Cannabidiol converts $NF_{\kappa}B$ into a tumor suppressor in glioblastoma with defined antioxidative properties.

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CONFLICT OF INTEREST

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ABSTRACT

Background: The transcription factor $NF_{\kappa}B$ drives neoplastic progression of many cancers including primary brain tumors (glioblastoma; GBM). Precise therapeutic modulation of $NF_{\kappa}B$ activity can suppress central oncogenic signalling 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 $NF_{\kappa}B$ 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 $NF_{\kappa}B$ subunit RELA and simultaneously prevents RELA-phosphorylation on serine-311, a key residue which 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 predictive biomarker for CBD-sensitive tumors.

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Conclusions: This evidence demonstrates how a clinically approved drug can convert $NF_{\kappa}B$ into a tumor suppressor and suggests a promising repurposing option for GBM-therapy.

KEYWORDS

$NF_{\kappa}B$ (Nuclear factor kappa-light-chain-enhancer of activated B cells), RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A; also designated p65 or NFKB3), stem-like GBM cells, GBM therapy, preclinical study.

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KEY POINTS

1. Cytoplasmic sequestration of $NF_{\kappa}B$ for therapeutic approaches has clinically failed
2. Cannabidiol (CBD) kills GBM cells by inducing DNA binding of a modified $NF_{\kappa}B$ 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 anti-convulsive 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 $NF_{\kappa}B$ subunit RELA, which results in GBM death. GBM have striking differences in canonical $NF_{\kappa}B$ 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 $NF_{\kappa}B$.

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INTRODUCTION

Transcription factors of the nuclear factor $_{K}$B (NF$_{K}$B) family have gained large attention as therapeutic targets for neoplastic disease.[1-4] In cancer cells NF-$_{K}$B integrates a range of environmental stimuli and promotes tumor progression by sustaining cell viability, inducing invasiveness and regulating metabolic adaption.[1,5,6] NF$_{K}$B, a heterodimeric molecule of RELA (p65) and p50 subunits, is also a central signalling hub for inflammation and coordinates responses to cytokines[2] like tumor necrosis factor-α (TNFα). In this canonical pathway NF$_{K}$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$_{K}$B, with limited clinical success.[1] It is becoming increasingly clear that control of posttranslational modification and subsequent fine-tuning of NF$_{K}$B-initiated transcription holds potential for clinical translation.[1]

Glioblastomas 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-$_{K}$B signaling.[4,11-14] Hence, in GBM NF-$_{K}$B represents a potentially efficient (but hitherto largely inaccessible) therapeutic target.[11,12,15]

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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 anti-convulsive 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 $NF_{\kappa}B$ signaling to initiate tumor cell death. The redox dependency of this pathway is shown and ROS level of GBM are introduced as predictive biomarker for CBD-sensitive tumors.

RESULTS

Pharmacogenomics uncover $NF_{\kappa}B$ (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.[21,22] These tmBTC allowed to identify therapeutic responses in a range of cells modelling 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 Fig. 1A).

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Next, we screened a panel of phytocannabinoids for anti-tumor effects and obtained best results with CBD (Supplementary Fig. 1B and C). Then we evaluated CBD-induced cytotoxicity in tmBTC (as compared to controls; Fig. 1A). Coherent with our data from assays with endocannabinoids (Supplementary Fig. 1A) we found that the phytocannabinoid CBD (Fig. 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*; Fig. 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 Fig. 1B; and Supplementary Table 1).

The pharmacogenomics screen (Fig. 1B; Supplementary Fig. 2A-C) suggested RELA and selenoprotein-controlled homeostasis of reactive oxygen species (ROS; schematic overview in Fig. 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 (Fig. 1D; of note *Rela* knockout, *Rela*^{KO}, did not affect tmBTC expansion in vitro, p=0.7451). In summary, our experiments showed that endocannabinoids or CBD suppress a range of genetically distinct tmBTCs, that CBD had the best tumor-cytotoxic activity in a panel of non-psychotropic

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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} *and Tp53*^{R172H}, *PDGFB, Rela*^{WT} cells under conditions of CBD or vehicle treatment (Fig. 1E). In agreement with previous reports[11,12,15] we observed that RELA expression promoted tumor viability (p-adj. = 8.7×10^{-9} in gene-ontology analysis). CBD-exposure versus vehicle-controls (in *Tp53*^{R172H}, *PDGFB, Rela*^{WT} cells) or RELA expression (*Tp53*^{R172H}, *PDGFB, Rela*^{KO} versus *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 (Fig. 1E). These were analyzed further and a representative functional annotation was obtained for the fraction of downregulated genes (p-adj. = 1.8×10^{-7}) showing that CBD-triggered RELA signaling attenuates levels of key glycolytic molecules important for tumor growth[23-26], 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.

