE-like motifs in the proximal (~2 kb) promoter of the human VEGFA gene. Two putative RORE sites (−1464 bp; −672 bp) were identified in proximity to a cognate BMAL1 binding site (E-box) (−1688 bp) [6,39] and estrogen receptor-responsive element (ERE) (−1542 bp) [40] (Fig. 5a, Supplementary Fig. 9). To test for DNA-binding, we amplified the predicted ROREs by PCR upon chromatin immunoprecipitation (ChIP) of the endogenous human VEGFA promoter. Cells (SW480, HCT116) were transfected with empty vector (EV) or REVERBA expression plasmid for 48 h, followed by ChIP. REVERBA Ab pulled-down DNA harboring the −672 bp RORE (Fig. 5b) (~2-fold; *p < 0.05 vs. no Ab control, Mann Whitney test, n = 4–5 per cell line), but not the −1464 bp RORE. Nuclear extracts from SW480 cells transfected with EV or REVERBA were incubated with biotin-labelled oligonucleotides containing the predicted ROREs. Electrophoretic mobility shift assay (EMSA) (Fig. 5c) confirmed binding of ectopic REVERBA to wild-type −672 bp RORE (~10-fold; *p < 0.05 vs. EV, Two-way ANOVA, n ≥ 3 per cell line), but not to the mutant probe.

The upstream region of the human VEGFA gene, covering ~2 kb until the start of the protein coding sequence, was then inserted into pGL3 luciferase reporter plasmid and transfected into SW480 cells for 48 h (Fig. 5d). REVERBA overexpression increased luciferase activity driven by the −2 kb VEGFA promoter (3.6 ± 0.3 vs. 1.1 ± 0.1 *p < 0.05 -fold vs. EV, Mann Whitney test, n = 3 per cell line). Similar results were obtained from HCT116 and HEK293T (as non-cancer control) cells (Supplementary Fig. 10). These data indicated that REVERBA binds and transactivates the human VEGFA promoter.

3.7. REVERBA cooperates with BMAL1 to increase VEGFA synthesis

To elucidate whether DNA-binding translates into expression of the endogenous VEGFA gene, SW480 cells were transfected with EV or REVERBA for 48 h, followed by RNA extraction and RT-qPCR analysis. Notably, REVERBA overexpression increased VEGFA mRNA (by 70%; *p < 0.05 vs. EV, One-sample t-test, n = 3) (Fig. 5e). ELISA confirmed augmented VEGFA secretion into the extracellular milieu upon overexpression of REVERBA (SW480: 82 ± 3 vs. 54 ± 5 pg/ml; *p < 0.05 vs. EV, Paired t-test; n = 3) (Fig. 5f). The positive effect of REVERBA on endogenous VEGFA expression was most prominent in SW480 cells, possibly due to already high basal VEGFA levels in HCT116 (555 ± 47 vs. 519 ± 13 pg/ml) and HEK293T (305 ± 23 vs. 165 ± 10 pg/ml) cells (Supplementary Fig. 10).

To interrogate the role of REVERBA in the regulation of VEGFA synthesis, we selected BMAL1 which, similar to MYC [12], binds to E-boxes adjacent to the predicted RORE [40] and up-regulates VEGFA transcription [7,8]. Indeed, BMAL1 overexpression in SW480 cells increased VEGFA promoter activity (Fig. 5g), VEGFA mRNA (Fig. 5h) and VEGFA protein (Fig. 5i). BMAL1 also augmented VEGFA promoter activity in combination with REVERBA (Fig. 6a). Similar results were obtained from HCT116 and HEK293T cells (Supplementary Fig. 11). Thus, REVERBA seems to cooperate with BMAL1.

