

Cannabidiol converts NF- κ B into a tumor suppressor in glioblastoma with defined antioxidative properties

Marie N. M. Volmar, Jiyong Cheng, Haitham Alenezi, Sven Richter, Alisha Haug, Zonera Hassan, Maria Goldberg, Yuping Li, Mengzhuo Hou, Christel Herold-Mende, Cecile L. Maire, Katrin Lamszus, Charlotte Flüh, Janka Held-Feindt, Gaetano Gargiulo, Geoffrey J. Topping, Franz Schilling, Dieter Saur, Günter Schneider, Michael Synowitz, Joel A. Schick, Roland E. Kälin,[†] and Rainer Glass[†]

Neurosurgical Research, Department of Neurosurgery, University Hospital, LMU Munich, Munich, Germany (M.N.M.V., J.C., H.A., S.R., A.H., M.G., Y.L., M.H., R.E.K., R.G.); Department of Medicine II, Klinikum rechts der Isar, Technische Universität München, Munich, Germany (Z.H., D.S., G.S.); Department of Neurosurgery, Division of Experimental Neurosurgery, Heidelberg University Hospital, Heidelberg, Germany (C.H.M.); Department of Neurosurgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany (C.L.M., K.L.); Department of Neurosurgery, University Hospital Center Schleswig Holstein, Kiel, Germany (C.F., J.H.-F., M.S.); Molecular Oncology, Max Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany (G.G.); Department of Nuclear Medicine, School of Medicine, Technical University of Munich, Munich, Germany (G.J.T., F.S.); Genetics and Cellular Engineering Group, Institute of Molecular Toxicology and Pharmacology, Helmholtz Zentrum Munich, Neuherberg, Germany (J.A.S.); Walter Brendel Center of Experimental Medicine, Faculty of Medicine, LMU Munich, Munich, Germany (R.G.); German Cancer Consortium (DKTK), Partner Site Munich and German Cancer Research Center (DKFZ), Heidelberg, Germany (R.G.)

[†]These authors contributed equally to this work.

Corresponding Author: Rainer Glass, PhD, Neurosurgical Research, Department of Neurosurgery, University Hospital, LMU Munich, Marchioninistr. 15, D81377 Munich, Germany (rainer.glass@med.uni-muenchen.de).

Abstract

Background. The transcription factor NF- κ B drives neoplastic progression of many cancers including primary brain tumors (glioblastoma [GBM]). Precise therapeutic modulation of NF- κ B activity can suppress central oncogenic signaling pathways in GBM, but clinically applicable compounds to achieve this goal have remained elusive.

Methods. In a pharmacogenomics study with a panel of transgenic glioma cells, we observed that NF- κ B can be converted into a tumor suppressor by the non-psychotropic cannabinoid cannabidiol (CBD). Subsequently, we investigated the anti-tumor effects of CBD, which is used as an anticonvulsive drug (Epidiolex) in pediatric neurology, in a larger set of human primary GBM stem-like cells (hGSC). For this study, we performed pharmacological assays, gene expression profiling, biochemical, and cell-biological experiments. We validated our findings using orthotopic in vivo models and bioinformatics analysis of human GBM datasets.

Results. We found that CBD promotes DNA binding of the NF- κ B subunit RELA and simultaneously prevents RELA phosphorylation on serine-311, a key residue that permits genetic transactivation. Strikingly, sustained DNA binding by RELA-lacking phospho-serine 311 was found to mediate hGSC cytotoxicity. Widespread sensitivity to CBD was observed in a cohort of hGSC defined by low levels of reactive oxygen species (ROS), while high ROS content in other tumors blocked CBD-induced hGSC death. Consequently, ROS levels served as a predictive biomarker for CBD-sensitive tumors.

Conclusions. This evidence demonstrates how a clinically approved drug can convert NF- κ B into a tumor suppressor and suggests a promising repurposing option for GBM therapy.

Key Points

1. Cytoplasmic sequestration of NF- κ B for therapeutic approaches has clinically failed.
2. Cannabidiol (CBD) kills GBM cells by inducing DNA binding of a modified NF- κ B subunit.
3. A predictive biomarker for CBD-sensitive GBM is highlighted.

Importance of the Study

Cannabinoids act as paracrine tumor suppressors in the brain, and exogenous cannabinoids may serve as brain tumor therapeutics. Non-psychotropic cannabinoids like cannabidiol (CBD) are well tolerated, penetrate the blood-brain barrier, and have anticonvulsive effects (Epidiolex). However, previously, it was not explored which cannabinoid compound would be therapeutically most efficacious for brain tumor treatment. Also, a central mode for drug action in glioblastoma (GBM) as well as GBM subset-specific therapeutic effects of cannabinoids remained to be determined. Here, we identify

CBD as the most potent GBM therapeutic. Remarkably, CBD promotes DNA binding and simultaneously prevents a posttranslational modification of the NF- κ B subunit RELA, which results in GBM death. GBM have striking differences in canonical NF- κ B signaling and redox levels, which co-control CBD-activated signaling pathways and serve as predictive biomarkers for CBD-sensitive tumors. This provides a practical basis for clinical testing of CBD as GBM treatment and indicates an entirely new way to tackle the tumor-supporting function of NF- κ B.

Transcription factors of the nuclear factor kappa B (NF- κ B) family have gained large attention as therapeutic targets for neoplastic disease.¹⁻⁴ In cancer cells, NF- κ B integrates a range of environmental stimuli and promotes tumor progression by sustaining cell viability, inducing invasiveness, and regulating metabolic adaptation.^{1,5,6} NF- κ B, a heterodimeric molecule of RELA (p65) and p50 subunits, is also a central signaling hub for inflammation and coordinates responses to cytokines² like tumor necrosis factor- α (TNF α). In this canonical pathway, NF- κ B is released from inhibitory chaperones in the cytoplasm and enters the nucleus in order to regulate larger numbers of genes.¹ Previously, pharmacological attempts for anti-inflammation aimed at cytoplasmic sequestration of NF- κ B, with limited clinical success.¹ It is becoming increasingly clear that control of posttranslational modification and subsequent fine-tuning of NF- κ B-initiated transcription holds potential for clinical translation.¹

Glioblastomas (GBM) are characterized by extreme invasiveness and heterogeneity, which complicates therapy.^{7,8} Hence, new GBM therapeutics with good blood-brain barrier (BBB) penetrance, which are able to reach tumor cells that escaped neurosurgical resection or irradiation, are in urgent demand.⁷⁻⁹ Concurrently, such compounds require predictive markers in order to address the needs of individual patients.^{8,10} Central aspects of the malignant phenotype of GBM, like treatment resistance, rapid proliferation, and infiltrative growth, as well as the induction of stem-like traits in GBM, have been linked with NF- κ B signaling.^{4,11-14} Hence, in GBM, NF- κ B represents a potentially efficient (but hitherto largely inaccessible) therapeutic target.^{11,12,15}

Neural precursor cells (NPCs) release endocannabinoids/vanilloids inducing brain tumor cell death,^{16,17} and this endogenous tumor suppressor mechanism may be recapitulated with the phytocannabinoid cannabidiol (CBD). This non-psychotropic substance is used as an anticonvulsive drug, named Epidiolex,¹⁸ and has established anti-tumor functions.^{19,20} However, currently it is not clear if CBD is the most efficacious phytocannabinoid for neuro-oncological treatment. Furthermore, it is not resolved if cannabinoids target all primary brain tumors or only distinct subsets of GBM. Finally, central aspects of the mechanism of anti-tumorigenic action of cannabinoids are unknown.

Our assays indicate that cannabinoid sensitivity of GBM cells is subject to inter-individual heterogeneity. From a panel of phytocannabinoids, CBD was evaluated as therapeutically most potent. Pharmacogenomics determined how CBD modulates NF- κ B signaling to initiate tumor cell death. The redox dependency of this pathway is shown, and ROS levels of GBM are introduced as a predictive biomarker for CBD-sensitive tumors.

Materials and Methods

Cell Culture

Biopsies from human primary and recurrent hGSC were obtained from planned resections (ethics licenses EA112/2001, EA3/023/06, EA2/101/08, and D-562-15).

