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Mitochondrial activity tunes nociceptor resilience to excitotoxicity

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SUMMARY:

The capsaicin receptor, TRPV1, mediates the detection of noxious chemical and thermal stimuli by nociceptors, primary sensory neurons of the pain pathway. Overactivation of TRPV1 leads to cellular damage or death through calcium entry and excitotoxicity. We have exploited this phenomenon to conduct a systematic analysis of excitotoxicity through a genome-wide CRISPRi screen, thereby revealing a comprehensive network of regulatory pathways. We show that decreased expression of mitochondrial electron transport chain (ETC) components protects against capsaicin-induced toxicity and other challenges by mitigating both calcium imbalance and the generation of mitochondrial reactive oxygen species via distinct pathways. Moreover, we confirm the regulatory roles of the ETC in sensory neurons through gain-of-function and loss-of-function experiments. Interestingly, TRPV1⁺ sensory neurons maintain lower expression of ETC components and can better tolerate excitotoxicity and oxidative stress compared to other sensory neuron subtypes, implicating ETC tuning as an intrinsic cellular strategy that protects nociceptors against excitotoxicity.

In brief

Excitation enables neuronal function; however, over-excitation and calcium overload can be deadly. This study reveals a cellular resilience program in which reduced expression of electron transport chain components protects against excitotoxic death, as seen in pain-sensing primary afferent neurons (nociceptors).

Graphical Abstract

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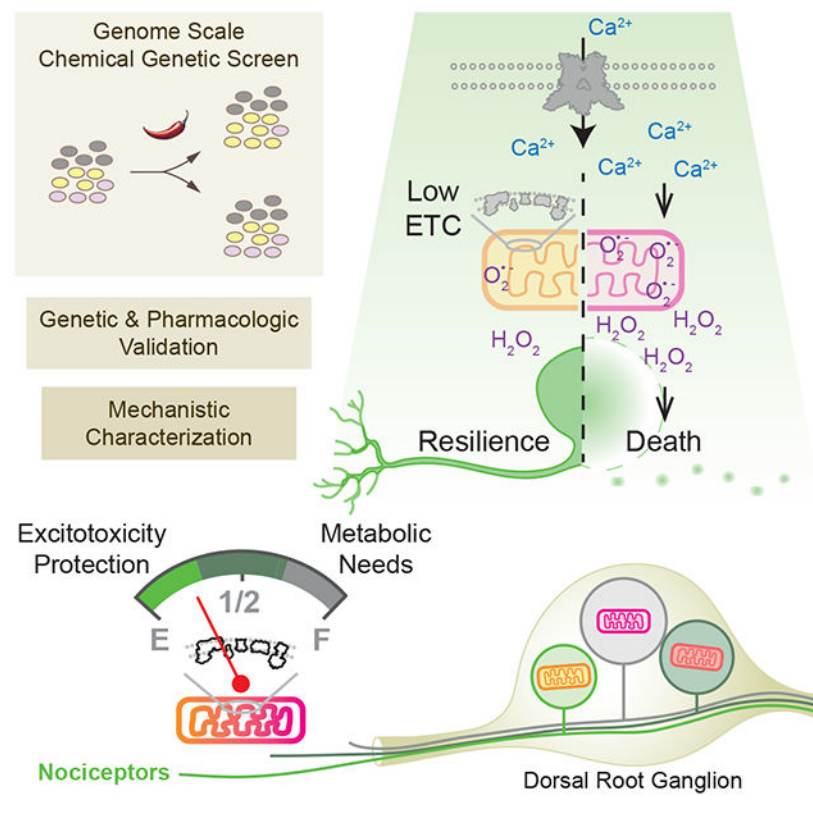
AUTHOR CONTRIBUTIONS

Conceptualization, L.Y., N.S.C., and D.J.; Methodology: L.Y.; Investigation: L.Y.; Writing - Original Draft: L.Y. and D.J.; Writing - Review & Editing: L.Y., N.S.C., and D.J.; Visualization: L.Y.; Supervision: D.J.; Funding acquisition: L.Y. and D.J..

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DECLARATION OF INTERESTS

D. Julius is a member of the Scientific Advisory Board of Rapport Therapeutics. The authors declare no other competing interests.



INTRODUCTION

Excitotoxicity describes the phenomenon whereby neurons are killed or injured following overactivation of excitatory receptors leading to calcium overload. This process is traditionally and most extensively studied in the context of glutamate-evoked toxicity in the central nervous system (CNS), which is implicated in ischemia and neurodegenerative disorders such as Alzheimer's and Parkinson's disease.^{1,2} Although excitotoxicity is associated with many disease conditions in the peripheral nervous system (PNS), its underlying mechanisms and contributions to neuropathology have received comparatively less attention and are thus less well understood.

Primary afferent nociceptors are a subset of peripheral sensory neurons that detect harmful (noxious) stimuli and transmit this information to the central nervous system to initiate pain sensations. Hyperexcitation of these neurons can result in chronic pain and/or afferent degeneration, as exemplified in cases of hereditary painful neuropathies caused by rare gain-of-function mutations in voltage-gated sodium channels.^{3–5} Hyperexcitability and degeneration are also observed in more common conditions associated with neuropathic pain, such as diabetes or post-herpetic neuralgia (shingles),^{6–8} but in such cases the link between hyperexcitability, excitotoxicity, and degeneration is less well established, in part reflecting a lack of mechanistic insight into cellular pathways underlying excitotoxic injury and death of nociceptors.

Capsaicin, the main pungent ingredient in ‘hot’ chili peppers, is neurotoxic for a major subclass of sensory nociceptors that express its receptor, the excitatory TRPV1 ion channel.^{9–12} TRPV1 is highly permeable to calcium ions,^{9,13} and, in this way, analogous to NMDA-type glutamate receptors, which play a key role in excitotoxicity of CNS neurons.^{1,2} Prolonged or repetitive exposure to capsaicin effectively kills TRPV1⁺ nociceptors or their afferent terminals, underlying its paradoxical use as a topical analgesic for treating certain types of chronic neuropathic pain, such as those associated with viral infection or diabetes.^{8,14}

Beyond its role as a receptor for pungent spices, TRPV1 is also activated by heat and inflammatory agents that drive pain hypersensitivity and excitotoxic stress, underlying its important role in both acute and persistent pain.^{15,16} Recent studies have linked TRPV1-dependent degeneration to corneal nerve damage in dry-eye-disease,¹⁷ further underscoring the importance of understanding how inflammation or other maladaptive conditions can promote peripheral nerve injury through activation of TRPV1 or other excitatory mechanisms.

While capsaicin has classically been used to selectively excite and kill nociceptors, even yeast cells can acquire vulnerability to capsaicin if they ectopically express *TRPV1*,^{9,18} suggesting the involvement of a conserved cell death pathway that can be systematically interrogated using facile non-neural cell culture models. With this in mind, we designed an unbiased, genome-wide CRISPRi screen to identify factors that influence capsaicin-evoked death in immortalized mammalian cells. Foremost among these ‘hits’ are components of the mitochondrial electron transport chain (ETC), suppression of which protects against capsaicin-induced excitotoxicity in both TRPV1⁺ cell lines and adult mouse sensory neurons. Pharmacologic and genetic experiments suggest that ETC suppression boosts cell resilience by two parallel pathways: reducing mitochondrial reactive oxygen species (ROS) and hence oxidative stress and modulating calcium overload. Interestingly, we find that TRPV1⁺ nociceptors from adult mice express ETC components at lower levels compared to other sensory neurons, conferring greater resilience to cell death due to calcium or ROS overload. Furthermore, we show that ETC suppression is protective against a broad range of excitotoxic insults. We therefore propose that tuning of aerobic respiration helps nociceptors mitigate risk associated with injury and other noxious events. These insights into cellular mechanisms controlling nociceptor excitotoxicity are relevant to understanding neuropathological consequences of diabetes, chemotherapy, and other conditions that disrupt normal, protective pain sensation.

RESULTS

Genome-wide CRISPRi screen reveals the ETC as a regulator of capsaicin-evoked death

Although many pathways have been suggested to account for TRPV1-mediated cell death, it is generally agreed that calcium influx is a required factor.^{11,19} Indeed, capsaicin-induced death of TRPV1-expressing HEK293T cells was abrogated by chelating or excluding extracellular Ca²⁺, demonstrating a similar requirement for Ca²⁺ influx in this heterologous system (Figure 1A). Moreover, both *Trpv1-EGFP*⁺ neurons (taken from *Tg(Trpv1-EGFP)MA208Gsat/Mmcd* mouse²⁰ and henceforth referred to as TRPV1⁺

neurons, Figure S1) and TRPV1⁺ HEK293T cells showed similar morphological features of necrosis in response to capsaicin (Figure 1B and Video S1 and S2). This capsaicin-induced toxicity was not ameliorated by small molecule inhibitors of apoptosis, autophagy, or other distinct cell death modalities such as ferroptosis, necroptosis, pyroptosis and parthanatos (Figure S2),²¹ compelling us to take a more comprehensive and unbiased approach to identifying pathways involved in capsaicin-evoked excitotoxicity.

Being able to recapitulate capsaicin-evoked death in an immortalized mammalian cell line enabled us to achieve this goal by conducting a genome-wide chemical genetic screen (Figure 1C).^{9,18} For this purpose, we engineered a TRPV1⁺ K562 clonal cell line expressing the required CRISPRi machinery (Figure S3A).²² These cells were transduced with a well-validated genome-scale single-guide RNA (sgRNA) library that targets 18905 genes with 5 sgRNAs/gene and 1895 non-targeting (NT) sgRNA controls.²³ Samples were collected from two biological replicates at the outset of the experiment (T0) and from either untreated or capsaicin treated cells at day 14, the experimental endpoint (Figure 1C and S3B). The effect of knocking down each gene on cellular survival was quantified by the relative abundance of targeting sgRNAs to NT controls for each gene.²² Using a 1% false discovery rate (FDR), we identified 1815 genes that regulated capsaicin-induced cell death by comparing untreated and capsaicin treated sample groups, and 2044 genes involved in cell growth by comparing T0 and untreated sample groups (Table S1 and S2). Among genes found to regulate capsaicin-induced cell death, 393 were regarded as “strong hits” with the absolute product of Log₂FC and -Log₁₀P > 4.

Among these “strong” candidate regulators, significant enrichment was seen in pathways that directly affect levels of TRPV1 on the cell surface by controlling membrane protein synthesis or intracellular protein transport, thereby validating the screen (Figure 1D and 1E). For example, knocking down a component of the endoplasmic reticulum membrane protein complex (EMC) led to reduced capsaicin toxicity by inhibiting the biogenesis of membrane proteins that include TRP channel family members (Figure 1E, 1F, and 1I).^{24,25} In contrast, knocking down flotillin (*FLOT*)-1, a protein that mediates clathrin- and caveolin-independent endocytosis,^{26,27} exacerbated capsaicin-induced cell death. In addition to validating our screen, these ‘hits’ suggest that cell death is mediated through activation of TRPV1 channels at the cell surface. Consistent with this, we found that a cell-impermeant TRPV1 activator (the DkTx spider toxin)²⁸ also confers cellular toxicity (Figure S3C).

The most enriched pathway identified in this screen highlights the electron transport chain (ETC) and its role in aerobic respiration (Figure 1D–1G, and Table S1). Specifically, knocking down expression of numerous components (25) or assembly factors (11) of the ETC strongly suppressed capsaicin-induced cell death (out of 393 “strong hits”). Our finding is somewhat counterintuitive and thus surprising since disruption of the ETC and resulting metabolic consequences are usually deleterious to cell health. Indeed, this conventional role of the ETC is confirmed by the control arms of our screen in which sgRNAs targeting the ETC were depleted at the untreated endpoint compared to T0 (Figure 1H). In contrast, we found that ETC disruption was beneficial in the presence of capsaicin, rescuing cells from capsaicin-evoked lethality (Figure 1G). Taken together, these results suggest that our

CRISPRi screen has revealed a regulatory strategy in which perturbation of the ETC weighs resilience to excitotoxic insult against the cell's overall metabolic needs.

To validate involvement of the ETC, we chose to examine stable knock-down clones targeting the mitochondrial complex I subunit, *NDUFC1*, because it scored highly as a rescue hit against capsaicin-induced toxicity yet had minimal effect on cell growth (Figure 1G and 1H). Indeed, knocking down *NDUFC1* protected cells against capsaicin-induced toxicity with minimal effects on growth (Figure 1I, 1J and S3D). In contrast, knocking down *NDUFC1* in parental cells that did not express *TRPV1* showed no survival difference, ruling out any off-target effect of capsaicin treatment in *NDUFC1*-deficient cells (Figure S3E). To further corroborate this unexpected link between ETC suppression and excitotoxicity, we tested the effect of ETC inhibition on capsaicin-induced cell death using a collection of well-defined small molecule inhibitors. Pretreatment with the complex I inhibitor, piericidin A (PierA) (1-10 nM), suppressed toxicity by capsaicin (Figure 1K). Similar protective effects were observed with low concentrations of the complex III inhibitor, antimycin A (AA, at 10 nM) (Figure 1K), and two other complex I inhibitors, rotenone and phenformin (Figure S3F–S3I). High concentrations of these inhibitors can promote apoptosis, but as previously reported,²⁹ only mild decreases in growth rates were observed at the relatively low concentrations that were protective against capsaicin lethality.

