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DUSP4 appears to be a highly localized endogenous inhibitor of epileptic signaling in human neocortex

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Abstract

Background: We previously identified the Mitogen Activated Protein Kinase (MAPK) pathway as focally upregulated in brain regions with high epileptic activity and showed that inhibition of MAPK signaling reduces epileptic spiking in an animal model. Here we examined how activators and inhibitors of the MAPK pathway are expressed in human epileptic cortex and how these could contribute to the localization of epileptic signaling.

Methods: We localized gene and protein expression in human epileptic neocortical tissues based on epileptic activities from 20 patients based on long-term intracranial recordings. Follow-up mechanistic studies by depolarization of human Sh-SY5Y cell line were used to model epileptic activity in the human brain.

Results: A clustering algorithm of differentially expressed genes identified a unique gene expression cluster distinct from other *MAPK* genes. Within this cluster was dual specificity phosphatase 4 (*DUSP4*), a potent MAPK inhibitor. In situ hybridization studies revealed focal patches of *DUSP4* mRNA in layer 2/3 brain regions associated with a dramatic reduction in *MAPK* signaling genes. In vitro depolarization led to the rapid and transient induction of DUSP4 protein, which, in turn, reduced MAPK activity. Activity-dependent induction of DUSP4 protein

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CRediT Authorization Statement

Kirchner, Allison: Conceptualization, methodology, validation, formal analysis, investigation, writing-original draft, visualization, funding acquisition. **Bagla, Shruti:** Conceptualization, methodology, investigation. **Dachet, Fabien:** Software, formal analysis. **Loeb, JA:** Conceptualization, methodology, writing-review and editing, visualization, supervision, project administration, funding acquisition.

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was transient and required MAPK signaling. Human epileptic brain regions with lower epileptic activity had lower MAPK activity and higher DUSP4 protein levels.

Discussion: DUSP4 is a highly localized, endogenous feedback inhibitor of pro-epileptogenic MAPK signaling in the human epileptic brain. Increasing DUSP4 expression could therefore be a novel therapeutic approach to prevent the development and spread of epileptic circuits.

Keywords

Epilepsy; Human Studies; Translational; MAPK signaling

1. Introduction:

Because the human brain has unique structural and molecular features not present in animal models, we developed a systems approach to study surgically resected neocortical human epileptic tissues (Kirchner et al., 2019; Loeb, 2010). Quantitative, long-term intracranial electrocorticography (ECoG) is used to identify seizure onset zones and interictal spiking regions. Tissues with different electrical activities in vivo are then compared to one another by subdividing each region for histology, genomics (Beaumont et al., 2012; Dachet et al., 2015; Lipovich et al., 2012), proteomics (Keren-Aviram et al., 2018) and metabolomics (Wu et al., 2017).

From this approach, we discovered that the Mitogen Activated Protein Kinase (MAPK) pathway is constitutively activated in specific cortical lamina in human epileptic brain (Beaumont et al., 2012). The MAPK pathway is a ubiquitous pathway found in all cell types throughout the body and is implicated in cell growth and cell cycle progression (Burotto et al., 2014; Roux et al., 2007; Shaul & Seger, 2007; Thomas & Huganir, 2004). Within this pathway, and specifically in the brain, MEK-ERK signaling has been implicated in long term potentiation and spine stability (Atkins et al., 1998; English & Sweatt, 1996; English & Sweatt, 1997; Thomas & Huganir, 2004). This suggests that MAPK signaling together with the frequent spiking that occurs between seizures, also known as interictal spiking, could produce a positive feedback loop that maintains the epileptic circuitry (Kirchner et al., 2019). Consistently, inhibition of the MAPK pathway using a MEK inhibitor in an animal model of interictal spiking prevented the development of epileptic spiking (Barkmeier et al., 2012).

While epilepsy is an often life-long condition, not all brain injuries produce epilepsy (Grant & Laskowitz, 2016; Hauser et al., 1993), not all brain regions are equally epileptic (Annegers, 1993), and, once formed, epileptic circuits are mostly stable making their surgical removal a successful treatment approach (Spencer & Huh, 2008). This suggests the existence of endogenous mechanisms to sequester or prevent the spread of epileptic circuits.

Here we describe evidence that a highly potent MAPK inhibitory protein called dual specificity phosphatase 4 (DUSP4) is an endogenous inhibitor of epileptic signaling in the human neocortex. Using an unbiased genomic approach, we identified *DUSP4* within a group of induced genes that clustered independently from previously defined *MAPK* genes in the human epileptic neocortex, suggesting a different spatial pattern of expression.

Consistently, we discovered small 'patches' of *DUSP4* mRNA expression in layers 2/3 of epileptic human neocortex that show a marked reduction of *MAPK* genes. Areas of increased DUSP4 protein expression show less MAPK activation and decreased interictal spiking. In vitro, DUSP4 acts as a potent MAPK antagonist that is rapidly and transiently induced after repeated depolarizations and dependent on MAPK signaling. These findings demonstrate that DUSP4 is an activity-dependent, negative feedback inhibitor of MAPK signaling expressed in focal brain regions that could act as a localized, endogenous inhibitor for the spread of epileptic signaling.

2. Materials and Methods:

2.1 Human epileptic neocortical tissue and ECoG:

Human epileptic neocortical tissue samples were obtained from 20 patients who underwent a two-staged surgery for drug resistant epilepsy. While these 20 patients have a variety of underlying brain pathologies and range in age from 11 months to 51 years (Dachet et al., 2020 in review) (Table 1), brain regions chosen for genomic analysis were all normal-appearing cortex without lesions (examined by a neuropathologist) and chosen based on their in vivo electrical properties. Samples were received following informed consent as part of a research protocol that was approved by an Institutional Review Board at Wayne State University and at the University of Illinois at Chicago. Patients first underwent long-term, invasive electrocorticography (ECoG) recording with electrodes placed directly on the neocortical surface of the brain to identify regions of seizure onset which were then removed in the second stage of the procedure (Kirchner et al, 2019). Medications were restarted the day prior to tissue resection, stopping all clinical seizures so gene expression changes are unlikely to be due to acute seizures. No additional tissue was removed for these studies, however, large en bloc resections often yield samples ranging from high to no epileptic activity based on the long-term recordings. Once extracted, $1cm^3$ tissue samples corresponding to a single overlying ECoG electrode were divided in half (Kirchner et al., 2019). One half was frozen on dry ice and stored at −80°C for molecular analysis while the other was fixed in 4% paraformaldehyde for 48 hours and later embedded in OCT for histological studies (Kirchner et al., 2019). Each tissue sample is identified by the ECoG electrode number and corresponding interictal activity, was calculated as the number of spikes in 10 minutes averaged from three independent ten-minute recordings using an automatic algorithm described previously (Barkmeier et al., 2011; Dachet et al., 2015; Maharathi et al., 2019). All data and tissue are stored with de-identified information in the University of Illinois Neuro-repository (UINR).

2.2 Microarray and gene clustering:

RNA was extracted from equal portions of layers I-VI for each human epileptic neocortical tissue sample using RNeasy Qiagen Lipid Kit according to manufacturer's instructions. Areas of high and low interictal activity were defined by ECoG recording. Tissue samples were chosen after they were verified by a neuropathologist to be devoid of structural or anatomical abnormalities. This was followed by microarray analysis using Agilent microarrays as described (Dachet et al., 2015). A dye-flip quadruplicate, two-color microarray analysis was performed to identify differentially expressed genes with a fold

change (FC)>1.2 and a false discovery rate (FDR) <1%, between areas of high and low interictal activity within each patient. Based on these parameters, we identified 1122 differentially expressed genes between high and low spiking regions from 20 patients (Dachet et al., 2020 in review). Differentially regulated MAPK pathway genes (158) were identified by ontological analysis. The probe expression profiles across the 40 samples were then clustered using 'R' software using the Pearson correlation p-value < 10^{-6} (R≥0.69, 40 samples) and visualized using the Cytoscape software (Smoot et al., 2011) The plugin AlegroMcode (AllegroViva Corporation, 2011) was used with default parameters to identify clusters. The clusters were labeled manually using some of the gene names within a given cluster. Gene expression within each cluster was standardized by the z-score then average-weighted with the number of degrees (number of correlations of each node) using the software 'R' (R Development Core Team 2010) (Dachet et al., 2019).