3.8. REVERBA and BMAL1 confer mBeva resistance in human CRC cells

VEGFA stimulates its own expression via VEGFA receptor-2 (VEGFR2) in a para- and autocrine fashion [40,41]. To test if neutralization of VEGFA by mBeva interrupts this positive amplification loop, cells (SW480, HCT116, HEK293T) were transfected with EV, REVERBA or BMAL1 together with −2 kb VEGFA promoter reporter plasmid for 24 h, followed by 48 h incubation in starvation (SM) or conditioned (CM) medium in absence or presence of mBeva (10 μg/ml) (Fig. 6b). Media preparation is described in Materials & Methods. Baseline luciferase activity was measured upon cultivation in SM and compared to the one achieved by CM, which, due to its cocktail of secreted growth factors (including VEGFA), served as a stimulant for the signalling response tested. REVERBA and BMAL1 increased the reporter activity under all conditions, whereas mBeva was unable to dampen this response (*p < 0.05 vs. EV, Two-way ANOVA, n = 3 per cell line).

To gain insight into related oncogenic pathways in addition to VEGFA, cells (SW480, HCT116) were transfected with REVERBA or BMAL1 together with reporter plasmids (Supplementary Fig. 12) detecting hypoxia, Ras or Wnt signalling. After 24 h, cells were serum-deprived (in SM) or stimulated (in CM) with or without mBeva (10 μg/ml for additional 48 h (as described for Fig. 6b). REVERBA and BMAL1 increased hypoxia- and Wnt-driven luciferase activity, while Ras-signalling was preferentially augmented by REVERBA (*p < 0.05, Two-way ANOVA, pair-wise subgroup analysis by Bonferroni post-tests as indicated by asterisks, n = 3 per plasmid and cell line). Again, mBeva did not significantly abrogate responses elicited by the two transcription factors in CM-stimulated cells as compared to the baseline of SM-exposed cells.

3.9. REVERBA inhibition, but not mBeva reduces proliferation of human CRC cells

The data above indicates that interference with REVERBA-BMAL1 signalling may be superior to VEGFA neutralization. To test this hypothesis, colorimetric MTT assays were performed to examine cell proliferation. SW480 cells were transfected with EV or REVERBA and cultivated for 1–7 days. REVERBA overexpression accelerated cell growth (~2-fold; *p < 0.05 vs. EV, Two-way ANOVA, n = 3) (Fig. 7a), whereas REVERBA knock-down by siRNA reduced proliferation (Fig. 7b). When cells were incubated in presence of hemin (30 μM), a physiological modulator of REVERB8s and other heme-binding proteins [31], growth was also diminished (by 80%; *p < 0.05 vs. vehicle, Two-way ANOVA, n = 3) (Fig. 7c). In contrast, mBeva did not alter proliferation rates (Fig. 7d). Likewise, BMAL1 overexpression or knock-down per se remained ineffective (not shown). Thus, targeting REVERBA, a feedback regulator of BMAL1, may have a greater effect on tumour cell growth compared with VEGFA neutralization.

Taken together, these results suggest that REVERBA, indirectly, e.g. via cooperation with BMAL1, facilitates a feed-forward amplification loop [7,8], which culminates in augmented VEGFA synthesis (as depicted in Fig. 8). This sequence of events may underpin a vicious cycle insensitive to mBeva, but instead susceptible to REVERBA-BMAL1 inhibition.

4. Discussion

In this work, we provide evidence that BMAL1 is a functional modulator and potential biomarker of resistance to anti-angiogenic therapy in CRC. This conclusion was elaborated using a translational approach combining preclinical, clinical and cellular mechanistic studies.

Consistent with other reports on VEGFA-pathway inhibition [42,43], Beva reduced CRC growth in two independent mouse models which were driven by active Wnt/Ras-signalling and, thereby, resemble the most common consensus molecular subtypes (CMS2/3) of human CRC [29]. However, instead of reducing vessel density, stromal reactions prevailed (e.g. desmoplasia, fibrosis) and bowel inflammation was mitigated. Uncoupling of tumour growth from angiogenesis may be caused by differential sensitivity towards or dependency on VEGFA, as a growth and survival factor in a given cell type [42,43]. Thus, beyond tumour and...
endothelial cells, other components of the tumour microenvironment, e.g. fibroblasts, macrophages or pericytes, may regulate VEGF signalling and secretion, and, thereby, contribute to the efficacy of Beva on CRC growth in vivo.