ETC regulates sensory neuron resilience to capsaicin-induced death

Our observations with *TRPV1*-expressing non-neuronal cell models prompted us to ask whether vulnerability of sensory neurons to capsaicin-induced death is also determined by their level of ETC expression. To test this directly, we manipulated ETC expression in mouse sensory neurons using orthogonal genetic strategies. To achieve loss-of-function, we conditionally ablated *Ndufs4* or *Uqcrcq* (complex I or III component respectively)^{30,31} from *TRPV1* lineage neurons (*Trpv1^{Cre}*),³² which reduced their viability while also imparting protection against capsaicin, as assessed in primary cultures of sensory neurons from dorsal root ganglia (DRG) (Figure 2A–2C and S4). This dual effect is consistent with both the essential role of the ETC for neuronal survival past 7 weeks of age³⁰ and the protective effect of reduced ETC expression that we observe when cells are challenged with capsaicin.

To achieve gain-of-function, we ectopically expressed *Ppargc1b* and *Esrra* to boost ETC biogenesis in cultured DRG neurons. These genes encode transcription co-factors PGC1 β and ERR α , which were chosen over other known ETC regulatory factors because they are both screen hits (Table S1).^{33,34} Indeed, sensory neurons that overexpressed *Ppargc1b*, *Esrra*, or both were significantly more sensitive to capsaicin-induced lethality compared to those transduced with empty vector (EV) controls, while the percentage of *TRPV1*⁺ neurons remained unchanged (Figure 2D–2F). Therefore, increasing ETC biogenesis weakened the resilience of *TRPV1*⁺ nociceptors to excitotoxicity. Together, these data show that ETC expression level dictates resilience against capsaicin-evoked excitotoxic cell death, whether assessed with proliferating non-neural cell lines or post-mitotic sensory neurons (Figure 1I, 1K, 2, and S3F–S3I).

Metabolic regulation of capsaicin-evoked calcium overload

To understand how ETC suppression protects cells from capsaicin-evoked death, we set out to investigate core functions of the ETC. The ETC is central to numerous cellular pathways, especially mitochondrial and cellular metabolism through production of ATP via oxidative phosphorylation (OXPHOS), which also recycles electron carriers (NAD^+ and FAD) required for macromolecular synthesis. To delineate effectors downstream of the ETC, we carried out mechanistic analyses using proliferating TRPV1⁺ cell lines.

Because increasing ETC biogenesis with the transcription factors PGC1 β and ERR α made neurons more vulnerable to capsaicin-induced toxicity, one might expect a similar outcome when OXPHOS is enhanced through other means. Indeed, we found that cells were sensitized to capsaicin-induced death when cellular respiration was increased by reducing glycolysis (Figure 3A).³⁵ Consistent with this, the mRNA splicing factor, LUC7L2, was also a sensitizing hit in our CRISPRi screen (Table S1), loss of which shifts metabolism from glycolysis to oxidative phosphorylation.³⁶ We next asked whether capsaicin application changes the ATP-to-ADP ratio in TRPV1⁺ HEK293T. Using the ratiometric fluorescent biosensor PercevalHR³⁷, we found no such effect (Figure 3B). Moreover, long-term imaging of cells undergoing capsaicin-evoked necrosis showed no change of PercevalHR signal throughout this process up until cell lysis (Movie S3). Taken together, these data indicate that a change in cellular ATP does not play a critical role in either capsaicin-evoked cell death or the protection afforded by ETC suppression.

Given the critical role of calcium in capsaicin-evoked excitotoxicity (Figure 1A), we asked whether there is a connection between calcium influx and ETC suppression. We found that *NDUFC1* knockdown or PierA treatment resulted in a partial reduction of capsaicin-evoked Ca^{2+} influx (Figure 3C and 3D), suggesting that ETC suppression can alleviate calcium overload. One possible mechanisms for downregulating TRPV1-mediated calcium influx would be to diminish levels of TRPV1 channels on the cell surface. Because the bulk reservoir of TRPV1 channels is intracellular (whether assessed in sensory neurons or transfected cells), quantifying cell surface localization by standard immunofluorescence staining is not possible. Instead, we leveraged our observation that FLOT1 regulates cell surface localization of TRPV1. Indeed, epistasis analysis of FLOT1 knockdown in K562 cells supports a relationship between ETC regulation of TRPV1-mediated Ca^{2+} influx and channel trafficking (Figure S5A–C).

To decouple metabolic pathways downstream of the ETC, we added pyruvate and uridine to the culture medium, which is known to rescue proliferation of cells that lack a functional ETC.^{38–40} We found that adding pyruvate and uridine circumvented the decrease in capsaicin-evoked Ca^{2+} influx caused by ETC suppression following either *NDUFC1* knockdown or PierA treatment (Figure 3C, 3D). Moreover, adding pyruvate alone was sufficient to reverse the PierA effect (Figure S5D), demonstrating involvement of a pyruvate-dependent process in regulating calcium influx.

Because the main role of pyruvate when added to ETC deficient cells is to regenerate NAD^+ ,⁴⁰ we asked whether direct manipulation of $[\text{NAD}^+]/[\text{NADH}]$ ratio can affect capsaicin lethality. Using a NADH oxidase from *Lactobacillus brevis* (*LbNOX*),⁴¹

we found that capsaicin-induced toxicity is exacerbated when *LbNOX* is targeted to the mitochondria (mito*LbNOX*) but not the cytoplasm (cyto*LbNOX*) (Figure 3E). As mito*LbNOX* was reported to double cellular $[NAD^+]/[NADH]$ whereas cyto*LbNOX* has no such effect,⁴¹ our data suggest that increasing $[NAD^+]/[NADH]$, especially in the mitochondria, exacerbates capsaicin-induced toxicity. This is consistent with the protection towards capsaicin-induced lethality afforded by suppressing ETC function and its effect on lowering $[NAD^+]/[NADH]$.⁴⁰ Moreover, our CRISPRi screen identified *SLC25A51*, the mammalian mitochondrial NAD^+ transporter,⁴² as a strong protective hit (Table S1), consistent with the protective effect of lowering $[NAD^+]/[NADH]$ in the mitochondria. Taken together, these data suggest that decreased $[NAD^+]/[NADH]$ contributes to the rescue effect of ETC suppression by alleviating calcium overload through flotillin-dependent decrease of cell surface TRPV1 - a process that can be overridden by pyruvate addition.

Interestingly, treatment with ETC inhibitors provided significant protection against capsaicin-evoked death even when cells were supplemented with pyruvate and uridine (Figure 3F–3H and S5E–S5H). Similarly, knocking out ETC components protected DRG neurons from capsaicin lethality when cultured in medium containing both pyruvate and uridine (Figure 2A–2C). Together, these results suggest the presence of another, NAD^+ -independent protective mechanism that is orthogonal to calcium regulation. Going forward, we included pyruvate and uridine in our experiments to isolate and characterize this NAD^+ -independent mechanism of cellular resilience.

Capsaicin- and ETC-dependent transcriptional changes

To identify the NAD^+ -independent component of ETC rescue, we took an unbiased transcriptome-based approach by comparing cellular responses to capsaicin (with pyruvate and uridine supplementation) in the absence or presence of PierA. We reasoned that if a factor mediates capsaicin-induced toxicity, then its expression may be altered by capsaicin treatment, and this change should be dampened by PierA. Pair-wise analysis between vehicle and capsaicin treated samples showed prominent transcriptional changes in “capsaicin responsive genes” after 4 hours of treatment (Figure 4A and Table S3). Gene ontology analyses showed that transcription factors were the dominant class of differentially expressed genes, many of which are known to respond to calcium and / or various forms of cellular stress. Importantly, PierA pretreatment dampened the transcriptional changes observed for these capsaicin-responsive genes (Figure 4B).

Cross-referencing top capsaicin-responsive transcription factors with the CRISPRi screen hits revealed one candidate encoding a small DNA binding protein called *MAFF*, whose expression is upregulated upon capsaicin treatment and dampened by PierA (Table S1 and S3, Figure 4B). MafF is recruited to antioxidant response elements during oxidative stress and either activates or represses transcription, depending on its binding partners.⁴³ Although MafF can also bind to calcium-responsive elements, our profiling was carried out in the presence of pyruvate to minimize the NAD^+ / calcium-dependent component (Figure 3C and 3D). Indeed, knocking down *MAFF* mitigated capsaicin-induced cell death without affecting Ca^{2+} influx (Figure 4C and 4D). Moreover, BACH1, a transcriptional repressor that is known to interact with MAFF,⁴³ emerged as a mild hit from both our CRISPRi

screen and differential expression dataset. Indeed, knocking down BACH1 also protected cells from in capsaicin-evoked toxicity, confirming the protective effect of interfering with this antioxidant transcriptional response pathway (Figure S5I). We therefore focused on elucidating the relationship between ETC suppression and oxidative stress as the other likely component of capsaicin-evoked excitotoxicity.

ETC suppression boosts resilience by lowering oxidative stress

As electrons pass down the ETC, they occasionally leak prematurely and generate reactive oxygen species (ROS) such as superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2).^{44,45} To examine the effect of capsaicin on mitochondrial ROS generation, we utilized two parallel approaches. One involved targeting a ratiometric fluorescent H_2O_2 biosensor, HyPer7,⁴⁶ to the mitochondrial matrix of TRPV1⁺ HEK293T cells, which revealed a steady increase of H_2O_2 in response to capsaicin that plateaued within 30 min (Figure 5A). No response was observed when vehicle was added to TRPV1⁺ HEK293T cells or to parental lines not expressing *TRPV1* (Figure 5A). As a second approach, we analyzed the intensity of mitoSOX, a mitochondrially targeted fluorescent indicator of superoxide, by flow cytometry. Consistent with the HyPer7 data, capsaicin elicited a dramatic increase of mitoSOX intensity in a TRPV1-dependent manner (Figure S6A). Moreover, omitting extracellular Ca^{2+} during capsaicin treatment prevented the increase of mitochondrial HyPer7 and mitoSOX signals, supporting the critical role of Ca^{2+} influx in both ROS generation and excitotoxicity (Figure 5B, S6B, and S6K).^{2,45}

The mitochondrial calcium uniporter (MCU) has been proposed to regulate glutamate toxicity.² By constructing a TRPV1⁺ MCU-deficient cell line,⁴⁷ we found that MCU is dispensable for capsaicin-induced cell death (Figure S6C). Consistent with this, sgRNAs that target MCU components did not emerge as hits in our CRISPRi screen (Table S1). Moreover, capsaicin-induced Ca^{2+} -dependent increase of ROS was also evident in cells that lacked MCU (Figure S6D), consistent with its dispensable role in capsaicin-evoked cell death. These data suggest that Ca^{2+} flux across the mitochondrial inner membrane is not critical for capsaicin-evoked ROS generation and lethality, or that calcium enters mitochondria to generate ROS through a MCU-independent mechanism, such as via the Ca/H exchanger LETM1 or Ca/Na exchanger NCLX in the mitochondrial inner membrane.^{48,49}

We next asked if ETC suppression affects capsaicin-evoked ROS generation. Indeed, pretreating cells with PierA or AA (supplemented with pyruvate and uridine) diminished the capsaicin-induced increase in HyPer7 and mitoSOX signals and rescued survival (Figure 5C, S6H, S6I, and S6K). Although AA is known to evoke ROS generation at high concentrations (1-100 μ M), no significant change in baseline HyPer7 ratio was observed before the addition of capsaicin in cells pretreated with low doses (10-20 nM) of AA used here (Figure 1K, 3G, and 5C). In any case, these data suggest that reduced generation of ROS constitutes the NAD^+ -independent mechanism by which ETC suppression protects against calcium overload and excitotoxicity. In support of this hypothesis, we found that pretreating cells with M40403, a superoxide dismutase (SOD) mimetic, prior to capsaicin application dampened the increase of HyPer7 and mitoSOX signals and rescued capsaicin-induced cell

death without altering calcium dynamics (Figure 5D–5F, S6E, S6F, and S6K). A similar protective effect was observed with mitoTEMPO, another mitochondrial-targeted SOD mimetic (Figure S6G). These results were complemented by the identification of *SOD1* as a sensitizing hit in our CRISPRi screen (Table S1). Thus, we conclude that capsaicin kills cells by increasing generation of mitochondrial ROS.