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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 (PKCζ), as confirmed by qPCR (Supplementary Fig. 2D). PKCζ phosphorylates RELA on serine-311 which is required for transactivation[27,28] (Supplementary Fig. 2E), while phosphorylation on this residue does not modulate nuclear localization or DNA binding of RELA.[27] 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 $\textit{Rela}^{S311A}$ (which cannot be phosphorylated) in $\textit{Tp53}^{R172H}$, *PDGFB,* $\textit{Rela}^{KO}$ cells, applied CBD (versus controls) and quantified cell viability (time resolution of rapid cell death was best in viability assays). We hypothesized that $\textit{Rela}^{S311A}$ may accelerate CBD-induced tmBTC death as compared to $\textit{Rela}^{WT}$-controls: $\textit{Rela}^{WT}$ cells require (time-consuming) downregulation of PKCζ for CBD-induced cytotoxicity, while this step is obsolete in $\textit{Rela}^{S311A}$ tmBTCs (hence, cell death is faster in $\textit{Rela}^{S311A}$ tmBTCs; see also cartoon in Fig. 1E). This was verified in a viability-assay (Fig. 1F); note that $\textit{Rela}^{KO}$ 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.

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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 samples[29] 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 (Fig. 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 h) CBD application, in agreement with CBD-pharmacokinetics[30], was sufficient to trigger pronounced hGSC death (Supplementary Fig. 3A, B). Strikingly, in CBD-sensitive hGSC CBD was superior to the chemotherapeutic temozolomide in an established concentration-range and time-course[31] in vitro (Fig. 2B). CBD-sensitive tumors were also identified after very short passaging in vitro (Supplementary Fig. 3C, Supplementary Table 3). Cell death in hGSC was executed by apoptosis or endoplasmic reticulum-stress and autophagy (Supplementary Fig. 3D – 3G). 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 range of transcription factors (binding with cognate gene promoter elements) including

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RELA.[32] Using this assay, we compared the CBD-insensitive GBM NCH421k with the CBD-sensitive GBM Line2 under control conditions (vehicle-treatment) versus CBD administration (Fig. 3A). Control levels were arbitrarily set as “1” (dotted line in Fig. 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. As a functional test, we applied a RELA inhibitor (SN50), which specifically prevents nuclear accumulation of RELA at this concentration (10 μM)[6], to our assays with CBD-sensitive GBM and found that this efficiently blocked CBD-induced cytotoxicity (Fig. 3B). Immunofluorescence corroborated that CBD promoted nuclear accumulation of RELA in human GBM, which is prevented by SN50 (Fig. 3C). Remarkably, nuclear RELA was detected 10 h after CBD application and persisted for up to 1 day while TNFα promoted a rapid (within 30 to 60 min) nuclear shift of RELA, which was reversed within 2 h. 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.

CBD kills GBM by sustained nuclear accumulation of RELA lacking phospho-Ser311

We investigated canonical $NF_{K}B$ signalling cues in CBD-sensitive and –insensitive hGSCs using RELA-reporter gene assays (Fig. 4A) and pharmacological stimulation (CBD, TNFα, CBD+TNFα or vehicle). In CBD-sensitive hGSC, TNFα (but not CBD) produced reporter activity and TNFα-triggered reporter signals were blunted by CBD pre-treatment. CBD-insensitive hGSC neither showed reporter

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activity in response to TNFα nor to CBD (or the combination of both; Fig. 4A). Altogether, we observed striking differences in the capacity of GBM cells for canonical $NF_{K}B$ signalling, which directly correlated with CBD sensitivity.

We found that CBD induced the delayed and prolonged nuclear accumulation (see Fig. 3C) and DNA binding (see Fig. 3A) of RELA, which caused hGSC death (see Fig. 3B) specifically in GBM with a capacity for canonical NFKB signaling (Fig. 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 labelled samples to determine levels of nuclear RELA (independent of phosphorylation) and RELA phosphorylated specifically on serine-311 (P-RELA) in hGSC exposed to CBD, TNFα, CBD+TNFα or vehicle-controls (Fig. 4B). In CBD-sensitive hGSC the application of CBD and / or TNFα promoted nuclear accumulation of RELA in all cases (rapid and transiently by TNFα; delayed and sustained by CBD). Strikingly, only TNFα induced nuclear P-RELA, which was abolished by pre-treatment with CBD. 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 (Fig. 4C). Coherent with our results from tmBTC we observed that PKCζ-inhibition strongly reduced hGSC viability (Supplemental Fig. 4A). In conclusion, we suggest a GBM-cytotoxic function for nuclear RELA lacking phospho-SER311 (graphically summarized in Fig. 4D).

In physiology $NF_{K}B$ responses are rapid and transient[3] and therefore we investigated the signaling cues promoting a persistent nuclear shift of RELA after

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CBD application. In agreement with our transcriptomic data (see Fig. 2A) we noticed that CBD downregulated key genes of the glycolytic pathway (Supplementary Fig. 4B) and accelerated mitochondrial respiration (Supplementary Fig. 4C, D). Glutamate dehydrogenase, which strongly promotes RELA nuclear accumulation[26,33], can integrate metabolic alterations and spur GBM cell death (Supplemental Fig. 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 $NF_{\kappa}B$-responsiveness and cell death specifically in CBD-sensitive GBM.