2.3 In situ hybridization (ISH):

Non-radioactive ISH was performed using Digoxigenin (Dig)-labeled RNA probes using the high epileptic spiking areas of human epileptic neocortical samples from 8 patients. Sense and antisense probes were generated from plasmids (*EGR1, DUSP4 and DUSP6*) and transcribed in vitro (Roche). Probes were hydrolyzed at 60°C using 60 mM Na_2CO3 and 40 mM $NaHCO_3$, pH 10.2. Hydrolyzed probes were purified on Sephadex G-50 spin columns and washed successively with 70% and 100% ethanol and then air-dried. Dried-up RNA pellets was dissolved in DEPC water and stored at −20°C. Sections with a 20 μm thickness previously defined as high-spiking human epileptic samples were used to examine the spatial expression of genes of interest. Sections were fixed in 4% paraformaldehyde (PFA) at room temperature (RT) for 10 min followed by three washes of phosphate-buffered saline + 0.1% Triton X-100, 5 min each, and treatment with 1 μg/ml proteinase K (Sigma) for 10 minutes at RT. After a second round of washing and fixation, sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 for 15 min. After another washing, the sections were incubated in pre-hybridization buffer (10mM Tris-HCl pH 7.5, 600mM NaCl, 1mM 0.5% EDTA, 25% SDS, 1X Dendhardt's solution, 50% Formamide and 300μg/ml yeast tRNA in DEPC Water) for 4 hours at 60°C. The sections were then incubated with 2 μg/ml probes in hybridization buffer (20mM Tris-HCl pH 7.5, 0.3 M NaCl, 5mM EDTA, 1X Denhardt's solution (Sigma), 50% formamide, 500 μg/ml yeast tRNA, 10% Dextran Sulphate, 10 mM NaPO4 in DEPC water) at 60°C overnight. The next day, the sections were washed in 1X SSC containing 50% formamide for 10 min at 65°C followed by treatment with 20 μg/ml RNase A (Sigma) in 10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA, at 37°C for 30 min to remove excess unhybridized RNA and sequential washes in 2x, 0.2x and 0.2x SSC washes, 20 minutes each at 65°C. The sections were then processed using Roche Washing/Blocking/Detection kit. They were incubated at 4°C in sheep anti-digoxigenin conjugated with alkaline phosphatase (Roche) overnight. The next day the signal was detected using BM Purple (nitroblue-tetrazolium-chloride (NBT)/5- bromo-4-chlor-indolyl-phosphate) as a substrate.

2.4 Cell culture, depolarizations, and electroporation:

Sh-SY5Y cells (p4-p17) purchased from ATCC were used in all in vitro studies. Cells were grown in high glucose DMEM (Gibco), supplemented with 10% Fetal Bovine Serum (FBS)

(Gibco), and 1% Penicillin-Streptomycin (Sigma). Cell lines were maintained in a sterile incubator at 37°C, 5%CO_2. For depolarization experiments, cells were treated with 100mM KCl in DMEM for 5 minutes. After which the KCl containing media was replaced with normal culture media. The depolarizations were repeated every two hours to create a sustained activated MAPK signaling profile that is similar to the one observed in the epileptic brain (Lipovich et al., 2012). Of note, in vitro KCl depolarizations have demonstrated similar activation of pathways in comparison to in vivo models of electroconvulsive shock (Kim Y. et al., 2008). Cells were harvested at various time-points as indicated by previous studies and as designated in figures. For transfections, cells were electroporated using the Neon Electroporation machine (1200 V, pulse number 2, pulse width 20) based on previously determined parameters for this cell line (Lipovich et al., 2012). *Silencer select* siRNA targeting *DUSP4* and control siRNA were purchased from ThermoFisher and used at a final concentration of 100nM. *DUSP4* plasmid and control pCMV plasmid were purchased from Roche and used at a final concentration of 5μg/mL.

2.5 Reagents:

Cycloheximide (Cell Signaling) was used at a concentration of 10μg/mL in DMEM for in vitro studies in Sh-SY5Y cells. MG132 (Cell Signaling) was used at 10μM in DMEM. PD184352 (PD18), a highly selective MEK1/2 inhibitor, was used at a concentration of 5μM in DMEM (Sebolt-Leopold et at., 1999). The vehicle for all experiments was DMSO.

2.6 Reverse transcription and quantitative PCR (qPCR):

For studies with human epileptic neocortical tissue samples, RNA was extracted from brain regions underlying each electrode using the RNeasy Qiagen Lipid MiniKit according to manufacturer's instructions. Of note, only layers I-IV were used for RNA extraction to guarantee that white matter was excluded. For in vitro experiments, RNA was extracted from the SY5Y cells using the RNeasy Qiagen MiniKit, according to manufacturer's instructions. Total RNA was then reverse transcribed using reagents purchased from ThermoFisher. Of note, equal amounts of total RNA were used for each experiment. For quantitative PCR (qPCR) reactions, Universal Taqman Master Mix (ThermoFisher) was used in combination with Taqman primer probes (ThermoFisher) for *GAPDH* (Hs02786624_g1), *DUSP4* (Hs01027785_m1), *DUSP6* (Hs0169257_m1), and *EGR1* (Hs0152928_m1). Results are represented as fold change from time matched control.

2.7 Western blots:

For the human epileptic neocortical tissue samples, alternating strips of frozen brain tissue (gray matter only; layers I-VI), underlying a single electrode, were pooled, processed and fractionated as previously described (Beaumont et al., 2012). The tissue samples included for western blot analysis were chosen based on *EGR1* mRNA expression levels. Coomassie (Bradford) Assay was used to determine the protein concentrations in each fraction, using Bovine Serum Albumin (BSA) as the standard. 30μg of protein was loaded onto each lane and subjected to SDS-PAGE, followed by transfer onto a PVDF membrane (Millipore). Membranes were blocked and incubated overnight in primary antibodies against EGR1 (Abcam, AV32241) (1:500), DUSP4 (Cell Signaling, #5149S) (1:1000), diphospho-ERK1/2 (dp-ERK) (Cell Signaling, #4370) (1:1000), ERK1/2 (ERK) (Cell Signaling, #4695)

(1:1000), and beta-actin (Cell Signaling, #3700) (1:3000) at 4°C in 5% milk in 1% tween in Tris buffered saline. HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG secondary antibodies (BioRad) were used and the signal was detected using ECL substrate (Thermo Scientific Super Signal West Pico Chemiluminescent Substrate). The proteins described are a similar molecular weight (42-44kDa) so Restore Stripping Buffer (Fisher Scientific) was used. Western blot results from the human epileptic neocortical tissue are from a minimum of 14 human tissue samples, 2 brain regions from 7 patients, performed in duplicate. Results are displayed as fold-change between the two brain regions within each patient. For in vitro studies, whole cell extracts were lysed in RIPA buffer (Sigma) supplemented with 1% Halt Protease Inhibitor. Protein was quantified using the Bradford assay. 10μg of each sample was loaded into individual lanes. Western blot results are displayed as the average from a minimum of three biological replicates, performed in duplicate. Results are displayed as fold change from the average control value per experiment. Controls were designated as 0-hour time-point for time-course experiments or mock transfection for electroporation experiments. Quantitation of band intensities was performed with local background subtraction using Metamorph software.