To further characterize mechanisms underlying capsaicin-evoked ROS generation, we tested the effect of two small molecule inhibitors, S1QEL1.1 and S3QEL2, which specifically suppresses $O_2^{\bullet-}$ or H_2O_2 generation from one of two major ETC sites without affecting OXPHOS.^{45,50,51} Interestingly, capsaicin-induced ROS generation and cell death were abrogated by S3QEL2 but not S1QEL1.1, suggesting that ROS is produced by site III_{QO} on complex III (Figure 5F–5H, S6J, and S6K). As site III_{QO} faces the inner membrane space, this is consistent with the identification of the inner membrane / cytosolically localized SOD1, but not matrix localized SOD2, as a sensitizing hit from our CRISPRi screen. To ask whether this mechanism is conserved in neurons, we pretreated DRG culture with S3QEL2 prior to capsaicin treatment and observed diminished neuronal death compared to vehicle controls (Figure 5I). Taken together, these data substantiated oxidative stress as a mediator of capsaicin-evoked excitotoxicity that is conserved between mitotic cells and neurons. Moreover, preventing Ca^{2+} -induced ROS production constitutes the main protective mechanism afforded by ETC suppression (Figure 5J).

Generalization to other modalities of excitotoxicity

Using the robust and facile model of capsaicin-induced excitotoxicity, we have shown that cellular resilience can be gained by diminishing activity of the ETC. Does tuning of the ETC enhance resilience to other modalities of excitotoxicity? We addressed this question by first asking if the DkTx spider toxin, a persistent and cell-impermeant TRPV1 agonist,²⁸ can kill TRPV1⁺ proliferating cells. This was observed to be the case (Figure 6A and S3C), and DkTx-evoked death was ameliorated by pretreatment with PierA (Figure 6B). Thus, ETC suppression provides protection against TRPV1-mediated excitotoxicity independent of the mode of channel activation.

Charcot-Marie-Tooth disease type 2C (CMT2C), a degenerative condition of peripheral neurons, is linked to mutations in another TRP channel, TRPV4.⁵² When expressed heterologously, the disease-associated gain-of-function mutant, R289C, can trigger cell death by increasing calcium influx through TRPV4 (Figure 6C).⁵² Indeed, we found that expression of TRPV4 R289C, but not wildtype TRPV4, was toxic to transfected HEK293 cells, and death was ameliorated by S3QEL2 (Figure 6D). Furthermore, persistent activation of wildtype TRPV4 by the selective agonist, GSK101,⁵³ also promoted excitotoxic death, which was similarly mitigated by S3QEL2 or AA (Figure 6E–6G). We noted that GSK101 in combination with the GOF mutation R289C produced significant lethality such that rescue by S3QEL2 and AA were less effective overall (Figure 6F and 6G).

Finally, to represent another category of physiological challenge experienced by sensory afferents, we examined the effect of α -hemolysin (Hla), a prototypic pore-forming pro-algesic toxin of *Staphylococcus aureus* that preferentially elicits calcium responses in TRPV1⁺ neurons through a TRPV1-independent mechanism.^{54,55} We found that chelating

Ca²⁺ with EGTA protected K562 cells from Hla-evoked lytic cell death (Figure 6H and 6I), as did pretreatment with PierA, M40403, or S3QEL2 (Figure 6J–6L). Thus, suppressing the ETC and reducing oxidative stress protects cells from Hla-induced toxicity. Together, these findings demonstrate that ETC suppression is generally protective against distinct instigators of cell death that elicit persistent elevation of intracellular Ca²⁺ and subsequent ROS overload.

Nociceptors are inherently more resilient to excitotoxicity

TRPV1⁺ nociceptors are targeted by a wide variety of noxious stimuli, as exemplified by DkTx and Hla toxins described above. We therefore wondered whether these or other excitotoxic insults have exerted evolutionary pressure on this subset of sensory neurons, resulting in differential vulnerability compared to other sensory neuron subpopulations. To test this hypothesis, we used a calcium ionophore (ionomycin) to elicit sustained calcium influx in all neurons cultured from dissociated mouse DRG. Time-lapse imaging showed that media containing 10 μ M ionomycin and 10 mM Ca²⁺ induced cellular swelling and necrosis (Figure 7A, Video S4). To our particular interest, TRPV1⁺ sensory neurons consistently fared better against this broadly acting excitotoxic challenge, demonstrating their greater resilience compared to other sensory subtypes (Figure 7A and S7A). We found the same to be true in response to oxidative stress where TRPV1⁺ neurons showed preferential survival following exposure to the mitochondrial ROS generator mitoParaquat (mitoPQ) (Figure 7B).⁵⁶

In light of the ETC resilience model that we propose here, we reanalyzed a published RNAseq dataset for expression of ETC components by different sensory neuron subtypes.⁵⁷ Interestingly, we found that these components, as well as *Esrra*, *Ppargc1b* and other transcription factors that govern ETC biogenesis, are expressed at lower levels in TRPV1⁺ neurons compared to other sensory subtypes (Figure 7C). Indeed, this was confirmed at the protein level, where lower amounts of two ETC components was observed in TRPV1⁺ neurons as quantified by immunohistochemical analysis of both dorsal root and trigeminal ganglia (Figure 7D and S7B). Consistent with lower ETC expression, TRPV1⁺ sensory neurons were more resilient to apoptosis induced by high concentrations of ETC inhibitors, suggesting less relative dependency on the ETC (Figure 7E). Furthermore, TRPV1⁺ sensory neurons showed significantly lower basal ROS levels with fewer highly stressed outliers as measured with the ROS sensitive dye, mitoNeoD⁵⁸ (Figure 7F and S7C).

DISCUSSION

Neurons are post-mitotic and thus the consequences of death are dire compared to proliferative cells. Primary afferent sensory neurons can regenerate their peripheral terminals, a process that is dependent on local calcium transients as well as energy provided by mitochondria,^{59–62} but nerve terminal regeneration is predicated on survival of the soma, underscoring the importance of regulating mitochondrial function to suit these survival and regenerative activities. Our comprehensive chemical genetic and pharmacologic dissections now supports an unexpected aspect of mitochondrial function in which ETC tuning in sensory neurons fosters resilience. Our investigation began by using capsaicin as a model,

but we also show that this conclusion pertains to other forms of excitotoxic and oxidative insults (Figure 6, 7A and 7B).

While previous studies have implicated mitochondria and ROS in excitotoxicity, our findings bring a different perspective to this area by viewing the problem from the standpoint of cellular resilience mechanisms that buffer such insults. Specifically, we propose that TRPV1⁺ sensory neurons establish a metabolic ‘sweet spot’ in which reducing ETC expression protects them from excitotoxicity while accommodating energetic demands of maintaining electrical excitability over long and arborized processes that innervate distant receptive fields. This balancing act between environmental resilience and cellular metabolism may limit the extent to which these neurons are protected in pathological situations that lead to painful neuropathy, including disorders such as diabetes that alter metabolic homeostasis.⁶ It will also be interesting to determine whether low ETC expression, which is protective under normal physiological conditions, will render nociceptors more vulnerable in maladaptive scenerios.

Metabolic tuning as a regulatory mechanism

Given its critical role in ATP production and NAD⁺ regeneration, impairing ETC activity generally has negative consequences for cellular health, as we observed with both proliferating cell models and neurons (Figure 1H and 2B).³⁰ However, we show that maintaining low ETC activity can be beneficial in the face of excitotoxicity, revealing interesting parallels to other physiologically challenging scenarios. For example, knocking down expression of ETC components delays aging in many model organisms.^{63–65} Another more extreme example was recently described for early oocytes, which eliminate their complex I altogether to avoid ROS accumulation during dormancy, when energy expenditure is presumably minimal.⁶⁶ Furthermore, the idea that ETC activity plays a regulatory role is consistent with recent observations that species-specific developmental rates are determined by mitochondrial function and metabolic rate.^{67,68} While the abovementioned studies focused on steady state ROS production, our study highlights the role of ETC suppression in regulating calcium-dependent ROS generation, which is especially relevant to excitotoxicity and pathological conditions that accompanied painful neuropathies.

We also uncovered an unexpected regulatory effect of the ETC on calcium overload. Specifically, we found that pyruvate addition can negate the effect of ETC regulation on calcium overload but not ROS, implying that these outcomes represent distinct modulatory pathways (Figure 3C, 3D, 3F–3H, and 5C). Based on hits from our CRISPRi screen, we explored FLOT1-dependent endocytosis as a potential mechanism that underlies the pyruvate-dependent downregulation of TRPV1-mediated calcium influx (Figure S5A–S5C). Flotillins are known to mediate the assembly and endocytosis of large protein complexes that reside in sphingolipid/cholesterol rich microdomains on the plasma membrane.^{26,27} As many ion channels, including Kv2.1, have been reported to cluster and/or localize to lipid rafts,^{69–71} this pathway may act broadly, beyond TRPV1, to regulate cell surface expression of signaling molecules that control excitability of primary afferent nociceptors. Interestingly, previous studies have shown that changes in pyruvate/lactate regulation can alter the excitability of peripheral or central neurons,^{72,73} and SUMOylation has been

proposed as one such downstream mechanism that regulates TRPV1 activation.⁷² Further analysis of endocytic and other pathways in relation to pyruvate and lactate metabolism may provide further insights into how and why dysregulated metabolic conditions, such as obesity or diabetes, are associated with chronic pain syndromes.

ETC tuning in different sensory subtypes

We show that a major class of nociceptors maintains lower ETC expression compared to other sensory subtypes, granting them greater resilience against excitotoxic and oxidative stress (Figure 7). We speculate that several factors, including ion channel physiology, pathogen tropism, and cellular properties, may be driving this difference among sensory neuron subtypes.

The TRPV1 ion channel has biophysical properties that may uniquely dispose cells to excitotoxicity. Compared to other excitatory ion channels in peripheral sensory neurons, TRPV1 is highly Ca^{2+} permeable (5-10 times more than Piezos, P2Xs, and other TRP channels expressed in the DRGs)^{13,74–76} and desensitizes much more slowly through a process that likely involves endocytosis⁷⁷ thus conferring a higher risk of calcium overload. Additionally, inflammatory mediators potentiate TRPV1 activation, further increasing the risk of excitotoxicity under maladaptive conditions.^{15,16}

Tropism of pathogens and toxins may also contribute to selective pressure that drives differential resilience. As noted above, Hla secreted by *Staphylococcus aureus* preferentially attacks TRPV1⁺ neurons to induce calcium influx, action potential firing, and pain sensation.^{54,55} We showed that ETC tuning can enhance resilience against such toxin-evoked calcium overload (Figure 6H–6L). TRPV1⁺ neurons are now recognized to initiate or orchestrate a range of efferent functions, such as innate immunity¹⁶, tissue repair, and organismal metabolism,^{78,79} via the peripheral release of neuropeptides. Enhanced resilience that protects the integrity of these neurons may be beneficial for maintaining key physiological processes beyond pain sensation.

At the same time, nociceptors may have lower energetic demands as they usually fire non-repetitively or at much lower frequency compared to other subtypes, including mechanoreceptors.⁵⁷ For example, A β low threshold fibers that specialize in detecting high-frequency vibratory stimuli can fire at over 500 Hz.⁸⁰ As calcium influx during action potential firing is more transient and reduced compared to that associated with TRPV1 activation, excitotoxicity during high frequency firing is likely less of a risk and outweighed by the higher ETC activity needed to support the energetic needs of these light touch neurons. Thus, different sensory neuron subgroups may adjust their metabolic states to suit their diverse functional roles.

While our findings suggest that lower ETC expression is an intrinsic characteristic of adult nociceptors, it remains to be determined whether this metabolic set point is established during development or tuned in response to physiologic or environmental challenges. For example, does this set point depend on the expression and activation of ion channels such as TRPV1 or other receptors that define the properties of the nociceptor? Furthermore, while we have shown that manipulating ETC activity in a range of cell types (both neural and

non-neural) protects against calcium-mediated excitotoxicity, still at issue is whether this represents an intrinsic program that different cell types use to enhance resilience *in vivo*.

Mechanisms of capsaicin-induced excitotoxicity

All previous studies on TRPV1-mediated excitotoxicity agree that, akin to glutamate toxicity, calcium overload is a key step leading to cell death. We demonstrated that calcium overload causes massive ROS production from III_{Q0} of the mitochondrial ETC, which is directly responsible for cell death (Figure 5D–5I). Although we do not pinpoint the exact ROS species involved, the effective rescue of lethality using SOD mimetics (Figure 5E and 5G) and our identification of SOD1 as a sensitizing hit suggest that O₂^{•−} may be the most relevant culprit. As O₂^{•−} is converted to H₂O₂ by SODs in cells, we noted overlapping hits between our screen and other CRISPR and shRNA screens that used exogenously added H₂O₂ to induce oxidative stress.⁸¹ Conversely, genes or pathways unique to our screen may play a role in the detoxification of O₂^{•−} and warrant further investigation.