Genetic Characterization of hGSC

Genomic DNA (gDNA) and total RNA were isolated in parallel; DNA libraries were prepared using the TruSeq Custom Amplicon Low Input Kit (Illumina Inc.); amplicon-based enrichment panel (TruSeq Custom Amplicon Low Input) and genome-wide copy number variation (CNV) profiles were analyzed.

Transgenic Mouse Models and In Vivo Studies

Mice were bred and raised at the animal facilities of the LMU and TUM according to the German law on animal welfare and approved by the "Regierung von Oberbayern" in Munich, Germany. ARRIVE guidelines were followed for all animal experiments.

For in vivo CBD application and Kaplan-Meier read-out, tumor growth was verified by histological inspection of random samples and subsequently mice were i.p. injected (every other day, for 21 days) with CBD (15 mg/kg) or vehicle (5% Tween 80, 5% ethanol in 0.9% saline).

CRISPR/Cas9 Screening

Mouse glioma cells were infected with the genome-wide gRNA lentiviral library²¹ at a multiplicity of infection (MOI) of 0.3. Purified libraries of treated/control samples were sequenced on Illumina HiSeq2500 by 50-bp single-end sequencing.²²

Transcriptomics

Sequencing data can be found at the EMBL-EBI ArrayExpress Archive (<https://www.ebi.ac.uk/arrayexpress/>) under accessions: E-MTAB-9341, MTAB-9343, and E-MTAB-9353.

Statistical Analysis

Data distribution was presented by mean values and standard deviation of the mean; numbers of independent experiments or individual animals were indicated in the figures, legends, or in the manuscript text. Student's *t* test, 1-way/2-way ANOVA with Tukey post hoc test, or ANOVA with Bonferroni correction were used as indicated; in survival experiments, Kaplan-Meier curves were used and log-rank (Mantel-Cox) test was applied to determine statistical significance; primary endpoint was development of neurological symptoms clearly indicative of hGSC. *P* values are indicated as **P* < .05, ***P* < .005, and ****P* < .0005 in all results.

Results

Pharmacogenomics Uncover NF- κ B (RELA) Tumor Suppressor Functions

We set-up a panel of transgenic mouse brain tumors cells (tmBTC) harboring genetic defects recapitulating

driver-mutations of human GBM.^{23,24} These tmBTC allowed to identify therapeutic responses in a range of cells modeling the inter-individual heterogeneity of GBM.⁷ First, we quantified cell death in tmBTC after exposure to NPC-conditioned media (containing tumor-cytotoxic endocannabinoids) and found that endocannabinoids suppress distinct tmBTC subsets (Supplementary Figure 1A).

Next, we screened a panel of phytocannabinoids for anti-tumor effects and obtained the best results with CBD (Supplementary Figure 1B, C). Then we evaluated CBD-induced cytotoxicity in tmBTC (as compared to controls; Figure 1A). Coherent with our data from assays with endocannabinoids (Supplementary Figure 1A), we found that the phytocannabinoid CBD (Figure 1A) was cytotoxic for a range of tmBTCs. In order to decipher the signaling pathway controlling CBD sensitivity, we used pharmacogenomics screens with CBD-sensitive, *Tp53*-mutant tmBTC (*Tp53*^{R172H}, *PDGFB*; Figure 1B). In this experimental paradigm, genes are functionally inactivated by introducing one disruptive mutation per cell (with an overrepresentation of 100 cells per mutation). Vehicle-treated CAS9-expressing *Tp53*^{R172H}, *PDGFB* cells transduced with the CRISPR-library generated random mutations, but lacked genetic-enrichment in absence of any selective pressure (control). However, when library-transduced *Tp53*^{R172H}, *PDGFB* cells were exposed to CBD, mutations preventing cell death represented a selective advantage. Sequencing of gDNA identified the enriched genes (more than 10,000-fold enriched; skyscraper plot in Figure 1B; and Supplementary Table 1).

The pharmacogenomics screen (Figure 1B; Supplementary Figure 2A–C) suggested RELA and selenoprotein-controlled homeostasis of reactive oxygen species (ROS; schematic overview in Figure 1C) as central signaling cues for CBD-induced cell death. A functional role for RELA was corroborated after deletion of *Rela* in *Tp53*^{R172H}, *PDGFB* cells (Figure 1D; of note *Rela* knockout, *Rela*^{KO}, did not affect tmBTC expansion in vitro, *P* = .7451). In summary, our experiments showed that endocannabinoids or CBD induced cell death in a range of genetically distinct tmBTCs, that CBD had the best tumor cytotoxic activity in a panel of non-psychotropic phytocannabinoids and that RELA is an essential mediator of CBD-induced therapeutic effects.

Anti-Tumorigenic Action of RELA-Lacking Phosphorylation on Serine-311

Since the transcription factor RELA was essential for induction of CBD-mediated cytotoxicity, we examined differential gene regulation in this cell death pathway. To this end, we compared transcriptomic data of *Tp53*^{R172H}, *PDGFB*, *Rela*^{KO}, *Tp53*^{R172H}, *PDGFB*, and *Rela*^{WT} cells under conditions of CBD or vehicle treatment (Figure 1E). In agreement with previous reports,^{11,12,15} we observed that RELA expression promoted tumor viability ($P_{\text{adj.}} = 8.7 \times 10^{-9}$ in gene ontology analysis). CBD exposure vs vehicle controls (in *Tp53*^{R172H}, *PDGFB*, *Rela*^{WT} cells) or RELA expression (*Tp53*^{R172H}, *PDGFB*, *Rela*^{KO} vs *Tp53*^{R172H}, *PDGFB*, *Rela*^{WT} cells) controlled a larger number (397) of identical genes. Some of these (114) remained to be differentially regulated after CBD application when RELA was absent. We subtracted these 114 from the set of 397 genes, which

then uncovered a set of 283 genes that were concordant for RELA or CBD pathways. Hence, a large portion of the CBD-modulated genes (283/788) related to the RELA pathway (Figure 1E). These were analyzed further and a representative functional annotation was obtained for the fraction of downregulated genes ($P_{\text{adj.}} = 1.8 \times 10^{-7}$) showing that CBD-triggered RELA signaling attenuates levels of key glycolytic molecules important for tumor growth,^{25–28} while upregulated genes did not cluster for any specific biological process. All in all, transcriptomics highlighted that pathologically relevant genes of the RELA pathway were repressed after CBD application.

We used our transcriptomics dataset to identify molecules responsible for the CBD-induced repression of RELA pathways. Thereby we uncovered that CBD application downregulated the atypical protein kinase C ζ (PKC ζ), as confirmed by quantitative PCR (Supplementary Figure 2D). PKC ζ phosphorylates RELA on serine-311 which is required for transactivation^{29,30} (Supplementary Figure 2E), while phosphorylation on this residue does not modulate nuclear localization or DNA binding of RELA.²⁹ Subsequently, we investigated the role of RELA Ser311 phosphorylation in the CBD-initiated tumor cell death pathway. To this end, we expressed wild-type *Rela* or *Rela*^{S311A} (which cannot be phosphorylated) in *Tp53*^{R172H}, *PDGFB*, *Rela*^{KO} cells, applied CBD (vs controls), and quantified cell viability (time resolution of rapid cell death was best in viability assays). We hypothesized that *Rela*^{S311A} may accelerate CBD-induced tmBTC death as compared to *Rela*^{WT} controls: *Rela*^{WT} cells require (time-consuming) downregulation of PKC ζ for CBD-induced cytotoxicity, while this step is obsolete in *Rela*^{S311A} tmBTCs (hence, cell death is faster in *Rela*^{S311A} tmBTCs; see also cartoon in Figure 1E). This was verified in a viability assay (Figure 1F); note that *Rela*^{KO} prevented cell death (as expected). Altogether, several independent lines of evidence showed that CBD signals in the RELA pathway. A new and original observation from our assays is that CBD triggers a RELA-isoform lacking phosphorylation on serine-311, which affects tumor-maintaining genes in GBM (as indicated by our transcriptomics experiment) and compromises tumor cell viability.