2.8 Experimental Design and Statistical Analysis:

All statistical analyses were performed using Graph Pad Prism 8.2. When comparing two groups, t-tests were performed. When comparing three groups, multiple time-points, or treatments, one-way ANOVA or two-way ANOVA with repeated measures (RM) was performed. To account for multiple comparisons, the Sidak test for multiple comparisons was performed. For test specifics, please see the result section and figure legends. The *n* stands for the sample size and each *n* is specified in the results section. Outliers were identified via ROUT outlier analysis with Shapiro-Wilk follow-up tests to confirm normally distributed data. Unless specified, all error bars are displayed as standard error mean (SEM). Significance was designated as $p<0.05$. For experiments that involved the application of compounds, vehicle controls were always included.

3. Results:

3.1 Unbiased clustering of MAPK gene transcription from human epileptic neocortical tissue reveals two independently expressed clusters.

Paired surgical samples comparing regions of high and low epileptic activity from each of 20 patients who underwent neocortical resections for drug-resistant epilepsy were used to measure differential gene expression (Dachet et al., 2015; Dachet et al., 2020 *in review*). A total of 1122 genes were identified as differentially regulated. Ontological analysis revealed that 158 of the 1122 differentially expressed genes were members of the MAPK signaling pathway, with most of these induced in epileptic brain regions consistent with previous human and animal studies (Beaumont et al., 2012; Barkmeier et al., 2012). Interestingly, both known activators and inhibitors of the MAPK pathway signaling were upregulated. To better understand better this apparent contradiction, we used a clustering algorithm and visualized the results using Cytoscape. We found 2 prominent clusters (Figure 1A). The first group (shown in RED) contains many previously described genes associated with epileptic MAPK signaling in neocortical epilepsy, such as *EGR1* (Beaumont et al., 2012). The second

group (shown in GREEN) that shows a unique expression pattern contains genes known to regulate MAPK signaling, including the potent MAPK inhibitor, *DUSP4*. A sample-by-sample correlation analysis using the z-score for each cluster across the 40 samples demonstrates that the expression patterns for each of the two clusters were poorly correlated with each other (p=0.35) suggesting a lack of precise co-expression (Figure 1B).

3.2 DUSP4 and MAPK signaling genes show well-demarcated but inverted patterns of expression in the human epileptic brain.

To understand the relationship between these two induced gene clusters we compared the spatial expression of a subset of genes from each cluster in human epileptic brain regions. MAPK signaling genes *EGR1* and *DUSP6* from the first cluster were compared to the expression of *DUSP4* from the second cluster on serial sections. Non-radioactive ISH showed that *DUSP4* was highly expressed in focal patches in superficial layers of the neocortex (Figure 2). This is the same cortical region where we found constitutive MAPK signaling activation linked to high epileptic spiking in both human and rat neocortex (Beaumont et al., 2012; Barkmeier et al., 2012). Remarkably, these patches of increased DUSP4 signaling were associated with dramatic reductions in the *EGR1* and *DUSP6* genes previously associated with high epileptic spiking. Similar findings were seen from a total of 6 patients. To be certain that these regions of downregulated MAPK genes were not due to structural abnormalities or experimental artifacts, an adjacent section was also hybridized with probes for *GAPDH* showing normal cortical architecture and cellular compositions. We also saw heightened *DUSP4* expression within deeper sulci where the expression of *EGR1* and *DUSP6* were reduced (Figure 2, sample B). This remarkable, inverted pattern of *DUSP4* vs MAPK gene expression raises the possibility that DUSP4 acts as a highly localized inhibitor of MAPK signaling genes in human epileptic neocortical tissue.

3.3 DUSP4 inhibits MAPK signaling.

DUSP4 directly blocks MAPK signaling by dephosphorylating ERK (Misra-Press et al., 1995; Patterson et al., 2009). To confirm a potential inhibitory role of DUSP4 on MAPK signaling and the downstream induction of MAPK signaling genes, such as EGR1, we knocked down DUSP4 in human neuronal-like Sh-SY5Y cells. Cells with a decrease in DUSP4 protein showed an increase in MAPK signaling (Figure 3). Knockdown of DUSP4 resulted in a 2-fold increase in the ratio of dpERK/ERK, indicating an increase in activated MAPK signaling. Downstream, qPCR was used to demonstrate that knockdown of DUSP4 mRNA resulted in a 4.5-fold increase in the EGR1 mRNA (Figure 3E). Consistently, we found that overexpression of DUSP4 led to significant reduction in MAPK signaling (Figure 4). Overexpression of DUSP4 resulted in a marked reduction of both dpERK and total ERK levels. Taken together, these results demonstrate DUSP4 as a potent inhibitor of MAPK signaling in human neuronal-like Sh-SY5Y cells.

3.4 DUSP4 is an activity-dependent negative feedback inhibitor of MAPK signaling.

As a means to explore the role of the ongoing epileptic activity on both MAPK signaling and DUSP4 expression, we modeled this in vitro by repeatedly depolarized Sh-SY5Y cells. Repeated depolarizations with 100 mM KCl produce sustained MAPK/CREB activation (Lipovich et al., 2012) and resulted in a significant increase in *EGR1* mRNA peaking at 4

hours (Figure 5A). This in vitro model was developed to produce sustained MAPK/CREB activation that mimics what we have seen in human brain regions with frequent interictal epileptic discharges. Prior to the *EGR1* peak, we observed a significant, but transient increase in the *DUSP4* mRNA at 2 hours (Figure 5A) followed by a 6-fold transient increase in DUSP4 protein expression at 4 hours (Figure 5B, 5C), coincident with the *EGR1* mRNA peak. After 4 hours, a steady reduction in *EGR1* mRNA expression occurs that goes back to baseline in 24 hours. These findings, taken together with Figure 3, further suggest that DUSP4 functions as an activity-dependent negative feedback inhibitor of MAPK signaling.

We further explored the basis of the transient nature of DUSP4 protein expression and stability (Crowell et al., 2014; Peng et al., 2010) using cycloheximide, a S6 ribosome inhibitor, to prevent protein translation as well as MG132, a proteasome inhibitor to prevent protein degradation by the ubiquitin-proteasome pathway. Cycloheximide significantly reduced DUSP4 expression at 4- and 8-hour time points, while MG132 increased DUSP4 expression at 8 and 12 hours (Figure 6A, 6B). This indicates that DUSP4 protein expression is maintained by a balance between the rate of translation and degradation through proteasomal pathways.

Previous studies have also demonstrated that DUSP4 expression is regulated by MAPK signaling (Brondello et al., 1997; Peng et al., 2010). In order to determine if the activity dependent increase in DUSP4 expression is regulated by MAPK signaling, we applied a MEK inhibitor. In the presence of 5uM PD184352 (PD18), DUSP4 protein was significantly decreased at 4 hours of depolarizations ($p=0.0006$, two-way ANOVA, $n=3$) (Figure 6C, 6D). This suggests that DUSP4 functions as an activity-dependent feedback inhibitor of MAPK signaling.

3.5 Human brain regions with high DUSP4 protein have lower EGR1 and less epileptic spiking.

Given the remarkable inverse relationship between *DUSP4* and *EGR1/DUSP6* mRNA expression in superficial cortical layers in human epileptic cortex (Fig. 2), we further examined the relationship between DUSP4 protein, MAPK signaling, and epileptic spike frequency (number of spikes per 10 minutes) in human cortex. As shown previously (Beaumont et al., 2012), brain regions with high MAPK signaling demonstrate high expression levels of both EGR1 mRNA and protein (Figure 7). We defined these brain regions as high and low MAPK signaling based on EGR1 expression within each patient. Interestingly, DUSP4 protein expression is significantly reduced in a majority of these same regions of high MAPK signaling and is increased in regions with low MAPK signaling (Figure 7B, 7D) in 7 out of 7 patients.

Going back to the long term ECoG recordings, we found a relative increase in spike frequency in the high MAPK regions compared to low MAPK regions in paired samples from 5 out of the 7 patients Fig. 7E). Consistent with the in situ hybridization studies of DUSP4 mRNA in Fig. 2, these findings provide a functional correlation between epileptic electrical activity and MAPK signaling that are both reduced when DUSP4 protein is expressed at higher levels.