ROS levels are linked with TNFα signaling and control CBD sensitivity

Our pharmacogenomics screen indicated that both the $NF_{\kappa}B$ pathway and selenoprotein-synthesis control sensitivity to CBD (schematic in Fig. 5A; see also Fig. 1C). Selenoproteins are involved in maintenance of cellular redox-state[34] and there is extensive crosstalk between $NF_{\kappa}B$ / RELA signaling and ROS-generation.[35-37] To determine if a relation 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 (Fig. 5B). Next, we performed a differential expression analysis and found that GPX1/7 expression levels tightly connect with transcriptomic profiles for TNFα signaling (Fig. 5C). Hence, we investigated this further and asked if CBD-sensitive (and TNFα-responsive) or CBD-insensitive (and TNFα-unresponsive) hGSC would differ with respect to ROS levels. Importantly, we discovered a strong inverse correlation of ROS levels and CBD-induced cytotoxicity rates (Fig. 5D). We explored if the very

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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 ROS-measurements under conditions of CBD treatment versus 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 (Fig 5E). All in all, this highlighted considerable inter-individual differences in ROS levels in human GBM which were closely linked with a capacity for TNFα 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 Fig. 5A - E) or the NFΚB pathway in hGSC (Supplementary Fig. 6A) did not relate to CBD-triggered cell death, but ROS levels predicted CBD sensitivity (see Fig. 5D, E; Supplementary Fig. 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 (15mg/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 (Fig. 6A;

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this was also recapitulated in an immunocompetent, genetically induced GBM model; Supplemental Fig 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 (Fig. 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 (Fig. 6C, D; FACS gating strategies and calibration are shown in Supplementary Fig. 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 $NF_{K}B$-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 $NF_{K}B$ signaling in cannabinoid-pharmacology (including CBD) or for therapeutic targeting of $NF_{K}B$ in GBM, which considered cytoplasmic

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sequestration of $NF_{\kappa}B$ 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[16,17,37] 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 $NF\text{-}_{\kappa}B$ 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.

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Individual hGSCs differed in NF-κB signaling[1,11,15] and the ability for canonical NF-κB signaling appeared as a prerequisite for CBD sensitivity. Hence, assays for TNFα-induced signals may be one possibility to identify drug-sensitive tumors. Alternatively, molecular markers were recently discovered identifying Glut3-addicted hGSC[25] 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.[38] Within this concentration range ROS predominantly affect cellular sensors like nuclear factor erythroid 2-related factor 2 (NRF2) or RELA.[35,38] NRF2 and RELA have many antagonistic functions[35,38] 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-κB signaling cascade are redox sensitive.[36,37] This provides a basis to explain why ROS levels in hGSCs firmly correlate with responsiveness in the canonical NF-κB / 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 TNFα 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 neurooncology. Altogether, this suggests ROS as a biomarker for stratification of patients potentially benefitting from CBD-therapy. Furthermore, coadministration of brain barrier permeable ROS quenching agents like

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e.g. the antihypertensive drug captopril (a sulfhydryl donor) may improve the therapeutic efficacy of CBD.[39]

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 $NF_{K}B$ in oncology. All in all, this may support the clinical implementation of CBD as a promising drug for GBM and the development of new $NF_{K}B$-modulating compounds for cancer-therapy.

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ACKNOWLEDGEMENTS:

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MATERIALS AND METHODS

Cell culture

Biopsies from human primary and recurrent hGSC were obtained from planned resections (ethics licenses EA112/2001, EA3/023/06 and 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 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.

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injected (every other day, for 21 days) with CBD (15mg/kg) or vehicle (5% Tween80, 5% ethanol in 0.9% saline).

CRISPR/Cas9 screen

Mouse glioma cells were infected with the genome-wide gRNA lentiviral library[40] at an MOI of 0.3. Purified libraries of treated / control samples were sequenced on Illumina HiSeq2500 by 50-bp single-end sequencing.[41]

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, 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 was indicated in the figures, legends or in the manuscript text. Student's *t*, one-way/ two-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<0.05, **p<0.005, and ***p<0.0005 in all results.

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Fig. 1. Pharmacogenomics reveal a tumor suppressor function of NF$_{\kappa}$B (RELA) lacking phosphorylation on serine-311. (**A**) Transgenic mouse brain tumor cells (tmBTCs) harbored Tp53 point mutations or ablation of Tp53, Cdkn2a, Pten or Nf1 (indicated by "-"), overexpression of platelet-derived growth factor-B (P), epidermal growth factor receptor (E), mutant EGFR (vIII) or both (E+vIII); CBD-induced cytotoxicity (fold-change) was determined and compared to wild-type (WT) controls. (**B**) *Tp53*^{R172H}, PDGFB tmBTCs were investigated in a genome-wide pharmacogenomics screen; CBD cytotoxicity was prevented by distinct knockouts (skyscraper plot). (**C**) Pharmacogenomics indicated that NF$_{\kappa}$B and selenoprotein pathways (*Secisbp2l*) control tmBTC death. (**D**) CBD-induced cytotoxicity of *Tp53*^{R172H}, PDGFB cells was abrogated by conditional RelA knockout (KO, as compared to wild-type, WT, or heterozygous, het, controls). **(E)** RNAseq of *Tp53*^{R172H}, PDGFB, *Rela*^{KO} or *Tp53*^{R172H}, PDGFB, *Rela*^{WT} tmBTCs (with or without CBD application): GO-analysis showed that RELA promoted hGSC viability (p-adj. = 8.7×10^{-9}); 283 genes were coherently regulated by RELA or CBD application; CBD downregulated *Prkcz* (PKCζ), 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 One-Way ANOVA with Bonferroni´s pair-wise comparison test, in (D and F) by One-Way-ANOVA plus Tukey post-hoc testing (NS = not-significant; *$p<0.05$; **$p<0.005$; ***$p<0.001$).