CBD Is a Promising Therapeutic for Human GBM

The panel of tmBTC was instrumental for the pharmacogenomics studies avoiding limitations associated with the analysis of heterogeneous human samples³¹ and for *Rela* knockout experiments. Next, we compared results from CBD treatment of tmBTC with data from human primary GBM. Therefore, we generated a broad panel of GBM cells including cells with confirmed stem-like properties (hGSC; Supplementary Table 2) and quantified the

anti-tumor effects of CBD in cytotoxicity assays. CBD treatment efficiently triggered cell death in a larger set of hGSC (Figure 2A), while some hGSC remained CBD-insensitive. Quantification of viable cells after CBD treatment was consistent with cytotoxicity measurements identifying drug-sensitive or -insensitive hGSC and short (6 hours) CBD application, in agreement with CBD-pharmacokinetics,³² was sufficient to trigger pronounced hGSC death (Supplementary Figure 3A, B). Strikingly, in CBD-sensitive hGSC, CBD was superior to the chemotherapeutic temozolomide in an established concentration range and time course³³ in vitro (Figure 2B). CBD-sensitive tumors were also identified after very short passaging in vitro (Supplementary Figure 3C, Supplementary Table 3). Cell death in hGSC was executed by apoptosis or endoplasmic reticulum stress and autophagy (Supplementary Figure 3D–G). Overall, we found that pulsed CBD treatment initiated durable anti-tumor effects and that predictive criteria for CBD-sensitive GBM are required for successful clinical application.

RELA Is Essential for CBD-Induced Cytotoxicity in Human GBM

We investigated if RELA would also represent a central signaling cue for CBD-induced cell death in hGSC. Therefore, we quantified activation levels for a range of transcription factors (binding with cognate gene promoter elements) including RELA.³⁴ Using this assay, we compared the CBD-insensitive GBM NCH421k with the CBD-sensitive GBM Line2 under control conditions (vehicle treatment) vs CBD administration (Figure 3A). Control levels were arbitrarily set as “1” (dotted line in Figure 3A) and CBD-induced alteration (fold-change) from controls is shown. Interestingly, statistically highly significant differences were specifically obtained for RELA activity but not for any of the other transcription factors. CBD robustly induced promoter-binding activity of RELA in the CBD-sensitive Line2, while the opposite effect was observed in CBD-insensitive NCH421K cells. The RELA inhibitor SN50, which specifically prevents RELA nuclear accumulation when applied at a concentration of 10 μM ⁶ (as used in our assays), efficiently blocked CBD-induced cytotoxicity (Figure 3B). Immunofluorescence corroborated that CBD promoted nuclear accumulation of RELA in human GBM, which is prevented by SN50 (Figure 3C). Remarkably, nuclear RELA was detected 10 hours after CBD application and persisted for up to 1 day while TNF α promoted a rapid (within 30–60 minutes) nuclear shift of RELA, which was reversed within 2 hours. In summary, our genetic and pharmacological screens identified RELA as an essential mediator of CBD-induced therapeutic effects in both transgenic and human primary GBM.

Tp53^{R172H}, *PDGFB*, *Rela*^{WT} tmBTCs (with or without CBD application): GO (gene ontology) analysis showed that RELA promoted hGSC viability ($P_{\text{adj.}} = 8.7 \times 10^{-9}$); 283 genes were coherently regulated by RELA or CBD application; CBD downregulated *Prkcz* (PKC ζ), which phosphorylates RELA on Ser311; we hypothesized that CBD promotes nuclear accumulation of RELA-lacking phosphorylation on serine-311, which results in tumor cytotoxicity (cartoon in E). (F) *Rela*^{S311A} (preventing phosphorylation on serine-311) accelerated CBD-induced glioma cell death as compared to *Rela*^{WT}, or RELA-deficient (vector) controls. Dots in (A, D, and F) represent data from independent experiments; statistical significance in (A) was investigated by 1-way ANOVA with Bonferroni's pairwise comparison test, in (D and F) by 1-way ANOVA plus Tukey post hoc testing (NS, non-significant; * $P < .05$, ** $P < .005$, *** $P < .001$).

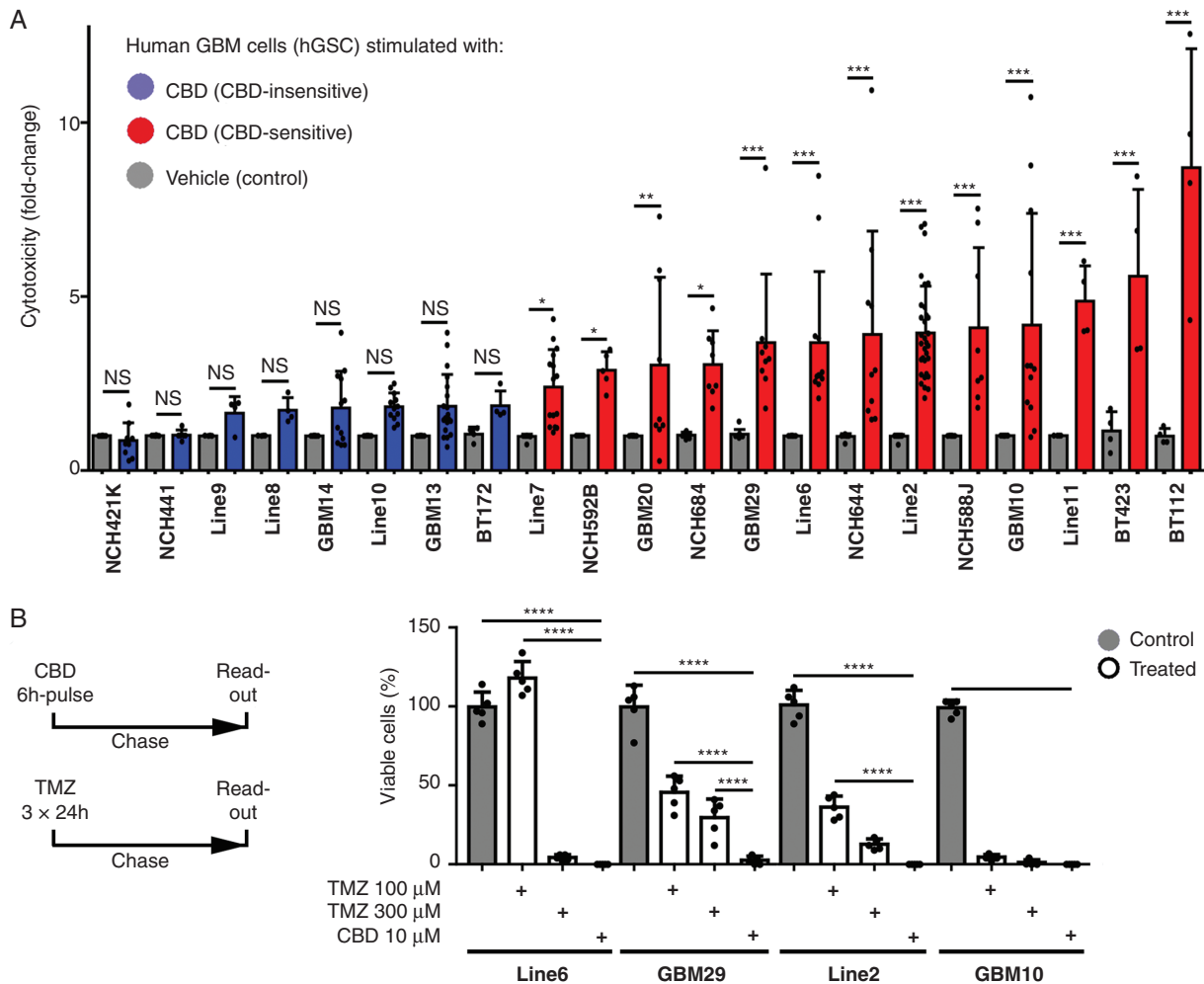


Fig. 2 CBD (cannabidiol) is a promising therapeutic for a large range of human GBM stem-like cells (GSC). (A) Human primary, stem-like hGSC cultures (recapitulating the inter-individual genetic heterogeneity of GBM) were either maintained under control conditions (0.01% DMSO) or treated with CBD (10 μ M) and cytotoxicity was quantified: Controls (gray bars) were arbitrarily set as "1" and fold-change of CBD-induced cytotoxicity is indicated. (B) Therapeutic effects of CBD were compared to the standard of care (the chemotherapeutic temozolomide; TMZ) in a pulse-chase paradigm; statistical significance in (A and B) was investigated by 1-way ANOVA with Bonferroni's pairwise comparison test (NS, non-significant, * P < .05, ** P < .005, *** P < .001, **** P < .0001).