Downstream of ROS generation, we identified the transcription factors, MafF and its binding partner BACH1, as regulators of capsaicin-evoked cell death. Our data suggest that disinhibiting the downstream target of MAFF-BACH1 promotes resilience to excitotoxicity. Given their roles in oxidative stress response, future characterizations of their targets may reveal executioner(s) of excitotoxic cell death. Interestingly, expression of MAFF in brain endothelial cells was recently shown to be age-dependent.⁸² As aging is a major risk factor of painful neuropathy, it will be interesting to ask if changes in MAFF expression contribute to decreased resilience during aging.

Capsaicin-induced death as a generalizable model of excitotoxicity

Our studies started with a reductionist model of capsaicin-evoked cell death in cultured proliferating cell lines, which allowed us to uncover ETC tuning as a conserved protective mechanism that we have explored in both human cell lines (HEK293T and K562) and mouse sensory neurons. Moreover, ETC tuning effectively protects against distinct challenges, ranging from a pathogenic pore-forming toxin to gain-of-function channelopathies associated with CMT2C (Figure 6). Our mechanistic analyses further revealed that calcium-dependent ROS generation from site III_{Q0} of the ETC is a conserved instigator of cell death for each of these cell types and excitotoxic challenges (Figure 5G–I, 6D, 6F, and 6L).

Consistent with our model, a recent study has shown that S3QEL2, but not S1QEL1.1, is protective against NMDA receptor-dependent toxicity of hippocampal neurons,⁸³ suggesting that divergent models of excitotoxicity may encompass common elements. At the same time, other studies have implicated different sources of superoxide generation,^{1,2} including nitric oxide synthase (NOS), NADPH oxidases (NOXs), or different modes of cell death (e.g., parthanatos or ferroptosis).^{84,85} Going forward, our approach of leveraging a genome-wide chemical genetic screen provides an unbiased strategy for identifying comprehensive networks of regulators that can be further analyzed to reveal both common and unique pathways contributing to excitotoxicity.

Limitations of the study

This study focuses on cellular resilience to excitotoxic cell death. Based on genetic and pharmacologic data, we propose that one resilience pathway involves a pyruvate-dependent regulation of membrane protein trafficking. A more definitive characterization of the underlying mechanism will require further genetic and biochemical analyses of relevant membrane trafficking machinery, such as the flottilins, EMC, and other proteins that have emerged from our CRISPRi screen, in neurons as well as non-neural cells. Future work is also needed to examine the relevance of these mechanisms to excitotoxic injuries at peripheral terminals, where SARM1 and calpain play a role in axon degeneration of PNS neurons.^{86,87} Moreover, how this resilience pathway is affected by pathological conditions that involves systemic metabolic dysregulations, such as diabetes, is required to better understand the involvement of excitotoxicity in painful neuropathies. Related to this, our observations on differential ETC expression are gleaned from mouse models, but currently available human DRG datasets do not have deep enough coverage of the relevant genes to draw direct comparisons to human physiology or disease.

STAR METHOD

RESOURCE AVAILABILITY

Lead contact: Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, David Julius (david.julius@ucsf.edu).

Material availability: Requests for cell lines and plasmids generated in this study should be directed to and will be fulfilled by David Julius (david.julius@ucsf.edu) or Lin Yuan (lin.yuan@ucsf.edu).

Data and code availability

- CRISPRi screen results and RNAseq data are available in this paper's supplemental information. Raw and processed datasets are deposited in the NCBI Gene Expression Omnibus (GEO) with accession number GSE302928. Other data reported in this study will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT SUBJECT DETAILS

Mice: All experiments performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of University of California San Francisco (UCSF). Experiments followed the ethical guidelines outlined in the NIH Guide for the care and use of laboratory animals. *Tg(Trpv1-EGFP)MA208Gsat/Mmcd* transgenic mouse line was generated by the MMRRC (#033029-UCD). *Ndufs4^{fl/fl}* was acquired from The Jackson Laboratory.³⁰ *Uqcrq^{fl/fl}* and *Trpv1^{Cre}* were generated as previously described and deposited with The Jackson Laboratory.^{31,32} Mice were housed under 12:12 light-dark cycle with ad libitum access to food and water. Both males and females were used in this study and no

difference was noticed between them. No notable differences in body size were observed across genotypes.

Primary adult mouse DRG culture: Primary DRG cultures from adult mice were used in Figure 1B, 2, 5I, 7A–B, 7E–F, S1B, S7A and S7C. DRG neurons were dissected from adult mice of both genders between 6 – 24 weeks of age. Dissected DRGs were collected into Leibovitz's L-15 medium (Thermo Fischer Scientific) on ice and dissociated by two steps of enzymatic digestions. DRGs were digested in 40 U/ml papain (Worthington Biochemical) for 15 min at 37°C in calcium- and magnesium-free (CMF) HBSS (UCSF Media Productions), followed by a second digestion with 3 mg/ml collagenase (Worthington Biochemical), 4 mg/ml dispase II (Sigma-Aldrich), and 0.5 mg/ml DNaseI (Sigma-Aldrich) in CMF HBSS. Enzymes were deactivated by addition of 10% horse serum (UCSF media Productions) and triturated in 0.5 mg/ml DNaseI in CMF HBSS. DRG neurons were enriched from debris and support cells by passing through a 100 µm strainer. For long-term cultures (Figure 2D–F), cells were further purified by centrifugation through a discontinuous Percoll (Sigma-Aldrich) gradient consisting of 1 ml 12.5% Percoll in 0.5 mg/ml DNaseI in CMF HBSS, 2 ml 25% Percoll in 0.5 mg/ml DNaseI in CMF HBSS, and 1 ml 50% Percoll in CMF HBSS for 20 min at 800 xg with slow deceleration. The interphase between 25% and 50% Percoll was collected and washed with 10 ml CMF HBSS twice by centrifugation at 300xg for 5 min. All centrifugation steps were performed at 4°C. DRG neurons were cultured in Ham's F-12 Nutrient Mix which contains 1 mM pyruvate in its formulation (Thermo Fischer Scientific) with 10% horse serum, 50 ng/ml Nerve Growth Factor 7S (Millipore Sigma), 5 µM FdU (Millipore Sigma), 5 µM uridine (Millipore Sigma) (DRG media), on Poly-D-lysine (Sigma-Aldrich) and laminin (Invitrogen) coated surfaces. For experiments that compared TRPV1+ and TRPV1– populations (Figure 7A–B, 7E–F, S7A and S7C), 50 ng/ml BDNF (STEMCELL technologies) was added to the DRG media. DRG culture was fed every 2 days with fresh DRG media.

Generation and culture of immortalized cell lines: All cell cultures were maintained at 37°C with 5% CO₂ in sterile humidified incubators. HEK293T cells were cultured in DMEM (UCSF Media Productions) with 10% Bovine Calf Serum (Hyclone) and used in Figure 1A–B, 3B, 5A–E, 5G–H, 6D, 6F–G, S2A, S3C, S3H, S6A–B, S6E–F, and S6H–K. K562 cells were cultured in RPMI (Thermo Fisher Scientific) with 10% Fetal Bovine Serum (FBS, Peak Serum) and 1% Pen strep (UCSF Media Production) and used in Figure 1C–K, 3C–G, 4, 6B, 6I–L, S2B–D, S3A–B, S3D–G, S3I, S5, and S6G. For Figure 3A, K562 cells were cultured in glucose-free RPMI (Thermo Fisher Scientific) supplemented with 10% dialyzed FBS (Sigma-Aldrich) and indicated concentrations of glucose or galactose. Wildtype and MCU KO MEF cells were generous gifts from Dr. Yuriy Kirichok⁴⁷ and were cultured in DMEM with 10% FBS and used in Figure S6C–D.

Stable HEK293T, K562, and MEF cell lines were generated from parental cell lines transduced with lentivirus carrying TRPV1, empty vector (EV), or cyto/mito*Lb*NOX with a fluorescent marker: pLenti+GFP-TRPV1 was used to make HEK293T TRPV1; pLenti+TRPV1-IRES-mApple was used to make K562 TRPV1 and MEF wt or Micu1 KO TRPV1; pLenti+ IRES-mApple was used to make MEF wt or or Micu1 KO EV clonal cell lines;

pWPI-IRES-GFP, cyto or mito*Lb*NOX-IRES GFP were used to make K562 TRPV1 EV, cyto, or mito*Lb*NOX-IRES GFP stable lines respectively). In general, cells were transduced with lenti virus carrying the abovementioned transgenes, after which fluorescent cells were enriched using Fluorescence Activated Sorting with SONY SH800 (Sony Biotechnology) at the UCSF Laboratory for Cell Analysis (LCA). Single cell clones were isolated by plating on 96 well plates and selected clones were further amplified. TRPV1 clones were functionally selected based on their response to capsaicin as assessed by Fura-2 AM imaging. K562 clones were derived from dCas9-KRAS stable cells (parental)²² and the expression of dCas9-KRAS in TRPV1 expressing clones was also confirmed by transduction with sgRNAs targeting CD81 and confirmed with surface staining of APC anti-human CD81 antibody (BioLegend, 349509, 1:100) followed by flow cytometry on an Attune NxT flow cytometer (Thermo Fisher Scientific) at the UCSF LCA. Stable knockdown clones were obtained after lentiviral transduction of individual targeting and NT sgRNAs followed by puromycin (2 µg/ml, Thermo Fisher Scientific) selection.

METHOD DETAILS

Compound treatment: Small molecules were reconstituted with DMSO with the following exceptions: CP-456733 and DFO were dissolved in water. Cells were treated as detailed below, all concentrations are of the final solution. Figure1A: Survival of TRPV1⁺ HEK293T cells after exposure to DMSO or 3 µM capsaicin for 5 hours in 0 or 2 mM EGTA (left) or in Ringer's solutions without Ca²⁺ or Mg²⁺ or with NMDG⁺ substituting Na⁺ (right) was measured and normalized to vehicle controls. Figure1I: Survival of knockdown clones after 24-hour treatment of DMSO or 0.2 µM capsaicin was measured. Figure1K: Survival of TRPV1⁺ K562 cells that received pretreatment of PierA for 72 hours or AA for 48 hours prior to a 24-hour exposure to 0.4 µM capsaicin was measured and normalized to vehicle conditions (not shown). Figure 2C: 100 µM capsaicin for 24 hours. Figure 2F: 20 µM or 2 µM capsaicin for 24 hours. Figure 3A: TRPV1⁺ K562 cells grew with indicated concentrations of glucose and galactose for 72 hours days and treated with 0.2 µM capsaicin or DMSO control. Figure 3C,D,E,G: TRPV1⁺ K562 cells were pretreated with 4 nM PierA for 72h, or 10 nM AA for 48 hours, and supplemented with 1 mM pyruvate (Pyr) and 50 µg/ml uridine (Uri). Figure 3E–G: 24-hour treatment of capsaicin at E: 0.1 µM; F and G: 0.2 µM. Figure 4A–B: K562 cells were pretreated with either vehicle or 4 nM PierA supplemented with 1 mM pyruvate and 50 µg/ml uridine for 3 days, then treated with vehicle or 0.4 µM capsaicin for 4 hours. Figure 4D: 1 µM capsaicin for 24 hours. Figure 5C: pretreating cells with 40 nM PierA or 20 nM AA supplemented with 1 mM pyruvate and 50 µg/ml uridine for 72 hours prior to HyPer7 assay. Figure 5D,E: 50 µM M40403 for 30 minutes followed by HyPer7 assay (D) or a 5-hour exposure of 6.25 µM capsaicin (E). Figure 5G–I: pretreat cells with 15 µM S1QEL1.1, 50 µM S3QEL2, or vehicle for 6 hours followed by HyPer7 assay (G) or an overnight exposure of 0.4 µM capsaicin (H) or 100 µM capsaicin with 2 mM CaCl₂ followed by a 24-hour recovery (I). Figure 6B: Survival of TRPV1⁺ K562 cells pretreated with 4 nM PierA supplemented with 1 mM pyruvate and 50 µg/ml uridine for 72 hours followed with 1.4 µM DkTx or vehicle treatment overnight. Figure 6D,F,G: HEK293T cells transiently transfected with EV, TRPV4 WT, or R289C were pretreated with vehicle or 50 µM S3QEL2 for 6 hours (D,F) or 20 nM AA for 48 hours (G) before adding vehicle (D,G) or 10 µM GSK101(F,G) with 2 mM CaCl₂ overnight. Figure

6I–L: Viability of K562 cells against 0.74 $\mu\text{g/ml}$ Hla (I, K, L) or 5 $\mu\text{g/ml}$ Hla (J) was assessed when cells were co-treated with 2 mM EGTA (I), pretreated with 4 nM PierA for 72 hours (J), 50 μM M40403 (K) or 50 μM S3QEL2 (L) for 6 hours. Figure 7A: 10 μM ionomycin with 10 mM CaCl_2 for 2 hours. Figure 7B: 10 μM mitoPQ for 48 hours or 3.3 μM for 72 hours. Figure 7E: 3-day treatment of 400 nM PierA or 200 nM AA.