4. Discussion:

While DUSP4 has been previously studied in cancer (Hijiya et al., 2016; Waha et al., 2010) and is highly expressed in neuronal tissue (Misra-Press et al., 1995), the role of DUSP4 in the nervous system has not been characterized. DUSP4 has been associated with aberrant synapse formation, suggesting a critical role in synaptic transmission that could be part of its role in epilepsy (Abdul Rahman et al., 2016). Using an unbiased approach, we discovered a remarkable, reciprocal relationship between DUSP4 and epileptogenic MAPK signaling gene expression patterns, suggesting that DUSP4 acts within focal brain regions to “turn off” MAPK signaling pathways. In vitro studies confirmed that DUSP4 expression reduces ERK activation and downstream MAPK gene transcription and is induced by ongoing activity.

One common attribute of most forms of focal epilepsy is that seizures start in localized brain regions based on underlying neuronal networks so that, once formed, there is little change in the network over time (Spencer & Huh, 2008). This is why surgical resections can lead to significant reductions or elimination of epileptic activities if seizure foci are successfully identified and resected. Exactly how these epileptic circuits remain localized is not known. Our observations here suggest that DUSP4 expression could be an endogenous inhibitor that quarantines these networks and keeps them from spreading more diffusely throughout the brain. Specifically, we found that brain regions with lower levels of epileptic activity and lower levels of MAPK gene inductions express higher levels of DUSP4 protein. This observation is supported by our previously published results demonstrating increased epileptic activity associated with constitutively activated MAPK signaling in humans and an animal model in superficial neocortical lamina (layers 2/3) (Barkmeier et al., 2012; Beaumont et al., 2012). How these regions of high spiking activity relate to seizure onset zones is still unclear and is a topic of ongoing studies.

The spatial organization of *DUSP4* expression in focal ‘patches’ within superficial neocortical lamina supports this role since these patches show a remarkable inverse expression of *DUSP4* and activating MAPK genes/proteins. Exactly how these patches form is not clear, but it can be speculated that they are protective in preventing the spread of neuronal synchrony to adjacent cortical regions. *DUSP4* mRNA expression was also noted to be increased within deeper cortical sulci that show reduced MAPK signaling. In fact, a recent study that mapped the propagation patterns of epileptic discharges in the human neocortex found that deep sulci, specifically the central sulcus, appear to act as a barrier for spike propagation (Maharathi et al., 2019). Taken together, our findings suggest that DUSP4 creates inhibitory brain regions along the cortical surface and within deep sulci that prevent the build-up and spread of epileptic activity.

While DUSP4 inhibits the MAPK pathway, previous studies have demonstrated that DUSP4 is itself regulated by MAPK activity (Brondello et al., 1997; Peng et al., 2010). Our studies confirm this in the setting of chronic depolarization since MEK inhibition resulted in a significant reduction of DUSP4 protein. This finding suggests that DUSP4 is an integral part of an activity dependent negative feedback loop in the epileptic brain, where DUSP4 expression is induced by activity-dependent MAPK signaling and then feeds back to inhibit

the MAPK pathway. This likely acts as a spatially segregated safety mechanism to prevent over excitation and to prevent the spread of epileptic activity.

Unexpectedly, in addition to reducing phosphorylation of ERK, our results showed that DUSP4 dramatically reduces total ERK protein expression (Figure 4). The regulation of ERK expression is not well studied, however, some studies have found that ERK activation can activate the SP1 transcription factor (Milanini-Mongiat et al., 2002), which can in turn induce the transcription of *ERK1* (Chu et al., 2005; Pagès et al., 1995). While these findings reveal potential new mechanisms into the maintenance of epileptic circuits, there are some limitations of this study. While using human tissue samples is highly relevant to human disease, there are inherent variabilities and limited sample sizes. We tried to reduce variability by normalizing each sample to an internal control.

Clinical and animal study data suggest that epileptic foci start out small and enlarge over time developing into full-blown epileptic circuits. Therefore short term inhibition of MAPK signaling through augmentation of DUSP4 expression could be a potential disease modifying or disease preventing treatment for epileptic circuits after a brain insult. Finding ways to induce DUSP4 could be a novel therapeutic approach. There are currently three FDA approved drugs that inhibit MAPK signaling, specifically MEK-ERK signaling, and are used in treating various cancers, in particular late stage melanoma (Grimaldi et al., 2017). However, the current MAPK inhibitors, when given systemically, cause serious side effects, especially in the GI tract and skin (Wang et al., 2007). A more targeted treatment that focuses on increasing DUSP4 expression selectively in the brain, could be more promising and result in less side effects.

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Significance Statement:

Epilepsy is a chronic debilitating disease. Once it develops, epileptic circuits often persist throughout life. Fortunately, in focal forms of epilepsy, these circuits can remain highly localized and are amenable to surgical resections, suggesting that endogenous mechanisms restrict their spread to other brain regions. Using a high-throughput genomic analysis of human epileptic brain regions, we identified DUSP4 as an activity-dependent inhibitor of MAPK signaling expressed in focal patches surrounding human neocortical epileptic brain regions. Our results suggest that DUSP4, through local inhibition of MAPK signaling, acts as an endogenous, spatially segregated safety mechanism to prevent the spread of epileptic activity. Augmenting DUSP4 expression could be a novel disease-modifying approach to prevent or treat human epilepsy.

Highlights:

- DUSP4 is expressed in focal patches surrounding human epileptic neocortex
- DUSP4 creates an activity-dependent negative feedback loop with MAPK signaling
- DUSP4 may function to restrict the spread of epileptic circuits