CBD Kills GBM by Sustained Nuclear Accumulation of RELA-Lacking Phospho-Ser311

We investigated canonical NF- κ B signaling cues in CBD-sensitive and -insensitive hGSCs using RELA-reporter gene assays (Figure 4A) and pharmacological stimulation (CBD, TNF α , CBD+TNF α , or vehicle). In CBD-sensitive hGSC, TNF α (but not CBD) produced reporter activity and TNF α -triggered reporter signals were blunted by CBD pretreatment. CBD-insensitive hGSC neither showed reporter activity in response to TNF α nor to CBD (or the combination of both; Figure 4A). Altogether, we observed striking differences in the capacity of GBM cells for canonical NF- κ B signaling, which directly correlated with CBD sensitivity.

We found that CBD induced the delayed and prolonged nuclear accumulation (see Figure 3C) and DNA binding

(see Figure 3A) of RELA, which caused hGSC death (see Figure 3B) specifically in GBM with a capacity for canonical NF- κ B signaling (Figure 4A). Next, we investigated if our model on CBD-mediated RELA modulation (and delayed, prolonged nuclear accumulation) was consistent within a panel of hGSC. Hence, we used quantitative image analysis of immunofluorescently labeled samples to determine levels of nuclear RELA (independent of phosphorylation) and RELA phosphorylated specifically on serine-311 (P-RELA) in hGSC exposed to CBD, TNF α , CBD+TNF α , or vehicle controls (Figure 4B). In CBD-sensitive hGSC, the application of CBD and/or TNF α promoted nuclear accumulation of RELA in all cases (rapid and transiently by TNF α ; delayed and sustained by CBD). Strikingly, only TNF α induced nuclear P-RELA, which was abolished by pretreatment with CBD.

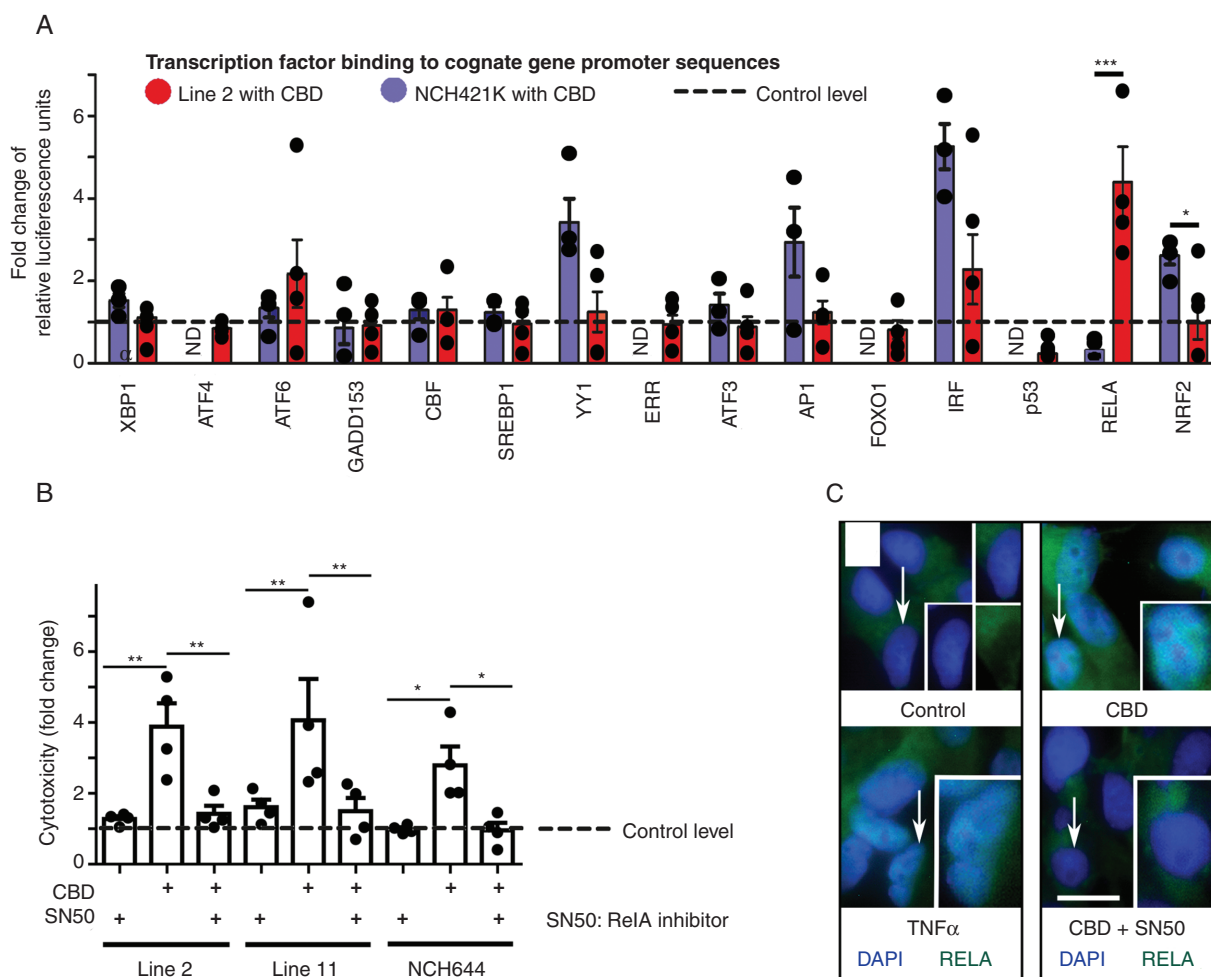


Fig. 3 RELA is essential for CBD (cannabidiol)-induced cytotoxicity of human GBM stem-like cells (GSC). (A) CBD-insensitive (NCH421K) or CBD-sensitive (Line2) hGSC were inspected for transcription factor binding-rates to cognate gene promoter sequences; fold-change after CBD treatment vs controls (arbitrarily set as “1,” dotted line) is indicated; note that highly significant changes were restricted to the NF- κ B subunit RELA. (B) CBD-induced cytotoxicity was quantified (as compared to controls, dotted line) with or without SN50 (RELA inhibitor) pretreatment; note that SN50 prevented CBD-induced cytotoxicity. (C) Immunofluorescence for RELA in Line2 hGSC under control conditions and after stimulation with TNF α or CBD; note that TNF α induced a rapid (1 hour) nuclear shift of RELA, CBD-induced nuclear RELA accumulation was slow (visualized at 16 hours) and persistent (for more than 10 hours); SN50 prevented the nuclear transfer of RELA. Scale in (c) indicates 10 μ m; dots (in A and B) represent data from independent experiments; statistical significance was investigated by 1-way ANOVA plus Tukey post hoc testing (* $P < .05$, ** $P < .005$, *** $P < .001$).

In contrast, CBD initiated the persistent nuclear shift of RELA that was unphosphorylated on serine-311. This was different from CBD-insensitive hGSC, in which CBD did not drive nuclear accumulation of RELA. All data were fully consistent with independent experiments using Western blotting (Figure 4C). Coherent with our results from tmBTC, we observed that PKC ζ inhibition strongly reduced hGSC viability (Supplementary Figure 4A). In conclusion, we suggest a GBM cytotoxic function for nuclear RELA-lacking phospho-SER311 (graphically summarized in Figure 4D).

In physiology, NF- κ B responses are rapid and transient³ and therefore we investigated the signaling cues promoting

a persistent nuclear shift of RELA after CBD application. In agreement with our transcriptomic data (see Figure 2A), we noticed that CBD downregulated key genes of the glycolytic pathway (Supplementary Figure 4B) and accelerated mitochondrial respiration (Supplementary Figure 4C, D). Glutamate dehydrogenase, which strongly promotes RELA nuclear accumulation,^{28,35} can integrate metabolic alterations and spur GBM cell death (Supplementary Figure 4E, F). In synopsis, our findings suggest that CBD induced prolonged nuclear accumulation and DNA binding of RELA-lacking phospho-Ser311 resulting in attenuated canonical NF- κ B responsiveness and cell death specifically in CBD-sensitive GBM.