Fig. 2. CBD is a promising therapeutic for a large range of human 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 µM) and cytotoxicity was quantified: Controls (grey 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 temozlomide; TMZ) in a pulse-

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chase paradigm; statistical significance in (A and B) was investigated by One-Way ANOVA with Bonferroni´s pair-wise comparison test (NS = not-significant; p<0.05*; P<0.005**; p<0.001***; p<0.0001****).

Fig. 3. RELA is essential for CBD-induced cytotoxicity of human 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 versus controls (arbitrarily set as "1", dotted-line) is indicated; note that highly significant changes were restricted to the $NF_{\kappa}B$ subunit RELA. (**B**) CBD-induced cytotoxicity was quantified (as compared to controls, dotted line) with or without SN50 (RELA inhibitor) pre-treatment; note that SN50 prevented CBD-induced cytotoxicity. (**C**) Immunofluorescence for RELA in Line2 hGSC under control conditions and after stimulation with TNFα or CBD; note that TNFα induced a rapid (1 h) nuclear shift of RELA, CBD-induced nuclear RELA accumulation was slow (visualized at 16 h) and persistent (for more than 10 h); SN50 prevented nuclear transfer of RELA. Scale in (c) indicates 10 µm; dots (in A and B) represent data from independent experiments; statistical significance was investigated by One-Way-ANOVA plus Tukey post-hoc testing (* p<0.05; **p<0.005; ***p<0.001).

Fig. 4. CBD-induced death of human GSC via sustained nuclear localization of RELA-lacking phospho-Ser311. (**A**) RelA activity in hGSC maintained under control conditions (Ctrl), stimulated with CBD (10 µM) or with TNFα (10 ng/ml); note that TNFα 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): 1h after stimulation with TNFα nuclear translocation and phosphorylation of RelA was restricted to CBD-sensitive hGSC and co-application of CBD reduced P-RELA levels; 16h after stimulation TNFα response had ceased but CBD induced

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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 One-Way-ANOVA plus Tukey post-hoc testing (**p<0.005; ***p<0.001).

Fig. 5. ROS levels in human GSC impact on NF$_{K}$B signaling and control CBD 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 activity of the TNFα 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): $r^2 = 0,8834$ ($p<0.0001$; F-test).

Figure 6. ROS level of human GSC-biopsies predict therapeutic response to 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 ± 10 mm^3) and

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analyzed by flow cytometry. 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 (grey symbols) were stringently separated from tumors. Dots in (B) represent data from individual tumors. Statistical significance in (C) was investigated by One-Way-ANOVA plus Tukey post-hoc testing (p<0.005**; ***p<0.001); statistical values (P) for Kaplan Meier studies are indicated (in C).