Note on capsaicin concentrations: For heterologous expression systems, most of the experiments were carried out at concentrations of capsaicin close to LD50 ($\sim 0.2 \mu\text{M}$) (Figure S3A). For neurons, lethality requires higher doses, and we empirically determined the best concentration to optimize the rescue or sensitizing effect of various treatments.

Supplemental figures: treatment conditions are indicated in the corresponding legends, including Figure S2, which used small molecule inhibitors of various cell death modalities (Z-VAD-FMK, ALLN, necrostatin-1, GSK872, U1026, E64D, AEBSF, cyclosporine A, Bafilomycin, TROLOX, IM-93, DFO, NSA, CP-456733, Liproxstatin-1, Ferrostatin-1, and ABT-888).

Time-lapse imaging for capsaicin or ionomycin toxicity: For capsaicin-induced toxicity, cultured primary sensory neurons or transfected HEK293T cells were imaged overnight in a temperature, humidity, and CO_2 controlled chamber on a Nikon inverted widefield fluorescent microscope at the Center for Advanced Light Microscopy (CLAM) at UCSF. An hour of baseline video was taken prior to the addition of capsaicin (Sigma-Aldrich) or vehicle. Images were acquired every 10 min at multiple locations of the chambered slide. For ionomycin-induced toxicity, primary DRG culture was imaged at 5 min / frame at multiple locations in duplicated wells. After 15 minutes (3 frames), 10 μM ionomycin or DMSO with 10 mM CaCl_2 was added and imaged for another 2 hours. The number of TRPV1-EGFP⁺ or TRPV1-EGFP⁻ neurons that survived treatment were counted manually, and statistics were tabulated using Excel (Microsoft) and graphed using Prism (GraphPad).

Molecular Cloning: Human TRPV1 cDNA was subcloned into pLenti-IRES mApple vector (generous gift from Dr. Martin Kampmann at UCSF), which was used to generate TRPV1⁺ immortalized cell lines. Mouse *Pgc1b* (Addgene1031) and mouse *Esrra* (Addgene 172152) were subcloned into pLenti IRES mApple vector for overexpression experiments. Individual sgRNAs (top scoring targeting sgRNAs or NT controls) were cloned from custom oligos (Integrated DNA Technologies) containing protospacers detailed in the Key Resources Table using an annealing protocol (<https://weissman.wi.mit.edu/crispr/>) into pU6+ Ef1alpha Puro-T2A-GFP (Addgene 111596). *LbNOX* and mito *LbNOX* were subcloned from pUC57 vectors (Addgene 75285, 74448)⁴¹ into pWPI IRES-EGFP vector (Addgene 12254). Site-directed mutagenesis was used to introduce R289C into human TRPV4 or to delete spyttag from pEXP5-aHL_spytag_his (Addgene 205343)⁹² before Hla purification. XL1-blue competent cells were used for all cloning described above.

Lentiviral production, transduction, and storage: Early passage HEK293T cells were used to package lentivirus: they were transfected using Transit Lenti transfection reagent (Mirus Bio) and Opti-MEM (GIBCO), boosted with ViralBoost

(Alstem, Inc) 24 hours after transfection, and harvested 48 hours post-transfection. K562 cells were transduced by “spinfection” for 2 hours (https://weissman.wi.mit.edu/crispria_cell_line_primer/). For neurons, harvested virus was concentrated 7-fold by precipitating with Lentivirus Precipitation Solution (Alstem, Inc) and reconstituted with DRG media. DRG culture was transduced on DIV2, fed every 2 days, and treated with capsaicin or vehicle on DIV9. Virus was stored at -80°C and used within 2 months.

CRISPRi screen: A genome-wide single-guide RNA (sgRNA) library hCRISPRi-v2 top5²³ was amplified using MegaX DH10B T1R Electrocomp cells and packaged into lentivirus as previously described²² (<https://weissman.wi.mit.edu/crispr/>). TRPV1⁺ CRISPRi K562 cells were transduced with library virus at a multiplicity of infection (MOI) <1 (22% transduction efficiency 2 days post-transduction) and a coverage of >1000 cells/sgRNA, which was maintained throughout the experiment. Replicates were maintained separately in spinner flasks for the course of the screen. Two days post-transduction, cells were selected with 0.75 $\mu\text{g}/\text{ml}$ puromycin for 2 days, resulting in 80-90% positive cells. After 24 hours of recovery from puromycin, a sample was collected from two biological replicates (T0), and remaining samples were further split into groups that received no treatment (NT) or pulsed capsaicin treatment (CAP). Cells were counted and split daily over the course of 14 days. CAP group received pulsed treatment of 0.2 μM (LD50) capsaicin until CAP cells had undergone 5-6 fewer doublings than UT cells.⁹³ Genomic DNA was isolated from T0, NT, and CAP samples using NucleoSpin Blood XL kit (Macherey-Nagel) and prepared for sequencing on an Illumina HiSeq4000 as previously described.²² Sequencing reads were aligned and counted using a previously described MAGeCK based pipeline.⁹¹ Data were graphed using RStudio and the ggplot2 and EnhancedVolcano packages.

Viability assays for immortalized cell lines: Viability of immortalized cell lines was performed in 96 well plate format according to manufacturers’ protocols and measured using a Biotek H4 plate reader (Agilent). For all experiments, 3-6 replicated samples of each condition were used. To establish standard curves under each condition (parental, TRPV1⁺, or KD cell lines; vehicle or highest concentration of pretreatment drugs), triplicated samples of counted cells (Countess II, Thermo Fisher Scientific) under each condition were generated on the same plate of each experiment. Estimated cell numbers were calculated when they were within linear range of the standards of the corresponding conditions. K562 cells were plated on 96 well plates with white walls and treated with capsaicin, DkTx, or Hla overnight. CellTiter-Glo (Promega G7570) was used to estimate total cell number (Figure 1I, 1K, 3A, 3E–3G, 4D, 6B, 6I–6L, S2B–D, S3A, S3D–G, S3I, S5C, S5F–H, S6G, and S6J). Lactate dehydrogenase (LDH) assays (Thermo Scientific C20300) were performed on the media supernatant taken after an overnight capsaicin treatment (Figure S5E). HEK293T cells were plated on Poly-D-Lysine coated 96 well plates with black walls, 5 hours after capsaicin or DkTx or 16 hours after GSK101 treatment, cell numbers were estimated using alamarBlue (Thermo Scientific DAL1025) fluorescence (Figure 1A, S2A, S3C, S3H, S6C and S6I) or cyQUANT Direct Cell (Thermo Scientific C35011) fluorescence to identify nuclei of live cells (Figure 5E, 5H, 6D, 6F, 6G and S6J). Data were obtained from the plate reader using Gen5 (Agilent), analyzed using Excel (Microsoft), and graphed with Prism 10 (GraphPad Software).

Immunoblotting: Cell lysate was extracted with PBST (0.5% Triton) with protease inhibitor cocktail (Roche) at 4°C for 30 min and supernatant was taken after centrifuging at 4°C 300g for 5 min. Proteins were resolved on 5-15% gels (bioRAD) and transferred onto PVDF membrane (0.2µm). For small molecular proteins, blots were fixed in 4% PFA for 30 min at RT, prior to blocking. This was followed by standard immunoblotting procedures and developed with ECL plus, ECL femto (Thermo Fisher Scientific), and imaged using a ChemiDoc Imager (Bio-Rad Laboratories). Primary antibodies: rabbit anti-Ndufc1 (Invitrogen, PA5-68240, 1:500), mouse anti-beta actin (Sigma-Aldrich, AC-15, 1:2000).

Calcium imaging and analysis: Fura-2 AM imaging was performed as previously described⁹⁴. Briefly, cells were washed with Ringer's solution (140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES and 10 mM glucose, pH 7.4 with NaOH; 290–300 mOsm kg⁻¹) once and loaded with 10 µg/ml Fura-2 AM (Thermo Fisher Scientific) in Ringer's solution containing 0.02% Pluronic F- 127 (Thermo Fisher Scientific) at room temperature in the dark for 1 hour. Following another wash with Ringer's solution, they were immediately imaged on an inverted microscope with 340 and 380 nm excitation (Sutter, Lambda LS illuminator) at a time interval of 2s. MetaFluor (Molecular Devices) was used for image acquisition and analyses. For Figure 3C–D, 4C, S5A–B, S5D, and S6F, 30–50 random K562 or HEK293T cells were selected from each video to analyze their response (emission ratio of 340/380) to indicated concentrations of capsaicin (for example, half maximum response was elicited by 33 nM capsaicin) and 10 µM ionomycin. Baseline (F_0) was calculated as the average 340/380 ratio prior to drug application, and ΔF was calculated as the stable 340/380 emission ratio after drug applications subtracted by the F_0 . Response to capsaicin was presented as a fraction of maximum response to ionomycin: $\Delta F_{\text{Cap}} / \Delta F_{\text{Ionomycin}}$. For Figure 2, 5I, 7E, and S1B, Fura-2 AM loaded DRG culture were used to determine TRPV1⁺%. Ringer's was added to control for activation of mechanosensitive neurons, followed by the addition of 10 µM capsaicin, and finally high-K⁺ Ringer's solution (same as Ringer's except with 5 mM NaCl and 140 mM KCl) to identify neurons.

Neurotoxicity assays: DRG cultures were plated on laminin (Invitrogen) coated 18-well chamber slides (ibidi GmbH, Cat No. 81816). On or after DIV2, primary DRG culture media was replaced with fresh media containing vehicle or compound treatments of indicated concentrations and durations. Capsaicin or vehicle treatments were supplemented with 2 mM CaCl₂ and incubated overnight, after which cells were washed with PBS, replaced with DRG media, and recovered for at least 24 hours before performing Fura-2 AM imaging to determine the percentage of TRPV1⁺ neurons (TRPV1⁺%) in each condition. TRPV1⁺% was calculated from the number of neurons that responded to 10 µM capsaicin out of the number of neurons responding to high extracellular K⁺. This was reported in Figures 2B, 2E, 5I, 7B and 7E. Percentage of TRPV1⁺ neuron that survived capsaicin or mitoPQ treatment (X) was calculated from the TRPV1⁺% after vehicle (Y) or capsaicin treatment (Z) under the same genetic or pretreatment conditions, where $X = Z(100 - Y) / Y(100 - Z)\%$ (see Figure S4) and was used for Figure 2C, 2F, 5I, 7B, and 7E. Videos were collected from at least 3 technical replicates (n) of each condition per biological

replicate (N). Technical replicates from the same condition of each biological replicate were aggregated into a single data point. This assay was devised because plate reader assays were unsuitable due to the small quantity of DRG neurons per animal, neuronal heterogeneity, and the presence of proliferating support cells. We also found that assays, such as propidium iodide staining, that mark nuclei of dead cells to be inaccurate because nuclei are often missing from necrotic cells. Due to variable percentage of TRPV1⁺ and baseline toxicity across biological replicates, we paired our analyses within each independent experiment.