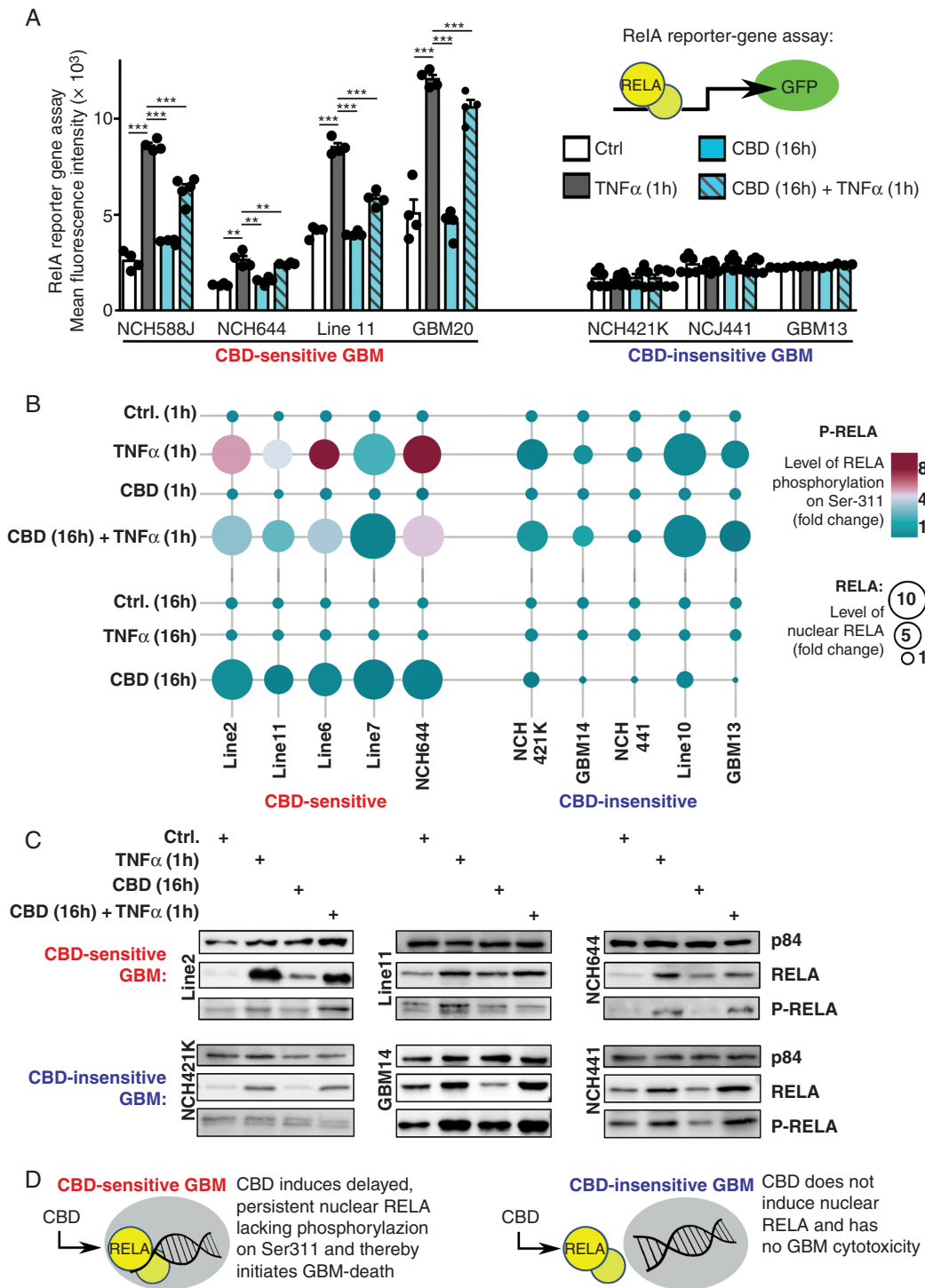


Fig. 4 CBD (cannabidiol)-induced death of human GBM stem-like cells (GSC) via sustained nuclear localization of RELA-lacking phospho-Ser311. (A) RelA activity in hGSC maintained under control conditions (Ctrl), stimulated with CBD (10 μ M) or with TNF α (10 ng/ml); note that TNF α application triggered strong reporter activity specifically in CBD-sensitive cells; CBD treatment did not induce any reporter gene activity. (B) Nuclear localization of RELA and nuclear phospho-Ser311-RELA (P-RELA) were quantified in panels of hGSC at two timepoints (and data visualized as bubble plot): 1 hour after stimulation with TNF α nuclear translocation and phosphorylation of RelA was restricted to CBD-sensitive hGSC and co-application of CBD reduced P-RELA levels; 16 hours after stimulation TNF α response had ceased but CBD induced strong nuclear accumulation of RELA-lacking phosphorylation on Ser311 specifically in CBD-sensitive hGSC. (C) Nuclear localization of RelA and nuclear phospho-Ser311-RelA (P-RelA) were detected by Western blotting of nuclear extracts (nuclear protein p84 served as loading control). (D) Schematic summary. Dots in (A) indicate data from independent experiments; statistical significance was investigated by 1-way ANOVA plus Tukey post hoc testing (** $P < .005$, *** $P < .001$).