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Figure 1

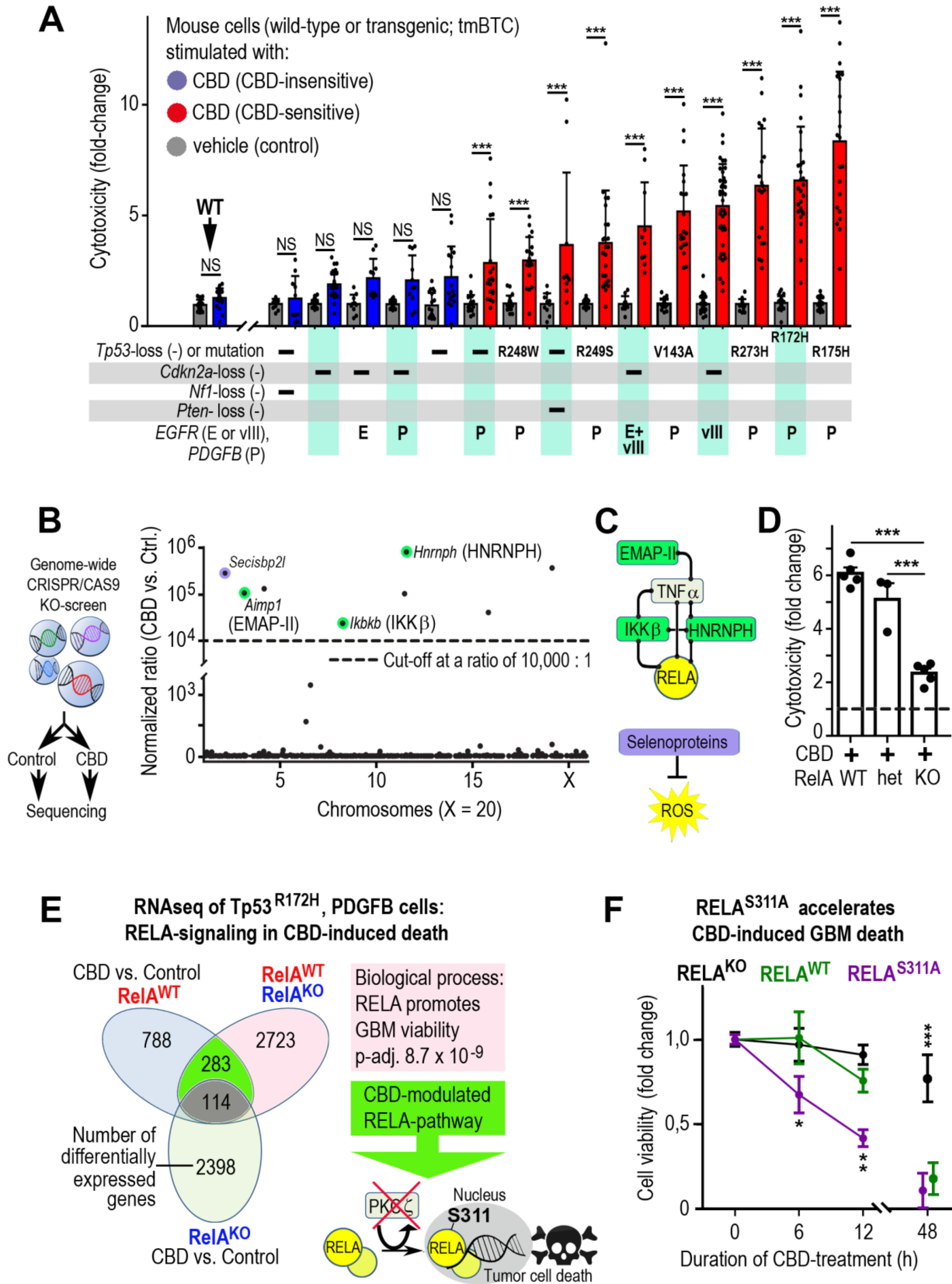

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Figure 2