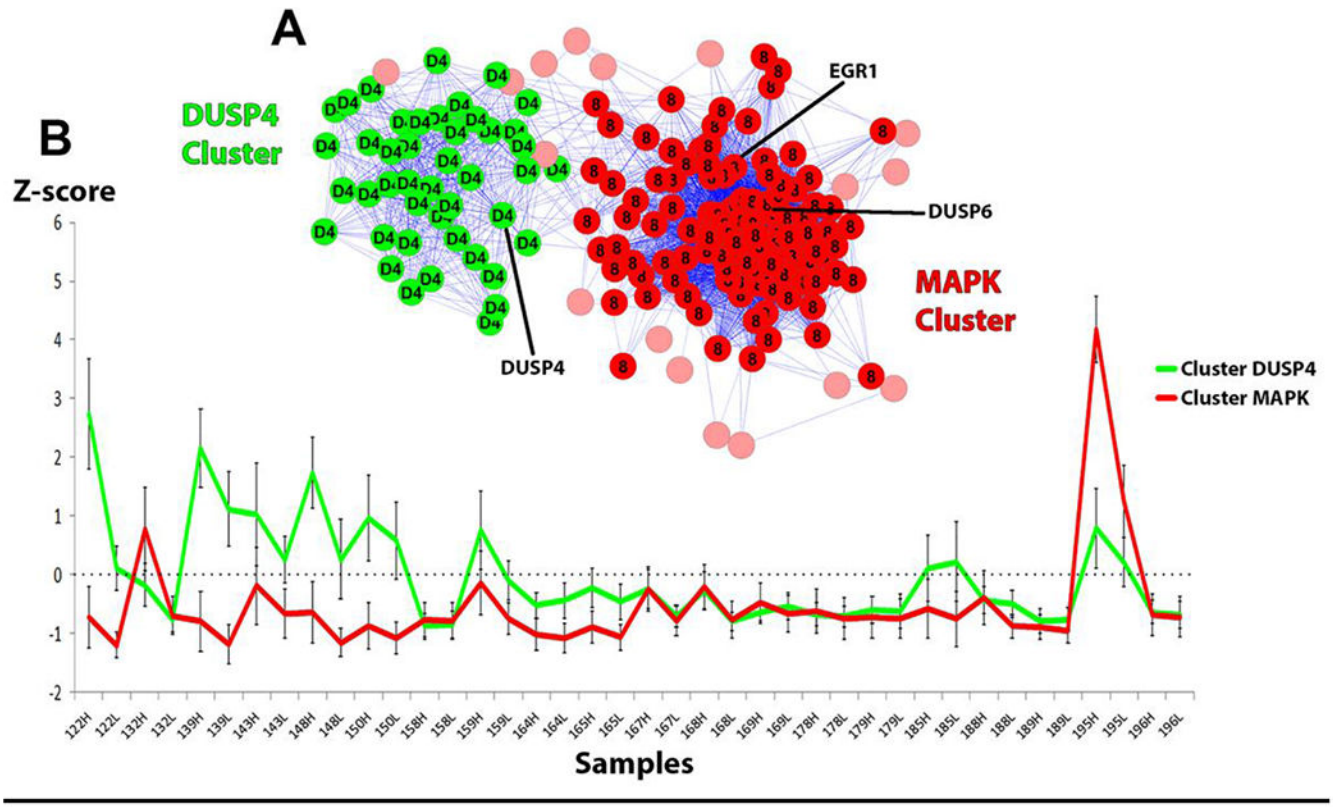

Figure 1: Identification and expression patterns of two distinct clusters of activity-dependent MAPK signaling genes in human neocortical tissue.

(A) Using AllegroMcode, we were able to separate MAPK genes into two discrete clusters with each gene represented as a colored circle. Cluster “DUSP4” in green is formed of genes marked “D4” and the cluster MAPK in red is formed of genes marked “8”. The cluster “DUSP4” includes the gene “DUSP4” while the cluster “MAPK” includes many genes previously implicated in epileptic brain regions including “EGR1” and ‘DUSP6’. The non-marked nodes colored pink correspond to genes that are not part of these clusters. Each link between genes correspond to a Pearson correlation p-value < 1E-6 (R≥0.69, 40 samples), the closer two genes are to each other, the more closely their expression profiles relate to one another. **(B)** The average expression pattern for each of these clusters is plotted for all 40 brain samples (H-high spiking; L-low spiking) from each of 20 epileptic patients using a z-score. Averages were weighted by the number of links so that a gene with a greater number of links will have greater significance. Vertical error bars correspond to standard deviations. A negative z-score value indicates an expression lower than the average; a positive z-score value indicates an expression higher than the average. This analysis shows a variable, non-identical, and often opposite expression pattern for the two clusters within each tissue sample.

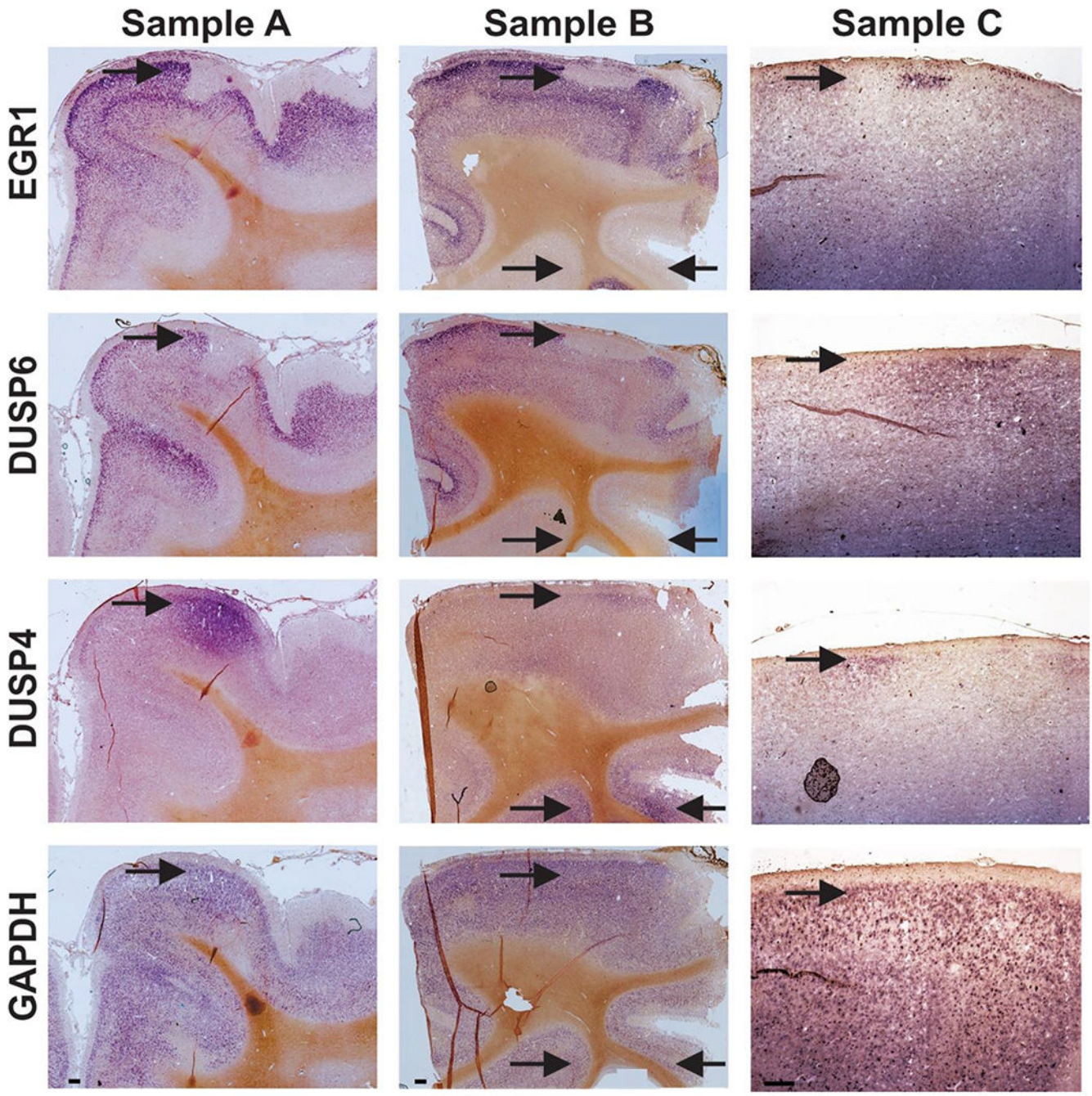

Figure 2: DUSP4 and MAPK genes are spatially and reciprocally expressed in human epileptic neocortex.

Images of non- radioactive in situ hybridizations from serial sections from 3 distinct neocortical regions demonstrate that MAPK signaling genes are significantly upregulated in superficial layers of the human epileptic neocortex. However, focal patches of *DUSP4* in these same superficial layers correspond precisely with the downregulation of the MAPK genes *EGR1* and *DUSP6*. *GAPDH* gene expression is used as a control to demonstrate that these patches are structurally intact. *DUSP4* was also seen to be highly expressed in deep cortical sulci, which also showed a marked reduction in MAPK genes. Arrows are used to

localize *DUSP4* expression in adjacent tissue sections. Scale bars in GAPDH images are all 500μm.