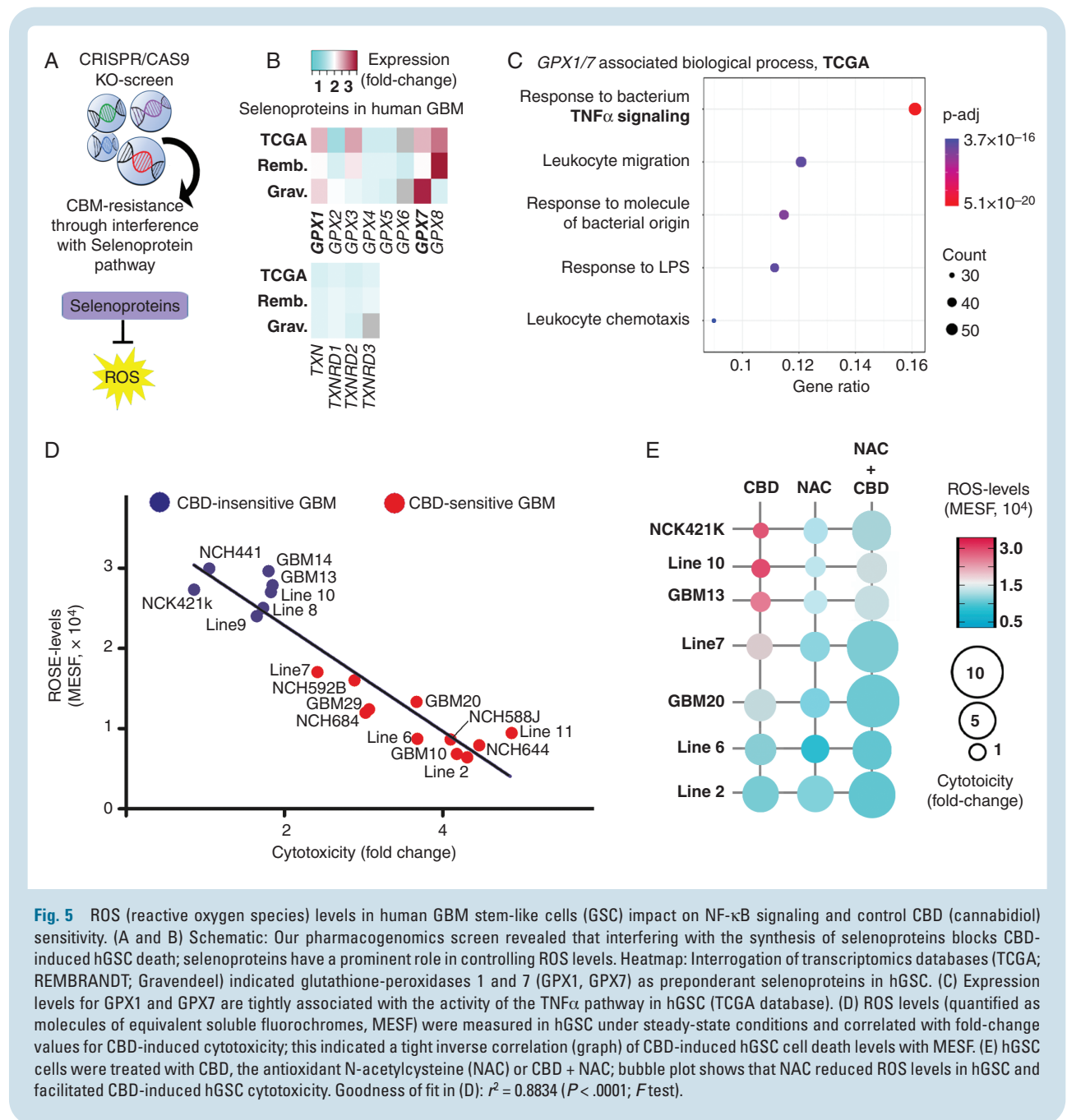


Fig. 5 ROS (reactive oxygen species) levels in human GBM stem-like cells (GSC) impact on NF- κ B signaling and control CBD (cannabidiol) sensitivity. (A and B) Schematic: Our pharmacogenomics screen revealed that interfering with the synthesis of selenoproteins blocks CBD-induced hGSC death; selenoproteins have a prominent role in controlling ROS levels. Heatmap: Interrogation of transcriptomics databases (TCGA; REMBRANDT; Gravendeel) indicated glutathione-peroxidases 1 and 7 (GPX1, GPX7) as preponderant selenoproteins in hGSC. (C) Expression levels for GPX1 and GPX7 are tightly associated with the activity of the TNF α pathway in hGSC (TCGA database). (D) ROS levels (quantified as molecules of equivalent soluble fluorochromes, MESF) were measured in hGSC under steady-state conditions and correlated with fold-change values for CBD-induced cytotoxicity; this indicated a tight inverse correlation (graph) of CBD-induced hGSC cell death levels with MESF. (E) hGSC cells were treated with CBD, the antioxidant N-acetylcysteine (NAC) or CBD + NAC; bubble plot shows that NAC reduced ROS levels in hGSC and facilitated CBD-induced hGSC cytotoxicity. Goodness of fit in (D): $r^2 = 0.8834$ ($P < .0001$; F test).

ROS Levels Are Linked With TNF α Signaling and Control CBD Sensitivity

Our pharmacogenomics screen indicated that both the NF- κ B pathway and selenoprotein synthesis control sensitivity to CBD (schematic in Figure 5A; see also Figure 1C). Selenoproteins are involved in the maintenance of cellular redox state,³⁶ and there is extensive crosstalk between NF- κ B/RELA signaling and ROS generation.^{37–39} To determine if a relationship between these signaling cues exists in human GBM, we interrogated different GBM databases and found that glutathione peroxidase 1 (GPX1) and GPX7 are the preponderant selenoproteins in human tumors

(Figure 5B). Next, we performed a differential expression analysis and found that GPX1/7 expression levels tightly connect with transcriptomic profiles for TNF α signaling (Figure 5C). Hence, we investigated this further and asked if CBD-sensitive (and TNF α -responsive) or CBD-insensitive (and TNF α -unresponsive) hGSC would differ with respect to ROS levels. Importantly, we discovered a strong inverse correlation of ROS levels and CBD-induced cytotoxicity rates (Figure 5D). We explored if the very robust correlation between steady-state quantities of cytoplasmic ROS and CBD-induced GBM cell death would highlight a functional relationship between ROS levels and CBD sensitivity. Hence, we recapitulated the cell death assays together with

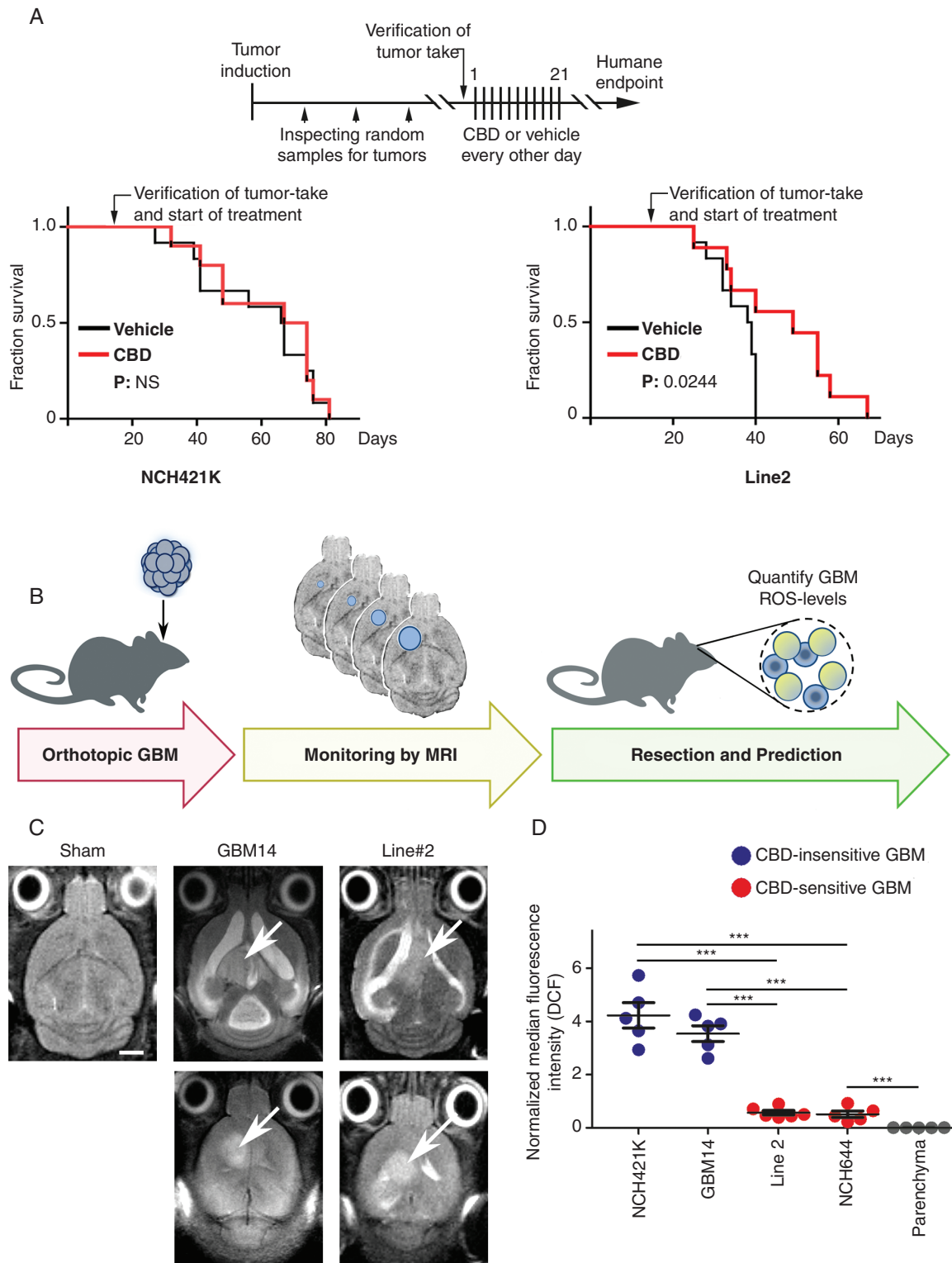


Fig. 6 ROS (reactive oxygen species) levels of human GBM stem-like cells (GSC) biopsies predict therapeutic response to cannabidiol (CBD). (A) CBD-mediated therapeutic effects were tested in immune-deficient orthotopic models after confirming tumor growth (see schematic); CBD treatment (red lines) prolonged survival (as compared to controls; black lines) specifically in hGSC that were identified as CBD-sensitive (by in vitro tests). (B) Summary of workflow for data in (C) and (D): CBD-sensitive or -insensitive GBM were orthotopically implanted, longitudinally observed by MRI (as compared to sham-injected controls), resected (when tumor-volume reached $50 \pm 10 \text{ mm}^3$), and analyzed by flow cytometry.