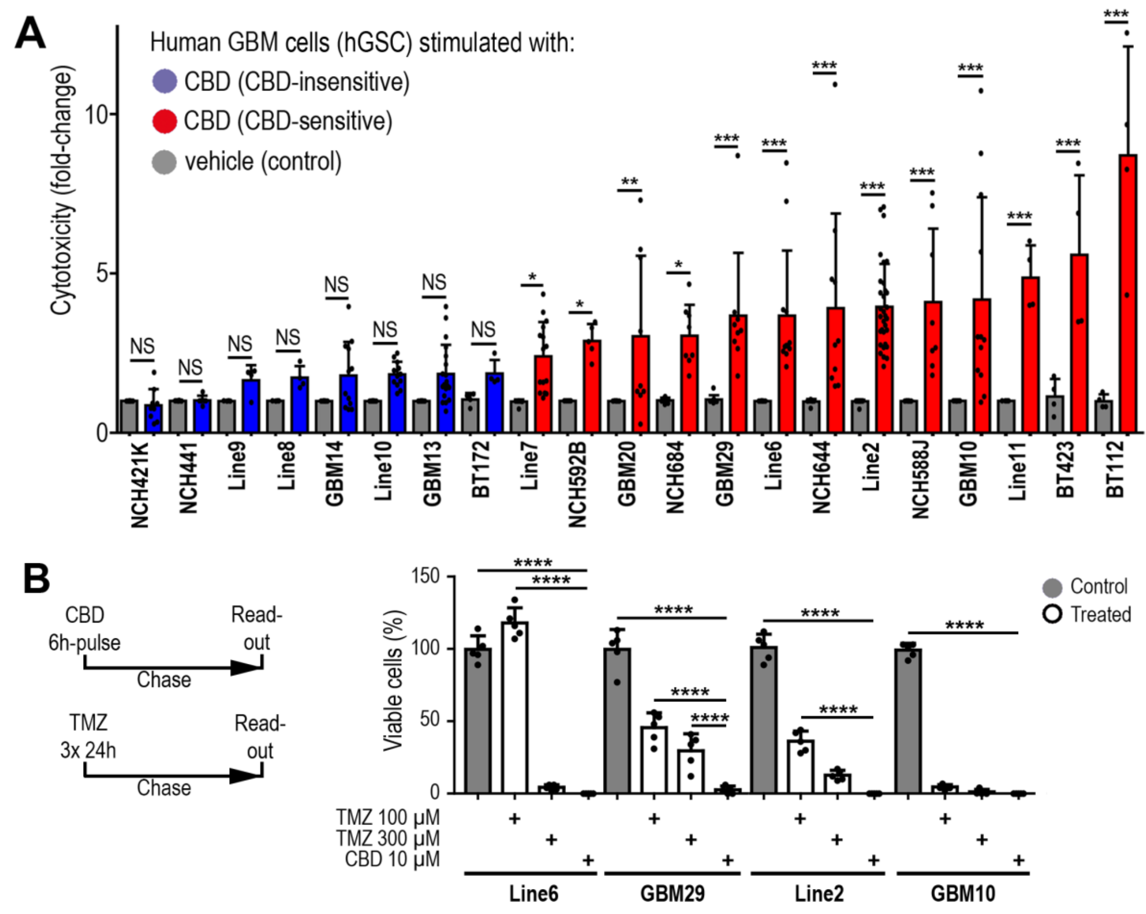

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Figure 3

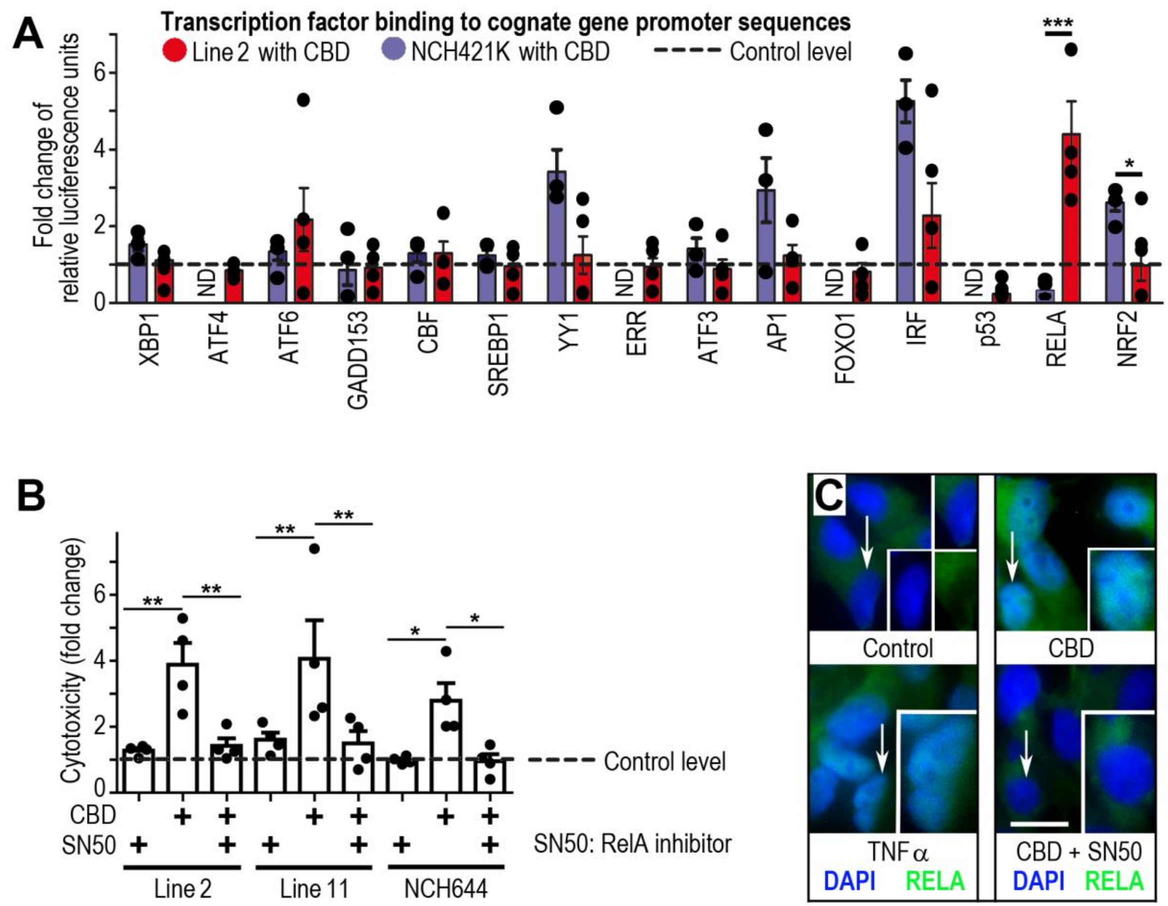