Previously recognised phenotypes in GEMMs confirm the co-existence of systemic cardio-vascular vs. local epithelial effects of BMAL1-dependent target genes (including VEGFA) [7,8]: As such, BMAL1 controls proliferation and migration of mesenchymal, neuronal,
epidermal and intestinal stem cells, thereby governs initiation and progression of tumours [44,45]. BMAL1 is also required for growth and survival of bone marrow and leukemic stem cells [46]. We were unable to detect major signs of necrosis or apoptosis in Beva-treated tumours. Instead, Ki67 data indicates that reduced proliferation of cancer (stem) cells is one mechanism whereby neutralization of VEGFA inhibits tumour growth in mice, and this process may be counteracted by a gain of function in BMAL1.

In both CRC mouse models, Beva failed to attenuate or even further up-regulated BMAL1 expression during treatment. Thus, high BMAL1 expression in murine CRCs may be a drug-mediated passenger effect, but not necessarily the causative driver of resistance to Beva. Experimental studies in Bmal1 transgenic or knock-out mice are required to further address this issue.

Our preclinical studies were confirmed by molecular and genomic analyses which revealed that SNPs in the BMAL1 (ARNTL) gene and high BMAL1 expression in tumour cells correlated with unfavourable clinical outcome in CRC patients that underwent combination chemotherapy with Beva. Compensatory up-regulation of alternative pro-angiogenic ligands and/or receptors in response to treatment have been postulated [2,47]. For example, additional VEGFA isoforms as well as VEGFB/C/D or placental growth factor allow by-passing of VEGFA-VEGFR2 signalling. Likewise, Notch signalling via delta-like ligand-4 evokes resistance by increasing VEGFR1 expression in the tumour-adjacent stroma [2]. Consistently, our data propose that resistance to Beva may be caused by intrinsic, intracellular "escape" pathways in tumour (stem) cells involving BMAL1 (and presumably other transcription factors) which lead to continuous VEGFA synthesis and secretion followed by persistent auto/paracrine stimulation of membrane-bound VEGFA-VEGFR2 signalling. Regarding the possible survival effects, one may speculate whether local gain of BMAL1-driven VEGFA production in tumour cells translates to other
VEGFA-sensitive stroma cell types in the microenvironment, including endothelial cells, and/or even spreads systemically via the vasculature.

Despite paying attention to exact daily timing of injections and endpoints in our preclinical studies, we cannot exclude circadian variations due to different sampling times of human tissues during routine surgery in the hospital. Future synchronised studies are warranted to address this limitation. In this context, the biological significances of the SNPs in the BMAL1 (ARNTL) gene in CRC remain to be defined as well. Clinical associations exist for sleep-wake disturbance in Alzheimer’s disease [48], bipolar disorder and seasonal depression [49] and metabolism associations exist for sleep-wake disturbance in Alzheimer’s disease [48], bipolar disorder and seasonal depression [49] and metabolism.

In addition, REVERBA and BMAL1 (presumably together with non-clock transcription factors [9]) augmented transcriptional responses to major oncogenic drivers of human CRC (such as Wnt and Ras) [29]. Hence, BMAL1-dependent regulation of DNA-repair mechanisms seems to be one common underlying mechanism how resistance to (radio)chemotherapy is established. So far, functional connections of BMAL1 with clinical response against targeted therapies (e.g., cetuximab in CRC) have not been described. Future studies are required to integrate experimental findings in mice and patients, these molecular data suggest a positive amplification loop where VEGF Ab fails to disrupt REVERBA/BMAL1-driven VEGFA-VEGFR2 signalling in tumour cells. This failure could be caused by an overabundance of secreted VEGF which simply overwhelms the capacity of Beva to neutralize this growth factor produced by the intracellular transcription machinery (Fig. 8). In addition, REVERBA and BMAL1 (presumably together with non-clock transcription factors [9]) augmented transcriptional responses to major oncogenic drivers of human CRC (such as Wnt and Ras) [29].