ROS measurements under conditions of CBD treatment vs co-application of CBD with the antioxidant *N*-acetylcysteine (NAC). Here, we found efficient attenuation of ROS levels by NAC, which consistently raised CBD cytotoxicity (Figure 5E). All in all, this highlighted considerable inter-individual differences in ROS levels in human GBM which were closely linked with a capacity for TNF α signaling and CBD sensitivity. The quantities of intracellular ROS and the extent of CBD-initiated cell death in hGSC were functionally connected and had a linear relationship. This experimental and synergistic bioinformatics evidence highlights ROS levels as a biomarker for CBD sensitivity.

ROS Levels Serve as a Predictive Biomarker for CBD Treatment

Classification of GBM by genetic subtypes, transcriptomics of established CBD targets and genomics of GBM (Supplementary Figure 5A–E), or the NF- κ B pathway in hGSC (Supplementary Figure 6A) did not relate to CBD-triggered cell death, but ROS levels predicted CBD sensitivity (see Figure 5D, E; Supplementary Figure 6B). To investigate if the predictive power of ROS was preserved in intracranially growing hGSC, we generated orthotopic xenografts of hGSC, when tumor growth was confirmed we applied CBD (15 mg/kg, i.p. injected every other day for 21 days) and determined overall survival as compared to vehicle-treated groups. We found that animals inoculated with NCH421K hGSC showed no response, while mice bearing hGSC named Line2 benefited from CBD treatment by prolonged survival (Figure 6A; this was also recapitulated in an immunocompetent, genetically induced GBM model; Supplementary Figure 7A). Our data from in vivo studies were congruent with our data from in vitro pharmacology and corroborated that GBM ROS levels (in vitro) predict therapeutic response to CBD. Therefore, we investigated if ROS levels in GBM biopsies may also have predictive power for CBD treatment. To this end, we used orthotopic hGSC models that were monitored by magnetic resonance imaging (MRI) throughout tumor expansion and subsequently FACS measured for ROS (Figure 6B). In all cases, we found that CBD-sensitive cells (with low ROS levels) could be readily differentiated from CBD-insensitive hGSC (with high ROS levels; of note: both were also distinguished from tumor-parenchymal cells), while variations in hGSC growth pattern had no impact on the reliability of ROS as a predictive biomarker (Figure 6C, D; FACS gating strategies and calibration are shown in Supplementary Figure 7B–D). All in all, our experiments demonstrated that CBD is a promising therapeutic for GBM when ROS measurements are used to stratify for CBD-sensitive tumors.

Discussion

The NF- κ B family member RELA is of central importance for pathological signaling in oncology and inflammation but remained largely inaccessible for clinical pharmacology.^{1–3} Here, we report that CBD, which is used in pediatric neurology (Epidiolex),¹⁸ has a previously unacknowledged effect on RELA signaling and thereby suppresses GBM. In particular, we show that nuclear translocation of RELA-lacking phosphorylation on serine-311 initiates a tumor-specific cytotoxic pathway. This newly identified pathway is fundamentally different from previous reports discussing a role for NF- κ B signaling in cannabinoid-pharmacology (including CBD) or for therapeutic targeting of NF- κ B in GBM, which considered cytoplasmic sequestration of NF- κ B as a central parameter.^{1,4,11,12,15,19,20} Our study indicates nuclear RELA-lacking phospho-Ser311 as a tumor-selective, drug-inducible trigger for GBM cytotoxicity.

Initially, we investigated the anti-tumor effects of NPC-released endocannabinoids^{16,17,39} and a range of phytocannabinoids. Our data showed that cannabinoid treatments of GBM require predictive markers and indicated CBD as the most efficacious phytocannabinoid against GBM. Together with a pharmacogenomic screen, this exhibited the paramount role of the NF- κ B subunit RELA in CBD-mediated tumor cytotoxicity, which was confirmed by additional pharmacological and genetic experiments. Collectively, *Rela* knockouts, pharmacological RELA inhibition, and transcription factor activity assays indicated an anti-tumorigenic role for RELA DNA binding after CBD stimulation of drug-sensitive GBM cells.

Our genetic and pharmacological studies with tmBTC indicated the molecular mechanisms for CBD sensitivity of GBM. Then we tested the pathological validity of our candidate mechanisms (RELA and ROS signaling) in human GBM cells with established pathological and genetic characteristics. Pharmacological manipulation, transcription factor binding assays, and quantification of RELA phosphorylation on Ser311 in hGSC confirmed the therapeutic relevance of this signaling cue for human pathology. Transcriptomics showed that CBD attenuated RELA signaling in transgenic glioma cells, which was confirmed by quantitative PCR in hGSCs. The CBD-induced nuclear shift of RELA (unphosphorylated on SER-311) was very persistent, and it is tempting to speculate that the sustained DNA binding of transcriptionally inactive RELA interferes with tumor homeostasis and viability.

Individual hGSCs differed in NF- κ B signaling^{1,11,15} and the ability for canonical NF- κ B signaling appeared as a prerequisite for CBD sensitivity. Hence, assays for TNF α -induced

Representative T_2 -weighted MR images for tumor-free controls (sham), CBD-insensitive (GBM14) and CBD-sensitive (Line2) hGSCs are shown in (C). (D) Ex vivo flow cytometry reliably indicated that CBD-sensitive GBM have low ROS levels, whereas CBD-insensitive hGSCs were identified by high ROS content; ROS levels in tumor parenchymal cells (gray symbols) were stringently separated from tumors. Dots in (B) represent data from individual tumors. Statistical significance in (C) was investigated by 1-way ANOVA plus Tukey post hoc testing (** $P < .005$, *** $P < .001$); statistical values (P) for Kaplan-Meier studies are indicated (in C).

signals may be one possibility to identify drug-sensitive tumors. Alternatively, molecular markers were recently discovered identifying Glut3-addicted hGSC²⁷ and since CBD strongly attenuated GLUT3 expression levels this may also appear as an interesting option. However, our pharmacogenomics study and subsequent flow cytometric experiments in vitro and ex vivo highlighted ROS as a predictive biomarker for CBD sensitivity of hGSC. ROS levels in hGSC could be readily distinguished from ROS in parenchymal cells, but remained within the boundaries that were previously detected under different redox-biological conditions.⁴⁰ Within this concentration range, ROS predominantly affect cellular sensors like nuclear factor erythroid 2-related factor 2 (NRF2) or RELA.^{37,40} NRF2 and RELA have many antagonistic functions^{37,40} and—consistently—they were found to be oppositely regulated after CBD application in our transcription factor activity assay. In addition, several molecules in the canonical NF- κ B signaling cascade are redox-sensitive.^{38,39} This provides a basis to explain why ROS levels in hGSCs firmly correlate with responsiveness in the canonical NF- κ B/RELA pathway and consequentially with CBD sensitivity. Our bioinformatics analysis of human GBM displayed a tight link between ROS control (by the selenoproteins GPX1, GPX7) and TNF α signaling. The in vitro and in vivo studies demonstrated the interdependence of ROS and CBD-triggered tumor death and affirmed the predictive power of ROS for CBD treatment in preclinical neuro-oncology. Altogether, this suggests ROS as a biomarker for stratification of patients potentially benefiting from CBD therapy. Furthermore, coadministration of BBB-permeable ROS-quenching agents like, eg, the antihypertensive drug captopril (a sulfhydryl donor) may improve the therapeutic efficacy of CBD.⁴¹

This is the first study investigating the role of RELA Ser311 phosphorylation in CBD-induced anti-tumor effects. We identified central mediators of drug action, suggested predictive criteria for CBD sensitivity, and opened up new perspectives for targeting NF- κ B in oncology. All in all, this may support the clinical implementation of CBD as a promising drug for GBM and the development of new NF- κ B-modulating compounds for cancer therapy.