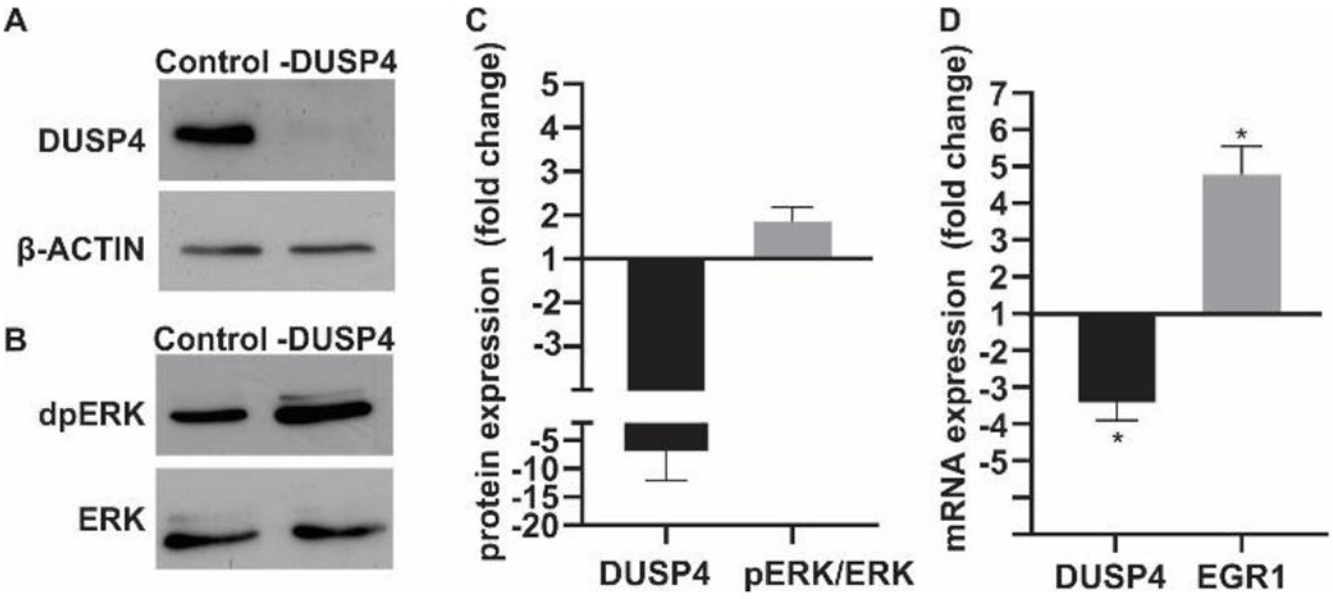

Figure 3: Downregulation of DUSP4 in vitro increases MAPK signaling and increases EGR1 mRNA.

(A) Targeted downregulation of DUSP4 using siRNA transfection in Sh-SY5Y cells significantly reduces DUSP4 protein expression in comparison to mock transfection (Control) by Western blot. **(B, C)** This results in a 2-fold increase in the ratio of diphosphorylated ERK (dpERK) to total ERK (ERK) ($p=0.116$, one sample t-test, n=3). Results are displayed as fold change in comparison to mock transfection. **(D)** Decreased in *DUSP4* mRNA following siRNA transfection (*$p<0.05$, one sample t-test, n=3) also results in 4.5-fold increase in *EGR1* mRNA (*$p<0.05$, one sample t-test, n=3). These results indicate that DUSP4 inhibits both MAPK signaling and the downstream transcription of MAPK signaling genes. Results are displayed as fold change and by comparing the gene of interest (*DUSP4* or *EGR1*) to *GAPDH*, followed by comparison to mock transfection.

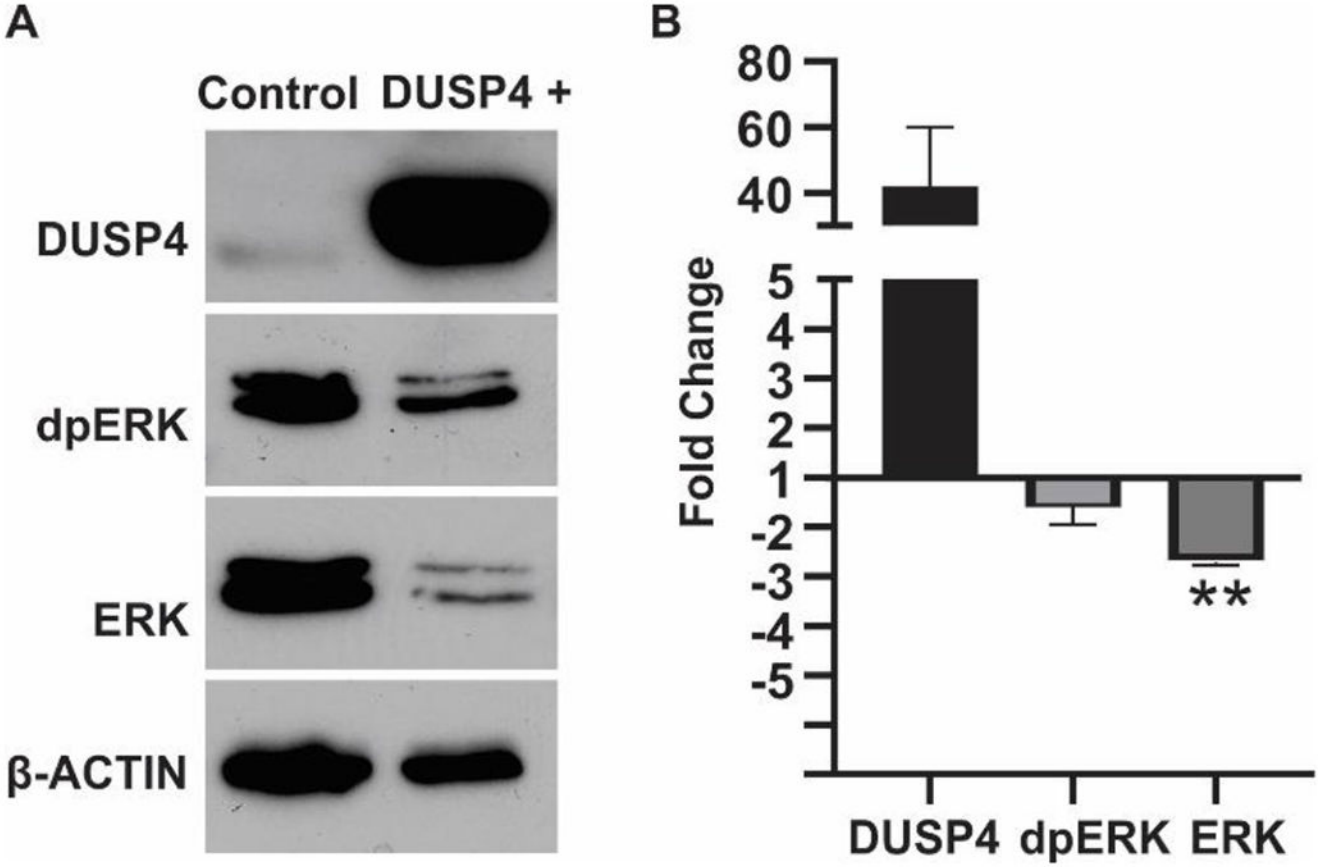

Figure 4: DUSP4 overexpression results in a decrease in dpERK and total ERK protein expression in vitro.

(A) Overexpression of DUSP4 (DUSP4+), compared to mock transfection as a control in Sh-SY5Y cells shown by Western blot analysis resulted in a significant increase in DUSP4 protein. **(B)** This led to a reduction in both dpERK/β-ACTIN ($p=0.197$, one sample t-test, $n=4$) and an even more significant decrease in total ERK/β-ACTIN (**$p<0.01$, one sample t-test, $n=3$). Results are displayed as fold change in comparison to control.