RNAseq: TRPV1⁺ K562 cells were pretreated with vehicle or 4 nM PierA for 72 hours in the presence of 1 mM pyruvate and 50 µg/ml uridine followed by vehicle or 0.4 µM capsaicin treatment for 4 hours. There were 3 technical replicates of each condition, each replicate used 1.6 million cells. RNA was extracted using RNeasy Micro kit (Qiagen) and all replicates had RIN = 10 (Agilent Bioanalyzer). Library preparation, sequencing (Illumina), and sequence alignment were carried out by Novogene Corporation Inc. Differential expression analyses, data visualization, and cross referencing to our CRISPRi screen were performed using RStudio with DESeq2, ggplot2, and EnhancedVolcano. RStudio with ComplexHeatmap was used to reanalyze and graph published dataset.⁵⁷

HyPer7, PercevalHR, and mitoNeoD imaging and analysis: HEK293T cells were transfected with mitochondrial-targeted HyPer7 (Addgene 136470),⁴⁶ or PercevalHR (Addgene 49082).³⁷ Live cells were imaged on an inverted Nikon Ti microscope at 37°C with CO₂ using NIS-Elements at UCSF CALM. HyPer7 was excited at 380 nm and 480 nm, and emission was collected at 525 nm over 45 min with 1 min intervals using a 40x objective (Figure 5). PercevalHR was excited at 480nm and 400nm, and emission was collected at 525nm over 13 min with 1min interval (Figure 3B) or 5 hours with 10 min intervals (Movie S3). In a typical independent experiment, 5 videos were obtained from each well, and each experimental condition was duplicated in two wells. Videos were analyzed using the NIS-Elements (v5.4) to obtain HyPer7 or PercevalHR ratio ($F = \text{fluorescent intensity of emission signals excited by 480nm/380nm for Hyper7 and 480nm/400nm for PercevalHR}$) of each cell over time. Hyper7 response to 20 µM capsaicin and 200 µM hydrogen peroxide (H₂O₂), or Perceval response to 10 µM capsaicin and 3.3 mM IAA was calculated by RStudio using the formula $\Delta F/F_0$ for HyPer7 and $\Delta F/(F_{\text{IAA}} - F_0)$ for PercevalHR where F_0 is the average of the baseline timepoints (before capsaicin addition) and $\Delta F = F_t - F_0$ at each time points. Primary DRG cultures from *Tg(Trpv1-EGFP)MA208Gsat/Mmcd* mice were stained with 10 µM mitoNeoD for 15 min in Ringer's and replace with fresh DRG media after washing. Live DRG neurons were imaged with bright field, GFP, and MitoNeoD (excited at 562 nm and emission was collected at 632 nm). Images were analyzed using a segmentation AI trained with bright field images of DRG neurons in NIS-Elements (v5.4) where GFP and mitoNeoD intensities of each neuron were obtained. Prism 10 was used for graphing and statistics. Summary data presented were from at least 3 independent experiments.

MitoSOX assay: Parental or TRPV1⁺ HEK293T cells were plated on 12-well plates and grown to 70% confluence on the day of capsaicin treatment. A fresh aliquot of lyophilized mitoSOX (Thermo Scientific) was reconstituted in DMSO and used at the final concentration of 50 µM during the last 20 min of capsaicin or vehicle treatment. Cells were

harvested on ice, centrifuged at 300xg for 5 min, and cell pellets were washed with PBS. Washed cells were resuspended in PBS with 10% FBS and analyzed on an Attune NxT flow cytometer (Thermo Fisher Scientific, UCSF LCA). Flow cytometry data were analyzed using FlowJo (FlowJo LLC) and Prism (GraphPad Software). Gating was determined using no mitoSOX and vehicle treatment controls and used for all samples of the same experiment.

Hla expression and purification: Recombinant Hla was expressed and purified as previously described.⁹² Briefly, pEXP5-aHL-6xHis was expressed in Rosetta2 DE3 pLysS Competent Cells grown in MagicMedia (Invitrogen, K6803) for 8 hours at 37°C. Cells were sonicated in 50 mM Tris pH8, 200mM NaCl, 10 mM Imidazole and Protease Inhibitor Cocktail (Roche). Hla was purified with Ni-NTA (GE, 30230), washed with 20 mM Imidazole, eluted with 300 mM Imidazole, and dialyzed into 10 mM HEPES pH7.6 and 140 mM NaCl for storage and assays.

Immunohistochemistry: TGs and DRGs were acutely harvested into ice-cold L-15 medium and transferred into 4% paraformaldehyde for overnight fixation. After washes with phosphate-buffered saline (PBS, Quality Biological, 119-069-491), the ganglia were allowed to settle in 30% sucrose (SigmaAldrich, S7903) at 4°C and were then cryopreserved in Tissue-Tek O.C.T. Compound (Sakura Finetek USA) for sectioning at 10 µm thickness. Sections were washed with PBST (i.e., PBS containing 0.3% Triton X-100 [Sigma-Aldrich, T8787]) and incubated with blocking buffer (PBST containing 10% bovine serum albumin (BSA) (Sigma-Aldrich A2153) and 10% normal goat serum (NGS) (Thermo Fisher Scientific, 16-210-064) at room temperature for 1 hour. Subsequently, sections were incubated with primary antibodies in PBST 1% BSA 1% NGS at 4°C overnight. Following 3 washes, the sections were incubated with secondary antibodies (Thermo Fisher Scientific) in blocking buffer at room temperature for 1 hr. Finally, the sections were washed and mounted with Fluoromount-G (SouthernBiotech, 0100-01). Images were taken on a Nikon inverted widefield fluorescent microscope (UCSF CALM). The same parameters were used to collect images of the same experiment. To quantify ETC expression, mean fluorescent intensities of TRPV1⁺ cells and their closest neighboring TRPV1⁻ cells were measured using Fiji. Primary antibodies: rabbit anti-TRPV1 (Alomone Labs, ACC-030, 1:400), chicken anti-GFP (abcam, AB13970, 1:1000), rabbit anti-Ndufs4 (Millipore Sigma, HPA003884, 1:100), rabbit anti-Cox6 (Millipore Sigma HPA014295; 1:100).

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed in GraphPadPrism. Specific tests are listed in the corresponding figure legends. Normality was assessed with the Shapiro-Wilk test (small samples), the D'Agostino-Pearson omnibus K² test (moderate to large samples), and/or the Anderson-Darling test, as appropriate. Data that met normality and equal-variance assumptions were analyzed with unpaired or paired two-tailed t-tests, or with one- or two-way ANOVA followed by the indicated multiple-comparison correction. Non-normal data were analyzed with the Mann-Whitney U test or the Kruskal-Wallis test followed by the appropriate post-hoc correction. All data are presented as mean ± standard deviation (SD). 'n' denotes technical replicates; 'N' denotes biological replicates (individual animals). Significance levels: n.s., not significant; P < 0.05; P < 0.01; P < 0.001; P < 0.0001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- A genome-wide screen identifies pathways regulating excitotoxicity
- Electron transport chain (ETC) is a major determinant of resilience to excitotoxicity
- Reducing oxidative stress and calcium entry underlie this protective mechanism
- Nociceptors have lower ETC expression and greater resilience to excitotoxicity

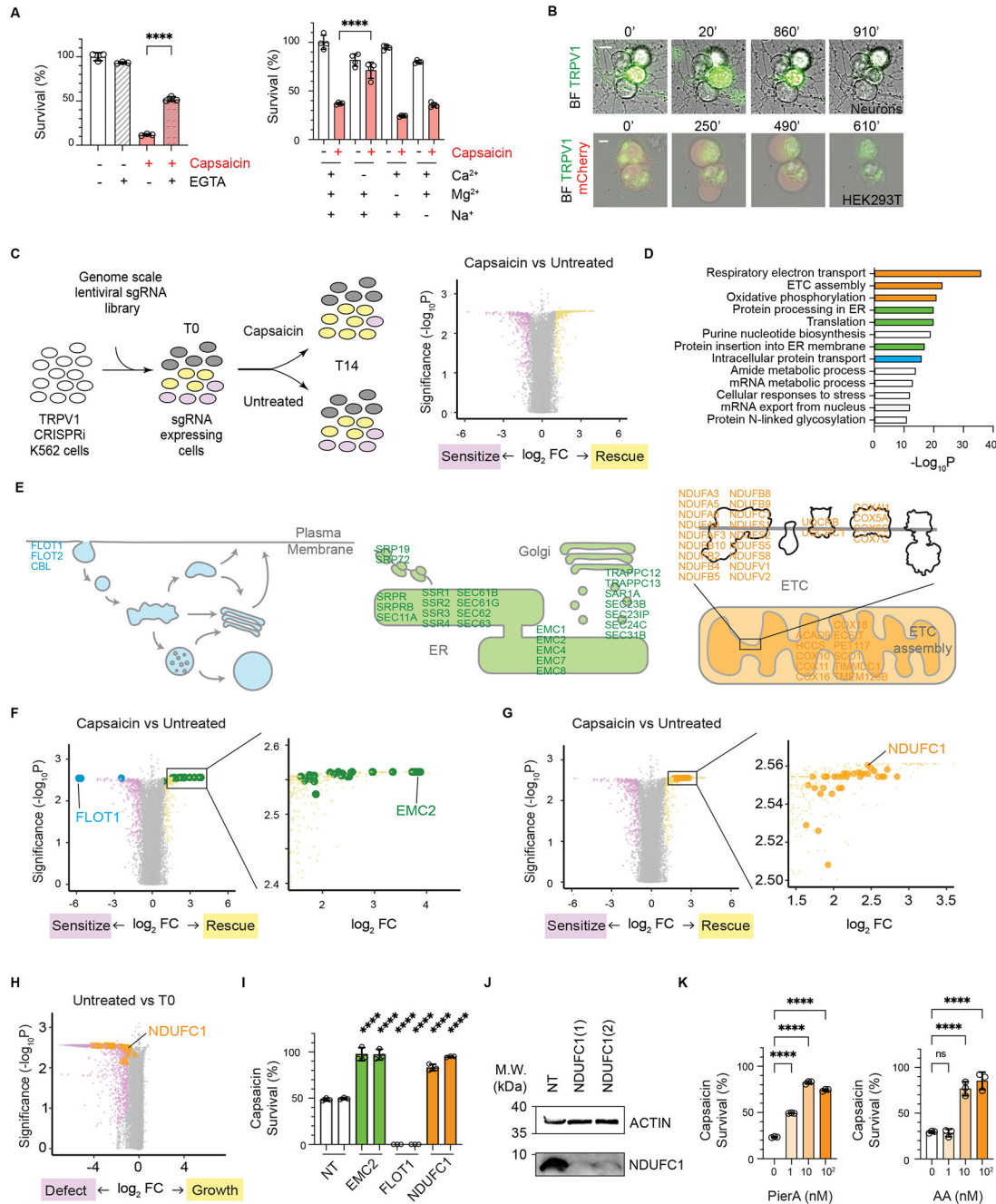


Figure 1. Genome-wide CRISPRi screen reveals the ETC as a regulator of capsaicin-evoked death.

(A) Survival of TRPV1⁺ HEK293T cells following capsaicin or vehicle treatment under different ionic conditions: (left) Extracellular calcium was either chelated with EGTA; (right) indicated ions were dropped out of Ringer-s solutions that cells were in throughout the viability assay.

(B) Representative images of DRG neurons from *Tg(Tipv1-EGFP)MA208Gsat/Mmcd* mice (top, Figure S1) and HEK293T cells transfected with plasmids containing GFP-TRPV1 and

mCherry (bottom) from time-lapsed imaging following the addition of capsaicin in minutes above each image. TRPV1⁺ neurons and cells showed features of necrosis, including soma swelling and the eventual rupture of plasma membrane. Scale bars: 10 μ m.

(C) K562 cells engineered with TRPV1 and CRISPRi machinery were transduced with a genome-wide sgRNA viral library. Duplicated samples were collected before treatment (T0) and after 14 days of no or pulsed capsaicin (LD50) treatment and sequenced for the abundance of barcoded sgRNAs. Volcano plot shows the relative abundance of all targeted genes and non-targeting controls when comparing capsaicin (CAP) to untreated (UT) samples.

(D) Most enriched Gene Ontology (GO) terms of CRISPRi screen hits of CAP compared to UT.

(E-G) Strong hits in the endocytic (blue), membrane protein biogenesis (green) and ETC (orange) pathways are illustrated in cartoons and highlighted on volcano plots.

(H) ETC hits from (G) is highlighted on the volcano plot that compares the relative abundance of sgRNAs from UT to T0.

(I) Knocking down indicated genes with two sgRNAs leads to protection (EMC2, NDUFC1) or sensitization (FLOT1) against capsaicin (LD50) comparing to non-targeting controls (NT) clones. Two sgRNAs of each target gene and NT controls were used to make stable clones, and the capsaicin survival was normalized to vehicle control of each clone (vehicle controls are not shown here, see full figure in Figure S3).

(J) Immunoblotting of lysates from *NDUFC1* knockdown clones made from two different sgRNAs and a NT control. Actin was used as loading control.

(K) Survival following capsaicin treatment was determined from cells pretreated with indicated concentrations of ETC inhibitors PierA or AA.

For **B,F,G**, $n \geq 3$ technical replicates representative of at least 3 independent experiments.

Data are mean $\pm$ SD. One-way ANOVA Tukey's or Dunnett's multiple comparisons tests.