These cooperative mode of action integrates well into previous evidence on BMAL1 regarding chemotherapy responsiveness: BMAL1 forms heterodimers with CLOCK or NPAS2 (and non-clock factors [9]) to regulate genes involved in DNA-repair, cell cycle checkpoints (p53 e.a.), cytoskeleton reorganization and drug metabolism. Many chemotherapeutics evoke DNA-damage (e.g. bulky adducts, double strand breaks). As such, BMAL1 protects against toxicity of cyclophosphamide [56], cisplatin and doxorubicin [57], while it sensitises to paclitaxel [58], gemcitabine [59] and doxorubicin [60] in cells, mice or patients. Further, BMAL1 cooperates with p53 to protect cells from UV stress-induced DNA-damage [61]. Clock-related gene expression also predicts clinical response to neoadjuvant chemoradiation in rectal cancer patients [62]. Hence, BMAL1-dependent regulation of DNA-repair mechanisms seems to be one common underlying mechanism how resistance vs. sensitivity to (radio)chemotherapy is established. So far, functional connections of BMAL1 with clinical response against targeted therapies (e.g., cetuximab in CRC) have not been described. Future studies are required to integrate experimental findings in mice and patients, these molecular data suggest a positive amplification loop where VEGF Ab fails to disrupt REVERBA/BMAL1-driven VEGFA-VEGFR2 signalling in tumour cells. This failure could be caused by an overabundance of secreted VEGF which simply overwhelms the capacity of Beva to neutralize this growth factor produced by the intracellular transcription machinery (Fig. 8). In addition, REVERBA and BMAL1 (presumably together with non-clock transcription factors [9]) augmented transcriptional responses to major oncogenic drivers of human CRC (such as Wnt and Ras) [29].

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to elucidate whether the impact of BMAL1 [57] on therapy outcome is specific for a given intervention modality or tumour context.

Pharmacological re-entrainment of the circadian clock in cancer cells improves tumour control, e.g. by seliciclib in mice [63] or cortisol in patients [64]. Likewise, cancer chronotherapy, the daily timing of chemotherapeutics administration (such as irinotecan) determines tolerability and clinical outcome [65]. In mice, REVERBA and BMAL1 were the best discriminators for chronotoxicity [66]. BMAL1, per se or via REVERBA, may be therefore proposed as a potential druggable target for preventing resistance to Beva in human CRC. CRY stabilisers have been developped, e.g. KL001, which inhibit the activity of BMAL1 [67]. REVERBA-agonists (SR9009/SR9011) exert strong cytotoxicity and facilitate oncogene-induced senescence in cell and mouse cancer models [68,69]. REVERBA-antagonist ARNS187 inhibits autophagy and reduces viability in breast cancer cells [70]. Consistently, we found that proliferation of CRC cell lines was attenuated by REVERBA siRNA and hemin, a physiological modulator of heme-binding proteins beyond REVERBs [31]. Thus, pharmacological intervention at the interlocked feedback loops of circadian rhythm transcription factors may represent an attractive approach to target cancer.

In conclusion, our data demonstrate that BMAL1 is frequently expressed in tumour tissues from CRC patients refractory to anti-VEGFA therapy with Beva. These data therefore support the monitoring of CRC patients for these markers, at the protein or genomic (SNP) level, to assess their risk for clinical non-response [18]. If combined with efficient screening procedures, biomarker-guided assessment may improve detection of early resistance and may prevent therapy failure.

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Author contributions
All authors contributed and critically reviewed and approved the manuscript. EB and ME defined the research theme. EB, FB, FE, FH, ISM and WW designed the methods and carried out the experiments. ATB, EB, FB, FE, FH, HJL, ISM and VWW analysed the data and interpreted the results. CW, FB and HJL performed statistical calculations. AM, CAW, JK and TG conducted and analysed immunostainings. ATB, EB, FB and ME drafted the manuscript. ATB, FL, HJL, JB, ME, NH, NS and RH provided samples and/or collected clinical data.

Writing assistance
None.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.07.004.

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