Keywords

GBM therapy | NF- κ B (nuclear factor kappa-light-chain enhancer of activated B cells) | preclinical study | RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A; also designated p65 or NF- κ B3) | stem-like GBM cells

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References

- Perkins ND. The diverse and complex roles of NF- κ B subunits in cancer. *Nat Rev Cancer*. 2012;12(2):121–132.
- Taniguchi K, Karin M. NF- κ B, inflammation, immunity and cancer: coming of age. *Nat Rev Immunol*. 2018;18(5):309–324.
- Tornatore L, Thotakura AK, Bennett J, Moretti M, Franzoso G. The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends Cell Biol*. 2012;22(11):557–566.
- Nogueira L, Ruiz-Ontañón P, Vazquez-Barquero A, et al. Blockade of the NF κ B pathway drives differentiating glioblastoma-initiating cells into senescence both in vitro and in vivo. *Oncogene*. 2011;30(32):3537–3548.
- Johnson RF, Perkins ND. Nuclear factor- κ B, p53, and mitochondria: regulation of cellular metabolism and the Warburg effect. *Trends Biochem Sci*. 2012;37(8):317–324.
- Barkett M, Gilmore TD. Control of apoptosis by Rel/NF- κ B transcription factors. *Oncogene*. 1999;18(49):6910–6924.
- Aldape K, Brindle KM, Chesler L, et al. Challenges to curing primary brain tumours. *Nat Rev Clin Oncol*. 2019;16(8):509–520.
- Touat M, Idbaih A, Sanson M, Ligon KL. Glioblastoma targeted therapy: updated approaches from recent biological insights. *Ann Oncol*. 2017;28(7):1457–1472.
- Ostrom QT, Gittleman H, Truitt G, Boscia A, Kruchko C, Barnholtz-Sloan JS. CBTRUS Statistical Report: primary brain and other central nervous system tumors diagnosed in the United States in 2011–2015. *Neuro Oncol*. 2018;20(suppl_4):iv1–iv86.
- Van Meir EG, Hadjipanayis CG, Norden AD, Shu HK, Wen PY, Olson JJ. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. *CA Cancer J Clin*. 2010;60(3):166–193.

11. Puliappadamba VT, Hatanpaa KJ, Chakraborty S, Habib AA. The role of NF- κ B in the pathogenesis of glioma. *Mol Cell Oncol*. 2014;1(3):e963478.
12. Cahill KE, Morshed RA, Yamini B. Nuclear factor- κ B in glioblastoma: insights into regulators and targeted therapy. *Neuro Oncol*. 2016;18(3):329–339.
13. Ohtsu N, Nakatani Y, Yamashita D, Ohue S, Ohnishi T, Kondo T. Eva1 maintains the stem-like character of glioblastoma-initiating cells by activating the noncanonical NF- κ B signaling pathway. *Cancer Res*. 2016;76(1):171–181.
14. Tchoghandjian A, Jennewein C, Eckhardt I, Momma S, Figarella-Branger D, Fulda S. Smac mimetic promotes glioblastoma cancer stem-like cell differentiation by activating NF- κ B. *Cell Death Differ*. 2014;21(5):735–747.
15. Soubannier V, Stifani S. NF- κ B signalling in glioblastoma. *Biomedicines*. 2017;5(2):29.
16. Stock K, Kumar J, Synowitz M, et al. Neural precursor cells induce cell death of high-grade astrocytomas through stimulation of TRPV1. *Nat Med*. 2012;18(8):1232–1238.
17. Glass R, Synowitz M, Kronenberg G, et al. Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival. *J Neurosci*. 2005;25(10):2637–2646.
18. Devinsky O, Cross JH, Laux L, et al. Trial of cannabidiol for drug-resistant seizures in the dravet syndrome. *N Engl J Med*. 2017;376(21):2011–2020.
19. Cristino L, Bisogno T, Di Marzo V. Cannabinoids and the expanded endocannabinoid system in neurological disorders. *Nat Rev Neurol*. 2020;16(1):9–29.
20. Pacher P, Kogan NM, Mechoulam R. Beyond THC and endocannabinoids. *Annu Rev Pharmacol Toxicol*. 2020;60:637–659.
21. Koike-Yusa H, Li Y, Tan EP, Velasco-Herrera Mdel C, Yusa K. Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nat Biotechnol*. 2014;32(3):267–273.
22. Trümbach D, Pfeiffer S, Poppe M, et al. ENCoRE: an efficient software for CRISPR screens identifies new players in extrinsic apoptosis. *BMC Genomics*. 2017;18(1):905.
23. Lee JK, Wang J, Sa JK, et al. Spatiotemporal genomic architecture informs precision oncology in glioblastoma. *Nat Genet*. 2017;49(4):594–599.
24. Brennan CW, Verhaak RG, McKenna A, et al. The somatic genomic landscape of glioblastoma. *Cell*. 2013;155(2):462–477.
25. Roberts DJ, Miyamoto S. Hexokinase II integrates energy metabolism and cellular protection: acting on mitochondria and TORCing to autophagy. *Cell Death Differ*. 2015;22(2):248–257.
26. Wolf A, Agnihotri S, Micallef J, et al. Hexokinase 2 is a key mediator of aerobic glycolysis and promotes tumor growth in human glioblastoma multiforme. *J Exp Med*. 2011;208(2):313–326.
27. Cosset E, Ilmjärvi S, Dutoit V, et al. Glut3 addiction is a druggable vulnerability for a molecularly defined subpopulation of glioblastoma. *Cancer Cell*. 2017;32(6):856–868.e5.
28. Wang X, Liu R, Qu X, et al. α -Ketoglutarate-activated NF- κ B signaling promotes compensatory glucose uptake and brain tumor development. *Mol Cell*. 2019;76(1):148–162.e7.
29. Duran A, Diaz-Meco MT, Moscat J. Essential role of RelA Ser311 phosphorylation by ζ PKC in NF- κ B transcriptional activation. *EMBO J*. 2003;22(15):3910–3918.
30. Leitges M, Sanz L, Martin P, et al. Targeted disruption of the ζ PKC gene results in the impairment of the NF- κ B pathway. *Mol Cell*. 2001;8(4):771–780.
31. Boettcher M, Covarrubias S, Biton A, et al. Tracing cellular heterogeneity in pooled genetic screens via multi-level barcoding. *BMC Genomics*. 2019;20(1):107.
32. Hložek T, Uttl L, Kadeřábek L, et al. Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion *in vivo* of CBD to THC. *Eur Neuropsychopharmacol*. 2017;27(12):1223–1237.
33. Kitange GJ, Carlson BL, Schroeder MA, et al. Induction of MGMT expression is associated with temozolomide resistance in glioblastoma xenografts. *Neuro Oncol*. 2009;11(3):281–291.
34. Betancur PA, Abraham BJ, Yiu YY, et al. A CD47-associated super-enhancer links pro-inflammatory signalling to CD47 upregulation in breast cancer. *Nat Commun*. 2017;8:14802.
35. Di Conza G, Tsai CH, Ho PC. Fifty shades of α -ketoglutarate on cellular programming. *Mol Cell*. 2019;76(1):1–3.
36. Brigelius-Flohé R, Flohé L. Selenium and redox signaling. *Arch Biochem Biophys*. 2017;617:48–59.
37. Sies H, Jones DP. Reactive oxygen species (ROS) as pleiotropic physiological signalling agents. *Nat Rev Mol Cell Biol*. 2020;21(7):363–383.
38. Morgan MJ, Liu ZG. Crosstalk of reactive oxygen species and NF- κ B signaling. *Cell Res*. 2011;21(1):103–115.
39. Pantano C, Reynaert NL, van der Vliet A, Janssen-Heininger YM. Redox-sensitive kinases of the nuclear factor- κ B signaling pathway. *Antioxid Redox Signal*. 2006;8(9-10):1791–1806.
40. Sies H, Berndt C, Jones DP. Oxidative stress. *Annu Rev Biochem*. 2017;86:715–748.
41. AbdAlla S, Langer A, Fu X, Qwitterer U. ACE inhibition with captopril retards the development of signs of neurodegeneration in an animal model of Alzheimer's disease. *Int J Mol Sci*. 2013;14(8):16917–16942.