**** $P < 0.0001$.

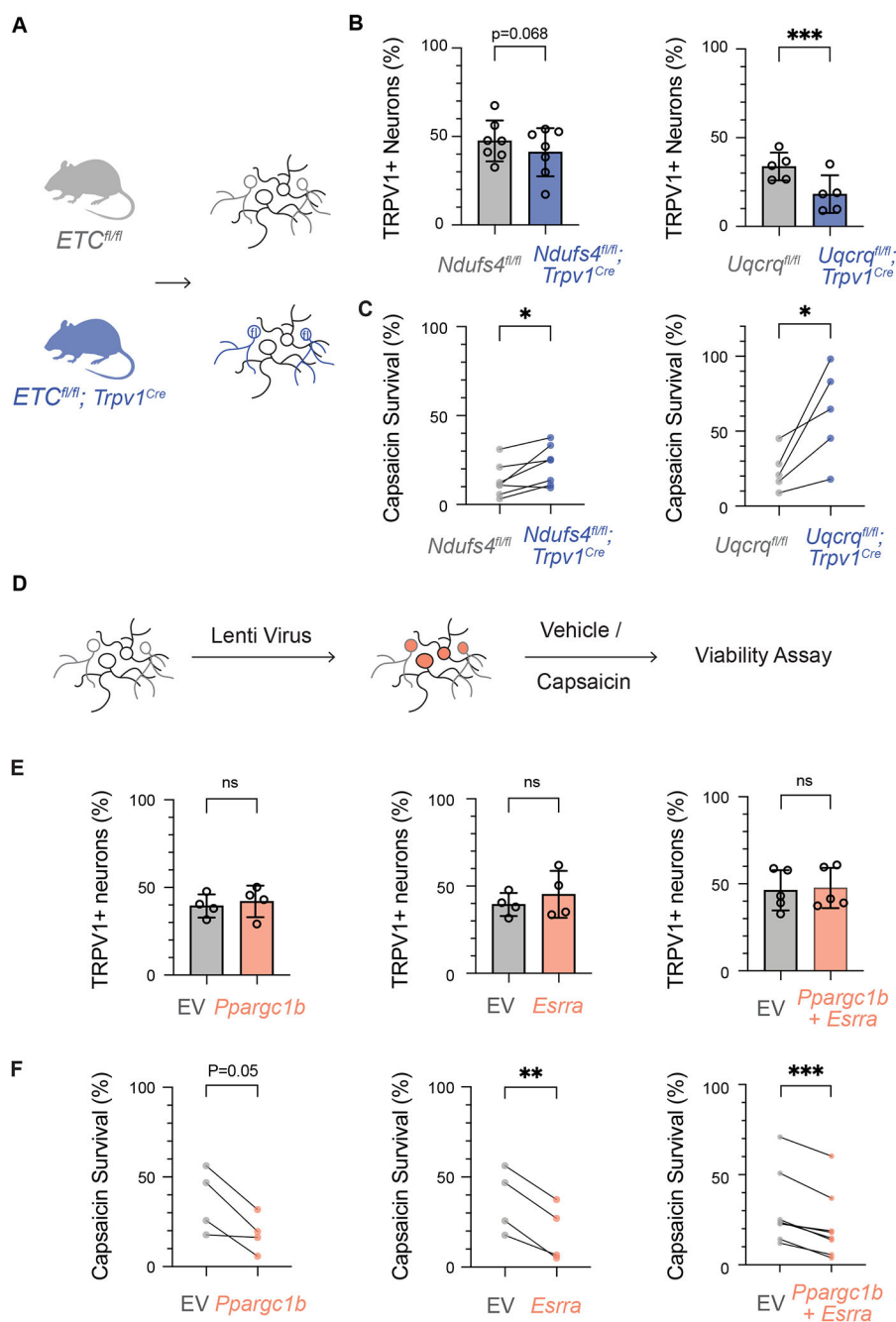


Figure 2. ETC regulates sensory neuron resilience to capsaicin-induced death.

(A) Schematics illustrates experimental design of (B,C).

(B,C) DRG cultures from littermates of the indicated genotypes were treated with vehicle or capsaicin. Percentage of TRPV1⁺ neurons in vehicle treated conditions and the percentage of TRPV1⁺ neurons survived capsaicin treatment were calculated from the number of TRPV1⁺ and total neurons at end points using Fura-2 AM assays (Figure S4). N= 7 or 5 pairs of animals for *Ndufs4* or *Uqcrcq* flox, calculated from least 3 technical replicates per animal.

(D) Schematics illustrates experimental design of (E,F).

(E-F) Primary DRG neurons were transduced with lentivirus carrying empty vector (EV) or indicated genes and treated with vehicle or capsaicin. Similar assay as (B,C). $N \geq$ biological replicates, calculated from at least 3 technical replicates.

Data are mean per animal and lines connect the same animal in C and F. Error bars are SD.

(B,E) Unpaired 2-tailed t-test. **(C,F)** Paired 2-tailed t-tests where each animal / biological replicate (N) were paired due to baseline variation; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$.

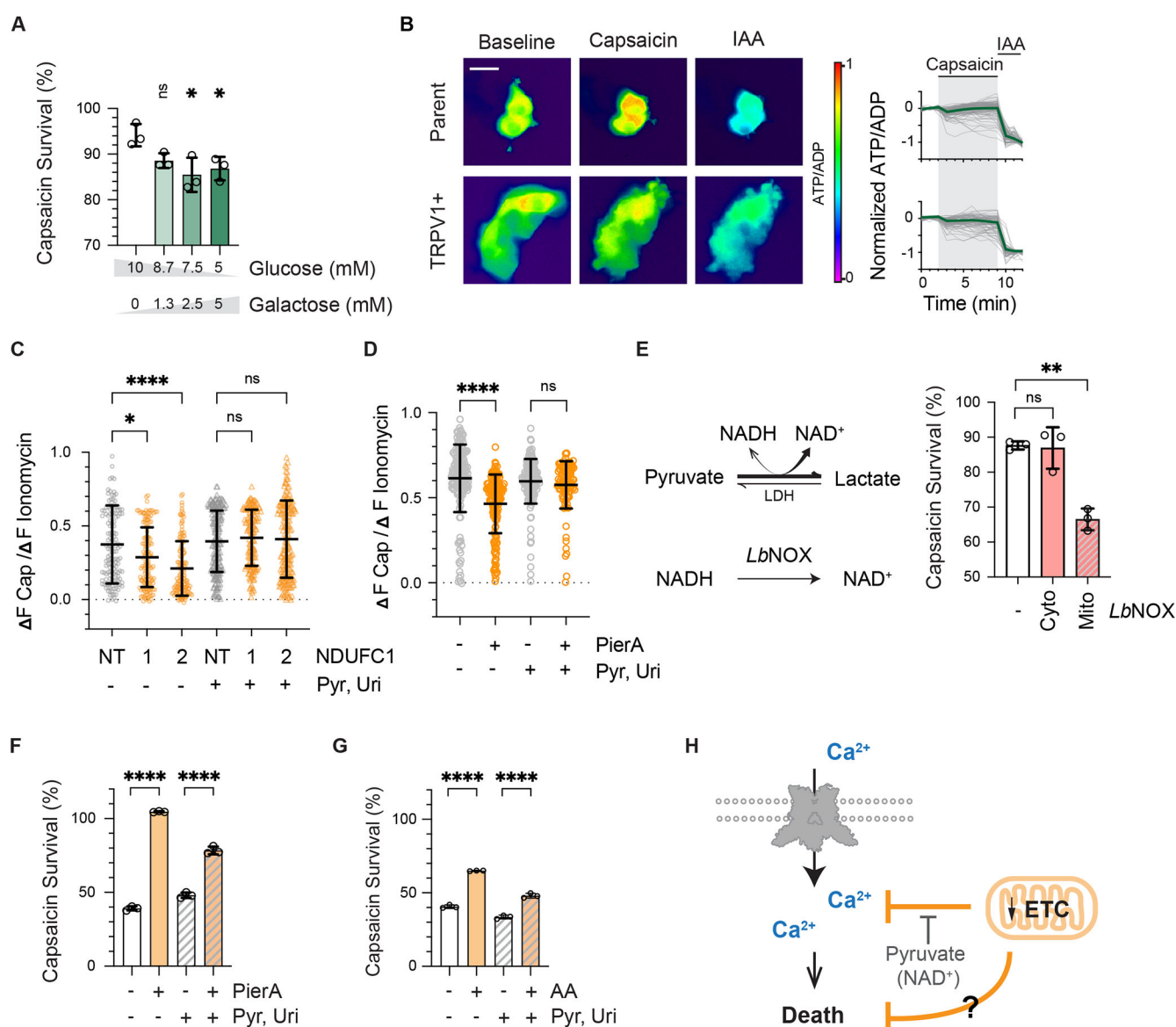


Figure 3. Metabolic regulation of capsaicin-evoked calcium overload.

(A) Capsaicin-induced toxicity in TRPV1⁺ K562 cells that had grown with indicated concentrations of glucose and galactose for 72 hours.

(B) ATP/ADP of HEK293T was measured with PercevalHR (pseudocolored rainbow scale; F = Ex480/Ex400) in response to 10 μM capsaicin at T₃ and 3.3 mM IAA at T₉ (revealing max response)³⁷. Normalized ATP/ADP (ΔF/ΔF_{IAA}) of each cell was plotted. Averaged kinetics in green. No difference in capsaicin response between parent and TRPV1⁺ cells. Scale bar: 10 μm.

(C,D) Calcium response was measured as change in Fura-2 AM ratio in response to capsaicin (33nM) as a fraction of maximum response to 10 μM ionomycin in (C) TRPV1⁺ K562 knockdown clones of *NDUF1* (using 2 different sgRNA) and a non-targeting (NT) control or (D) TRPV1⁺ K562 cells following the indicated pretreatment.

(E) Pyruvate accepts electrons from NADH to produce lactate and NAD^+ , a coupled redox reaction catalyzed by lactate dehydrogenase (LDH) that supports the proliferation of ETC-deficient cells⁴⁰. Oxidation of NADH to NAD^+ can be carried out by *LbNOX*.⁴¹ Viability of K562 stable lines that expressed empty vector, cyto, or mito*LbNOX* after capsaicin treatment and normalized to vehicle control.

(F,G) Following indicated pretreatments, survival against capsaicin was determined by normalizing cell number to vehicle controls of each condition.

(H) Schematic summary: ETC suppression imparts protection by reducing calcium overload, which can be negated by increased pyruvate (and thus NAD^+/NADH). ETC suppression is still protective of capsaicin toxicity in the presence of pyruvate, revealing an alternative rescue mechanism.

Data are mean $\pm$ SD * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. **(A,E-G)**: One-way ANOVA with Dunnett's (A,E) or Tukey's (F,G) multiple comparisons test. $n = 3$ technical replicates, representative of at least 3 independent experiments. **(C,D)**: Kruskal-Wallis test with Dunn's multiple comparisons test. $n =$ (C) 150; or (D) ≥ 107 cells from 3 independent experiments.

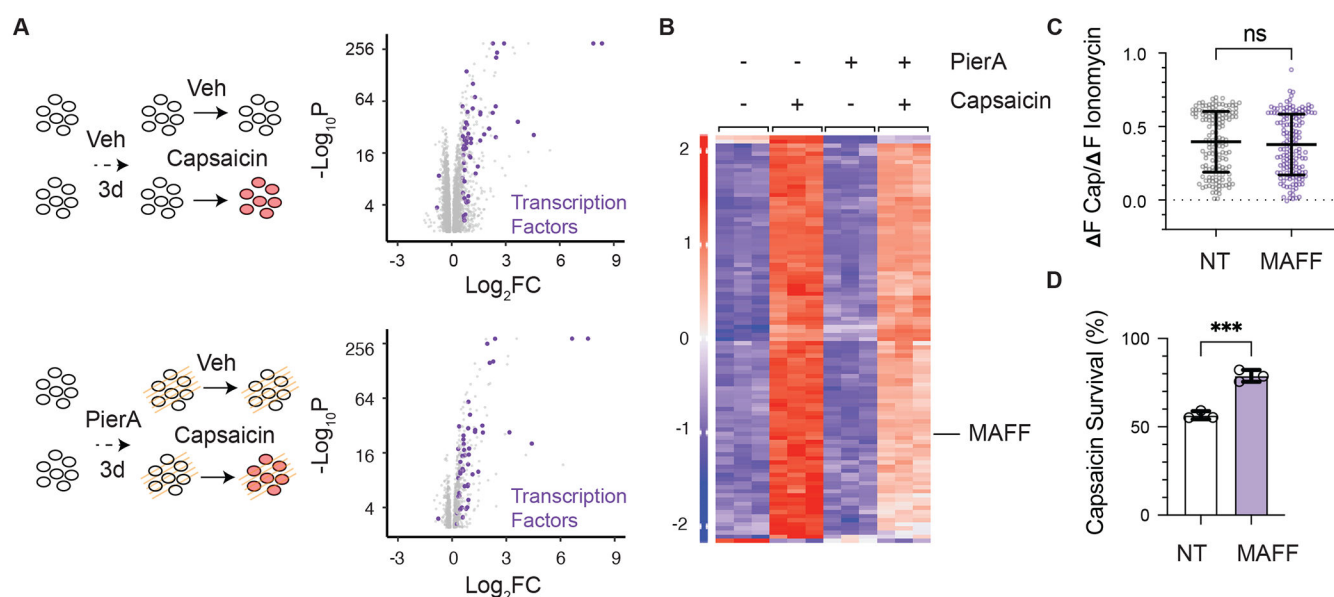


Figure 4. Capsaicin and ETC dependent transcriptional changes.