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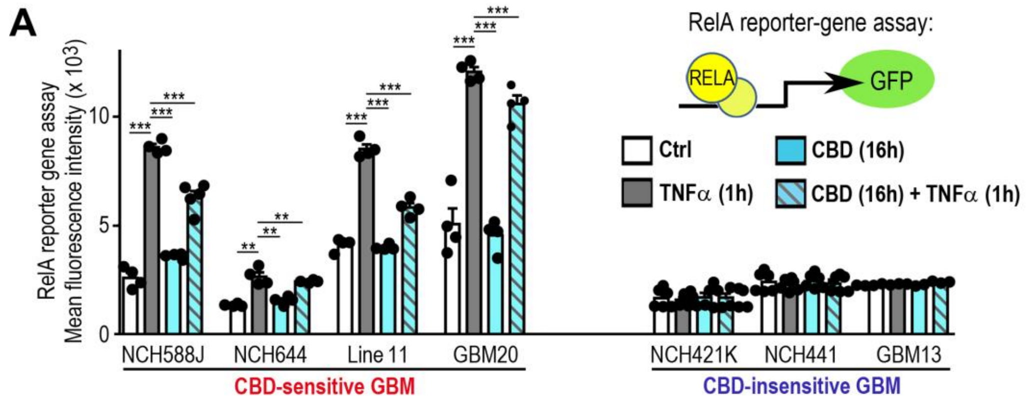
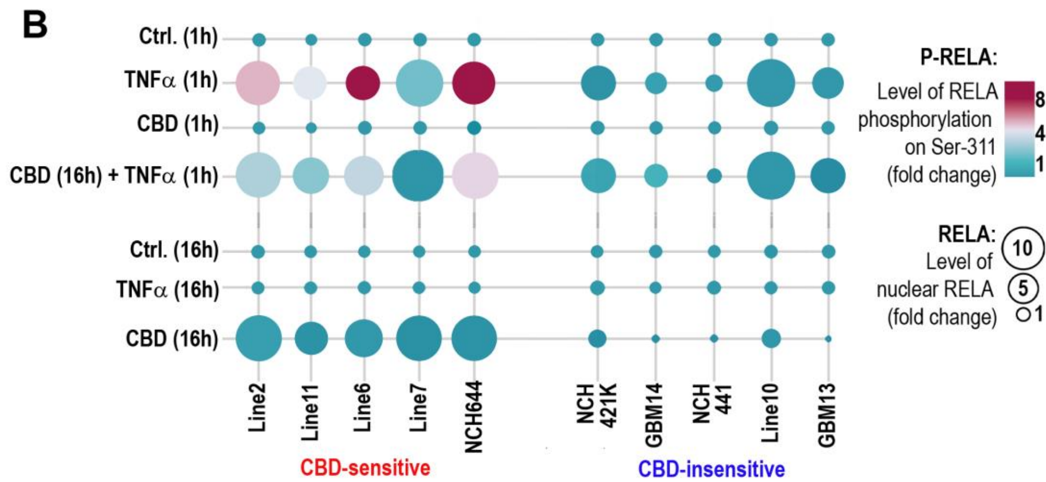
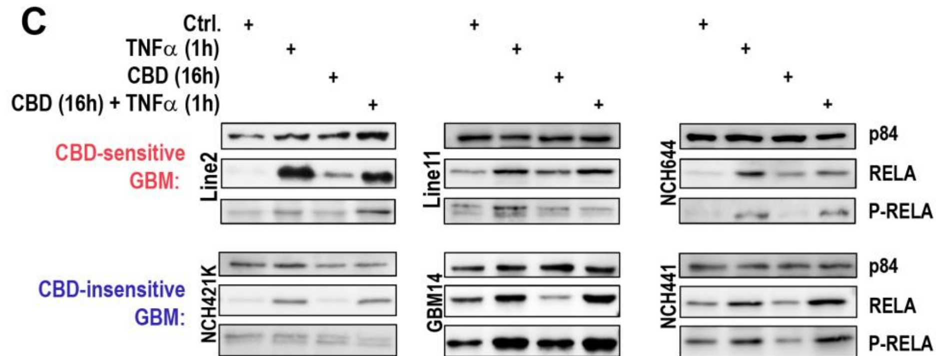
Figure 4