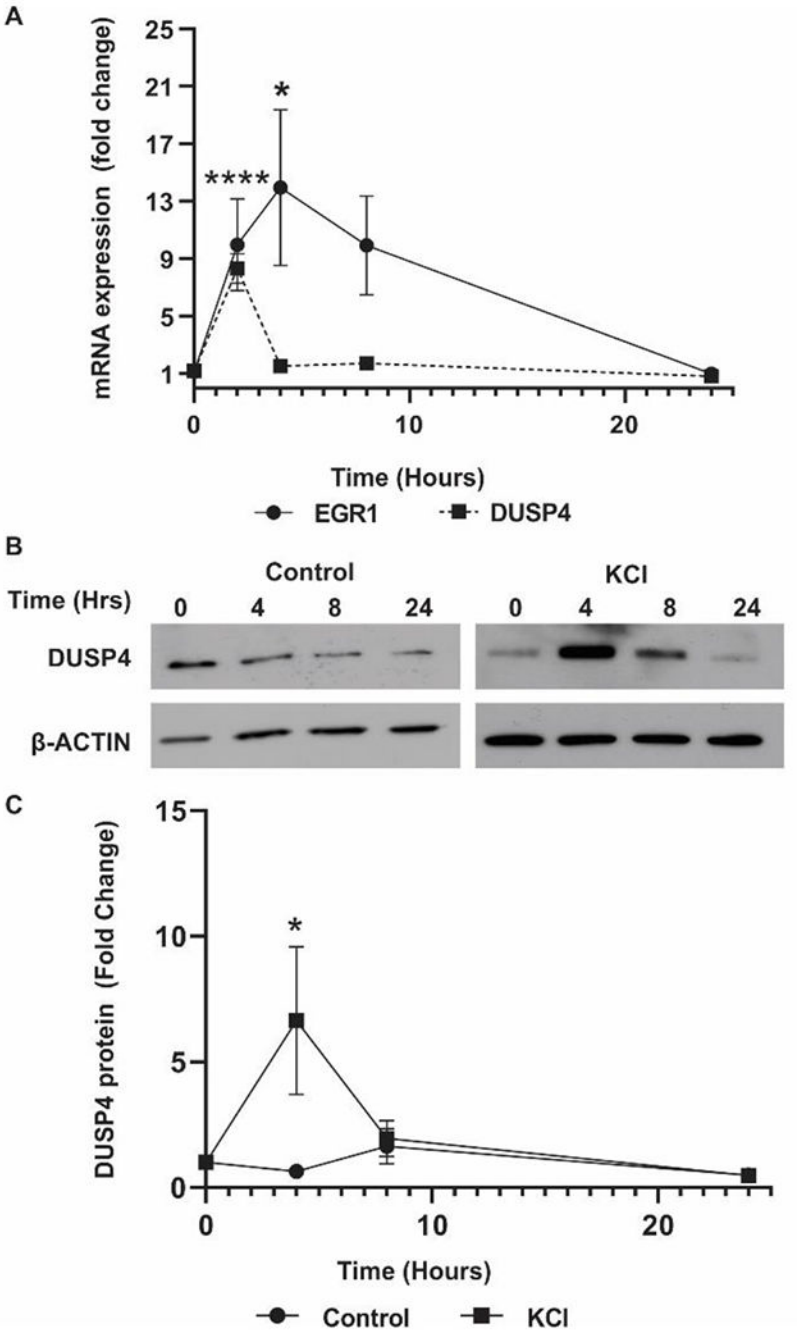

Figure 5: Repeated depolarization leads to a rapid, transient increase in DUSP4 mRNA and protein expression in vitro.

(A) qPCR from Sh-SY5Y demonstrates a rapid and transient increase in *DUSP4* mRNA levels at 2 hours following repeated 100mM KCl depolarizations (****p<0.0001, one-way ANOVA, n=3) and an induction of *EGR1* mRNA peaking at 4 hours (*p<0.05, one-way ANOVA, n=4). Of note, some time-points included in this graph were combined from separate experiments. **(B, C)** DUSP4 protein expression is significantly increased on Western blotting following repeated depolarization compared to control at 4 h (*p<0.05,

two-way ANOVA, n=3). Results are displayed as fold change in comparison to baseline DUSP4 expression.

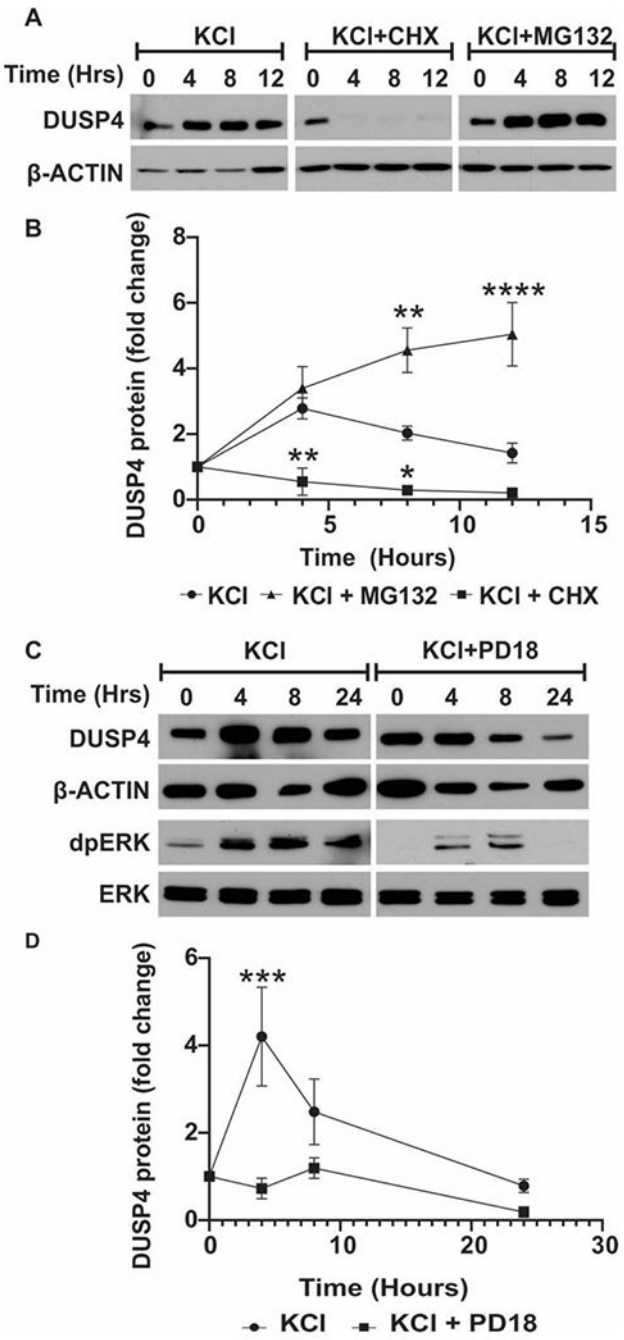

Figure 6: Activity-dependent DUSP4 protein expression requires both protein translation and stabilization, and MAPK signaling.

(A, B) Sh-SY5Y cells with repeated depolarizations treated with either 10ug/mL cycloheximide (CHX) or 10uM MG132 (proteasome inhibitor) showed a significant decrease at 4 h (**p<0.001, 2way ANOVA, n=3) and 8 h (*p<0.05, two-way ANOVA, n=3) and increase at 8 h (**p<0.01, two-way ANOVA) and 12 h (****p<0.0001, two-way ANOVA, n=3) in DUSP4 protein levels, respectively, compared to time-matched untreated controls by Western blotting. **(C, D)** Sh-SY5Y cells exposed to repeated depolarization

treated with 5mM PD18, a MEK inhibitor, had a significant decrease in DUSP4 expression at 4 hours (***$p<0.001$, two-way ANOVA, n=3) compared to controls on Western blotting. Results are displayed as fold change in comparison to baseline DUSP4 expression.