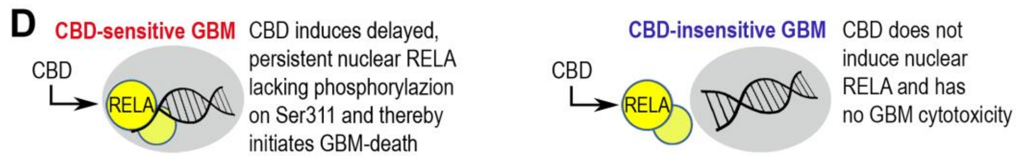


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Figure 5

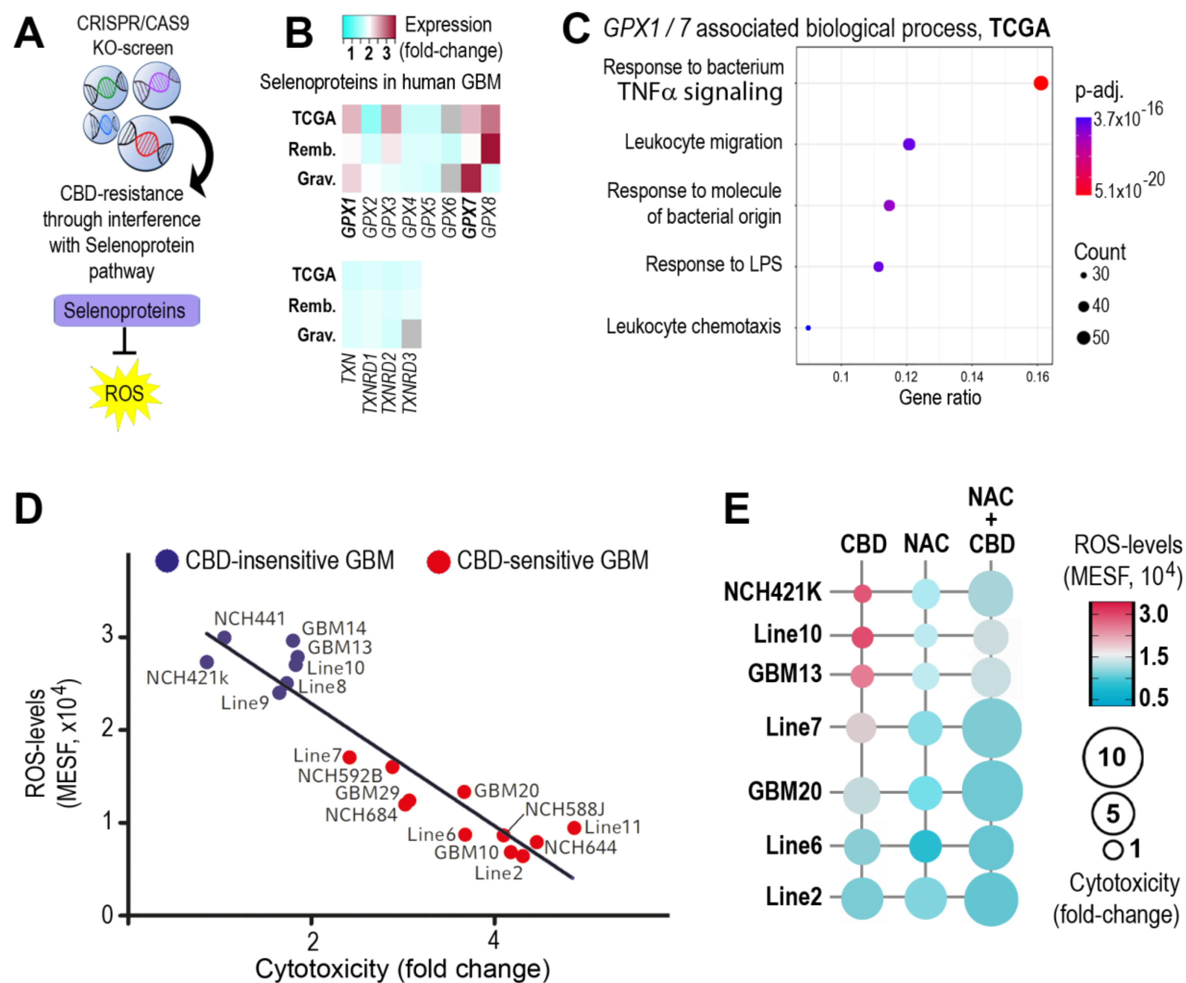

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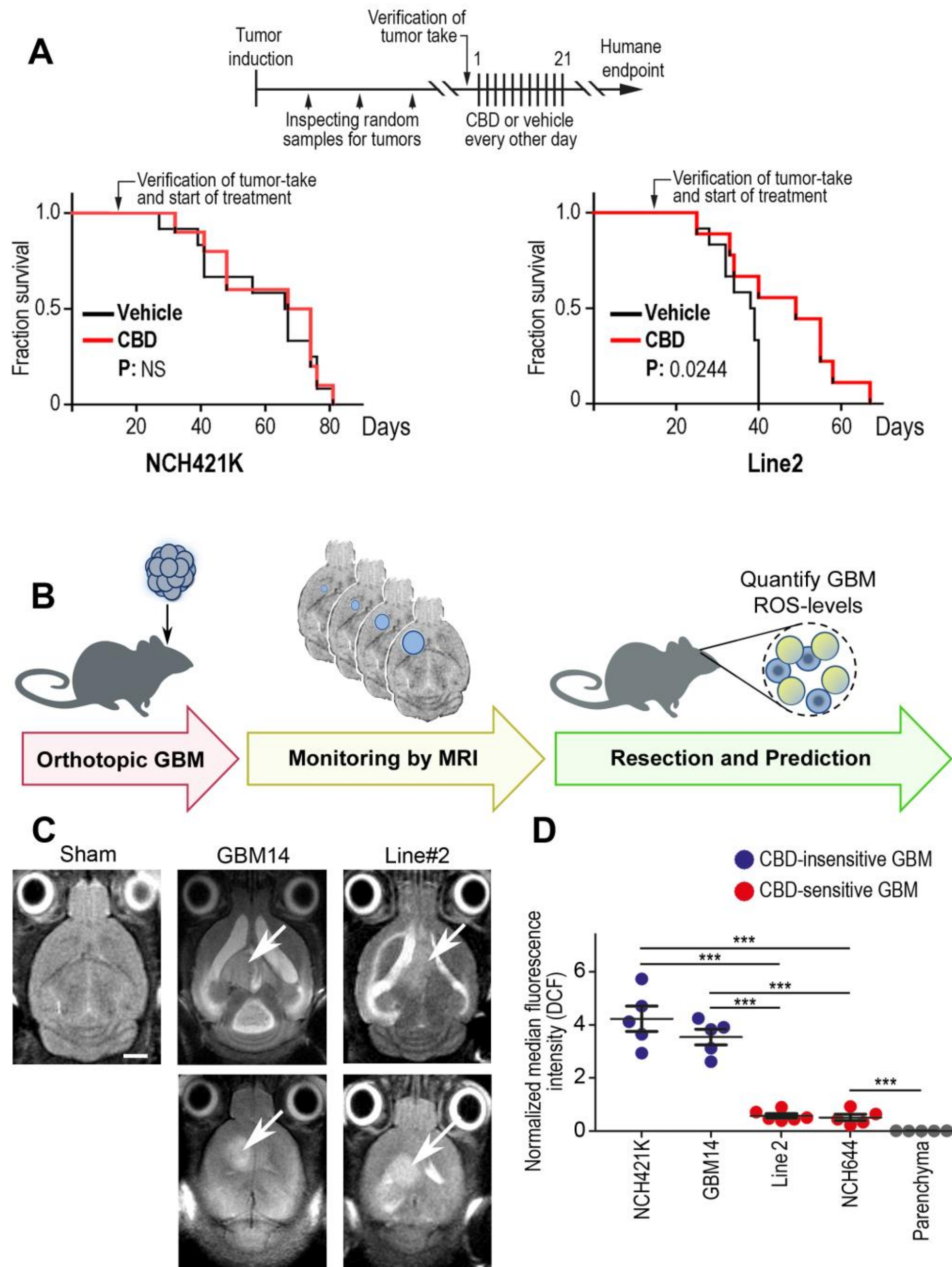
Figure 6



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