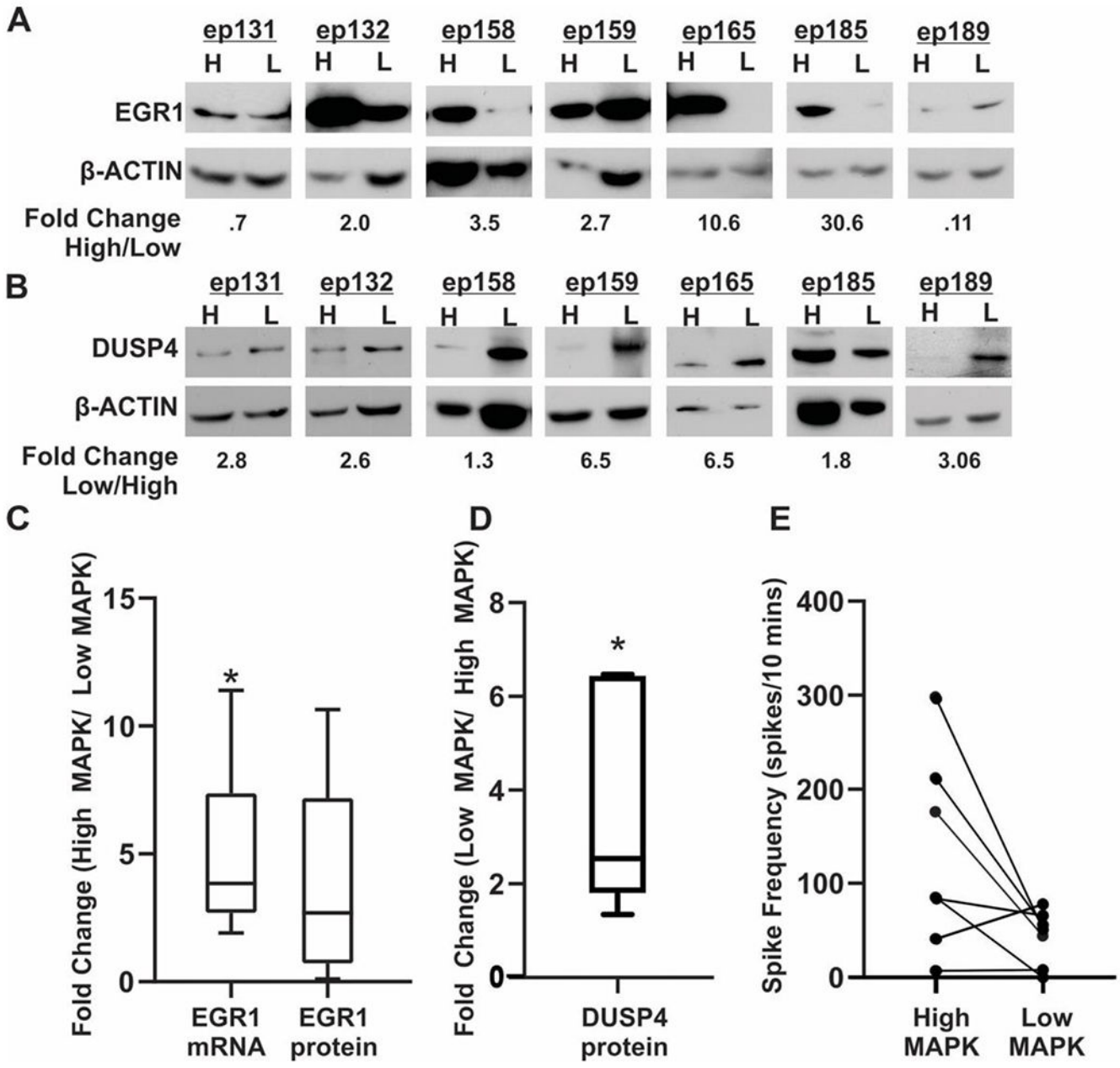

Figure 7: Human neocortical brain regions with increased epileptic spiking have higher MAPK signaling genes (EGR1) and lower DUSP4 protein expression levels.

(A) EGR1 and β-ACTIN protein levels were measured in paired samples from patients having both High MAPK signaling (H) and Low MAPK signaling (L) regions within each patient by Western blotting. High MAPK regions were defined based on their high expression levels of EGR1. **(B)** DUSP4 protein levels were increased in regions identified as Low MAPK signaling. **(C)** Quantitative analysis from qpCR and western blot comparing two neocortical brain regions from 7 patients with epilepsy to determine regions of high MAPK signaling. Regions were retroactively defined as high MAPK signaling when mRNA expression of *EGR1* was relatively increased in comparison to electrodes with lower

expressing *EGR1* mRNA levels (*p<0.05, one sample t-test, n=7). The same neocortical regions with an increase in *EGR1* mRNA also demonstrate an increase in EGR1 protein (p=0.0949, one sample t-test, n=7). Results are displayed as fold change, comparing the high MAPK signaling region to the low MAPK signaling region within each patient. **(D)** Quantitative analysis from western blots demonstrates increased DUSP4 protein in the previously defined low MAPK signaling regions (*p<0.05, one sample t-test, n=7). Results are displayed as fold change, comparing the previously defined low MAPK region to the high MAPK region within each patient **(E)** Spike frequency, defined as the average number of spikes in 10 minutes from in vivo human recordings, is increased in high MAPK signaling areas in comparison to areas with high DUSP4 expression. This indicates that increased DUSP4 expression correlates to a relative decrease in spike frequency in patients with neocortical epilepsy. The relative decrease in spike frequency in areas of high DUSP4 expression was seen in 5 out of 7 patients.

Table 1:

Patient data. ep, epilepsy patient; F, female; M, male; U, unknown; N/A, not applicable; m, microarray; wb, western blot; ish, in situ hybridization

Patient	Age (years)	Sex	Spike frequency (spikes/10 minutes)		Epilepsy Classification	Medications	Pathological Findings	Studies
			Low	High				
ep122	15	F	0	6	Complex Partial	zonisamide, diazepam	Polymicrogyria	m
ep131	14	M	N/A	N/A	Intractable epilepsy	zonisamide, lamotrigine, topiramate	Gliosis	wb
ep132	10	F	1	116	Intractable epilepsy	oxcarbazepine, lamotrigine, clobazam	Gliosis	m, wb
ep139	17	F	42	44	Intractable epilepsy	lamotrigine	Gliosis	m
ep143	11	F	0	5	Intractable epilepsy	clobazam, lamotrigine, oxcarbazepine	Heterotopia	m
ep148	7	F	0	425	Complex Partial	zonisamide, valium, oxcarbazepine	Gliosis	m
ep150	33	M	157	576	Intractable epilepsy	levetiracetam, phenytoin	Normal temporal lobe	m
ep158	1	M	0	85	Infantile spasms	zonisamide, levetiracetam, topiramate	Gliosis	m, wb
ep159	27	M	2	27	Intractable epilepsy	levetiracetam, phenytoin	White matter gliosis, hippocampal sclerosis	m, wb
ep164	3	F	66	141	Intractable epilepsy	valproic acid, topiramate	White matter gliosis, superficial heterotopia	m
ep165	3	F	56	212	Intractable epilepsy	valproic acid	Gliosis	m, wb
ep167	7	F	25	215	Intractable epilepsy	levetiracetam, topiramate	Cortical dysplasia	m
ep168	6	F	26	124	Infantile spasms	clobazam	Gliosis	m
ep169	8	M	3	172	Infantile spasms	oxcarbazepine, lamotrigine	Gliosis	m
ep178	1	F	2	80	Infantile spasms	lamotrigine, levetiracetam, topiramate	Gliosis, Cortical dysplasia	m
ep179	23	M	89	137	Intractable epilepsy	levetiracetam, lacosamide	Gliosis	m
ep185	56	F	51	299	Intractable epilepsy	lamotrigine, topiramate, lacosamide	Hippocampal sclerosis	m
ep186	5	F	N/A	N/A	Complex Partial	topiramate, oxcarbazepine, levetiracetam, phenytoin	Encephalitis, Gliosis	wb, ish
ep188	16	M	44	176	Complex Partial	levetiracetam, valproic acid	Gliosis	m, ish
ep189	11	F	2	66	Complex Partial	lamotrigine, levetiracetam	Cortical dysplasia	m, wb
ep195	7	M	25	150	Complex Partial	oxcarbazepine, levetiracetam, valproic acid, zonisamide,clobazam,	Gliosis	m
ep196	2	M	8	79	Infantile spasms	oxcarbazepine, topiramate	Gliosis	m