(A) Schematic of RNAseq experiment. RNAseq analyses were performed on 3 replicates of each condition. Differentially expressed genes of capsaicin versus vehicle treatments following (top) vehicle or (bottom) PierA pretreatment were plotted. Transcription factors are colored purple.

(B) Relative expression of top 100 significantly different genes in response to capsaicin was plotted based on the Z score of normalized counts (rlog transformation of the DEseq2 package).

(C,D) K562 cells that stably expressed NT or *MAFF*-targeting sgRNAs were tested for their calcium response to capsaicin (C) and viability after capsaicin treatment was quantified and normalized to vehicle (D).

Data are mean $\pm$ SD. **(C):** Mann-Whitney U test. $n = 150$ cells from 3 independent experiments;

(D): unpaired 2-tailed t-test. $n = 3$ technical replicates, representative of 3 independent experiments. *** $P < 0.001$.

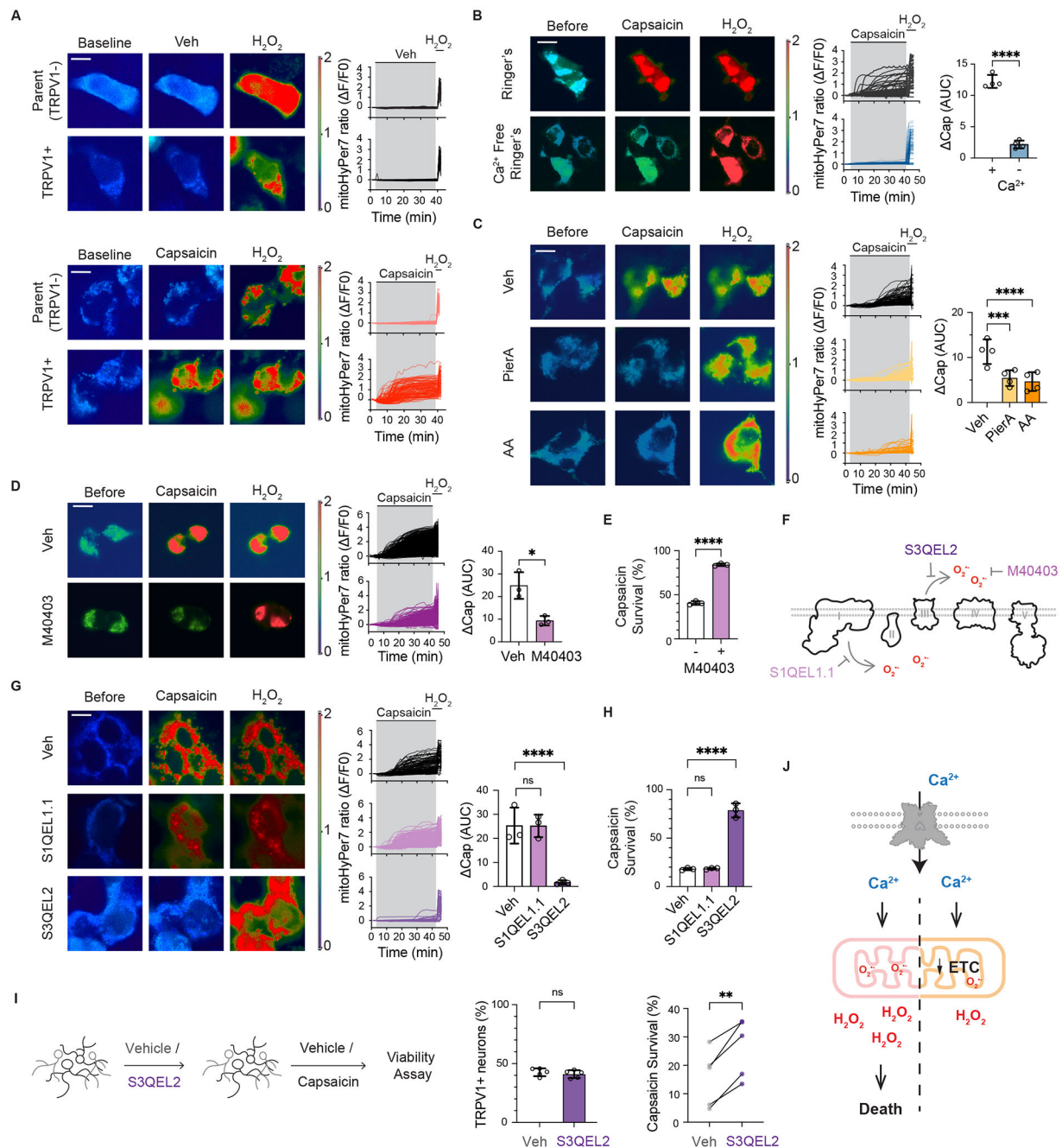


Figure 5. ETC suppression boosts resilience by lowering oxidative stress.

(A-D,G) Mitochondrial ROS generation kinetics in HEK293T cells. Capsaicin (20 μ M) was added at 3 min and H_2O_2 (200 μ M) at 42 min to elicit maximum response under indicated conditions. Representative images of the same cells at T_0 (before), T_{42} (max capsaicin response), and T_{45} (max H_2O_2 response) are shown in pseudo color of HyPer7 ratio ($F = \text{ex480/ex380}$, rainbow scale). Normalized HyPer7 ratio ($\Delta F/F_0$) over time of each cell are plotted for a representative experiment. Averaged capsaicin response of experimental replicates are summarized and quantified by area under curve (AUC). Scale bars: 10 μ m.

(A) Capsaicin or vehicle was added to parental (TRPV1⁻) or TRPV1⁺ cells.

(B) Capsaicin-evoked HyPer7 response in cells imaged in regular or calcium free Ringer's.

(C) Cells pretreated with ETC inhibitors PierA or AA (with pyruvate and uridine) were examined.

(D,E) HyPer7 response (D) and viability (E) of cells pretreated with a SOD mimetics M40403.

(F) Schematic summary: M40403 detoxifies O^{•-}; S1QEL1.1 and S3QEL2 block ROS production via sites I_Q and III_{QO} on complex I and III respectively without affecting OXPHOS.

(G,H) Cells pretreated with vehicle, S1QEL1.1, or S3QEL2 were treated with capsaicin and their HyPer7 response (F) and survival (G) were examined.

(I) Percentage of TRPV1⁺ neurons and their survival after capsaicin treatment following S3QEL2 or vehicle pretreatment (Figure S4).

(J) Schematic summary: Ca²⁺ influx through TRPV1 leads to mitochondrial ROS generation and cell death. ETC suppression protects cells by decreasing capsaicin-evoked ROS generation.

Data are mean ± SD. **(B-D,G)**: two-way ANOVA (B,D) with Dunnett's multiple comparisons (C,G). N = (B,C) 4; or (D,G) 3 as number of independent experiments (see Figure S6K). **(E)**: 2-tailed unpaired t-test. **(H)**: one-way ANOVA with Dunnett's multiple comparisons test. (E,H) n = 3 technical replicates, representative of at least 3 independent repeats. (I): 2-tailed paired t-test; N = 5 independent experiments. *P<0.05, **P<0.01, *** P<0.001, **** P<0.0001.

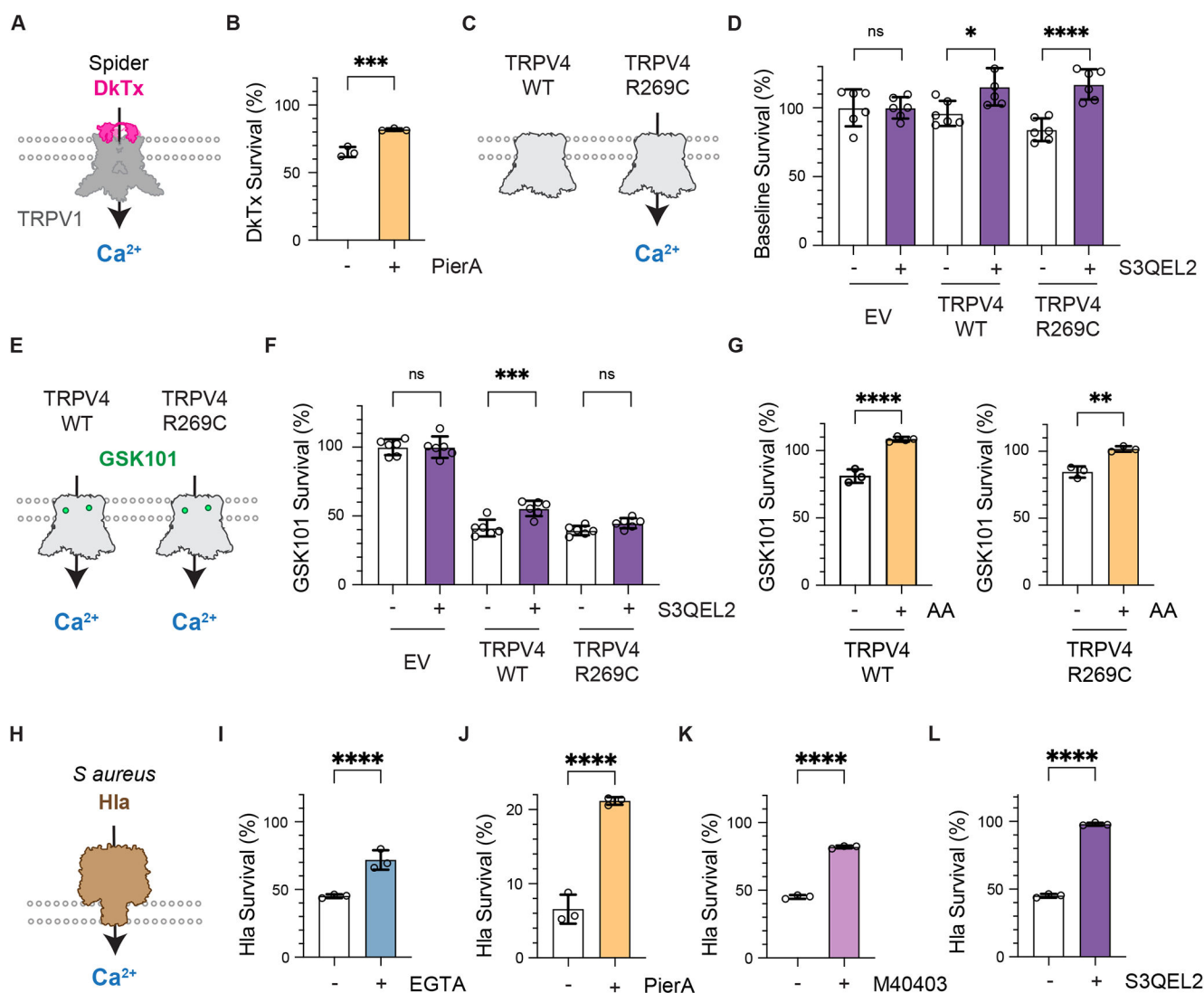


Figure 6. Generalization to other modalities of excitotoxicity.

(A) The spider toxin DkTx (pink) activates TRPV1 by binding to the extracellular loops, making it a distinct agonist from vanilloids including capsaicin.

(B) Survival against DkTx was tested in K562 cells pretreated with PierA or vehicle.

(C) Wildtype (WT) TRPV4 is closed in the absence of agonists. Disease-associated gain-of-function mutation R269C opens the channel and results in excitotoxicity without agonists.

(D,E,G) HEK293T cells transfected with TRPV4 WT, R269C, or empty vector (EV) control, were pretreated with vehicle or indicated ETC inhibitors (S3QEL2 or AA) prior to addition of vehicle (D) or TRPV4 agonist GSK101 (F,G).

(E) Both TRPV4 WT and R269C are activated by GSK101 (green).

(H) *S. aureus* secretes Hla (brown) that forms heptameric nanopores on plasma membrane and permeates Ca^{2+} at physiological extracellular concentrations (2 mM)

(I-L) Survival against Hla was tested in K562 cells co-treated with EGTA (F) or pre-treated with pre-treated with PierA (G), M40403 (H), S3QEL2 (I), or vehicle controls.

Data are mean $\pm$ SD. **(B,G,I-L)**: 2-tailed paired t-test. **(D,F)**: One-way ANOVA with Šídák's multiple comparisons test. *P<0.05, **P<0.01, *** P<0.001, **** P<0.0001.

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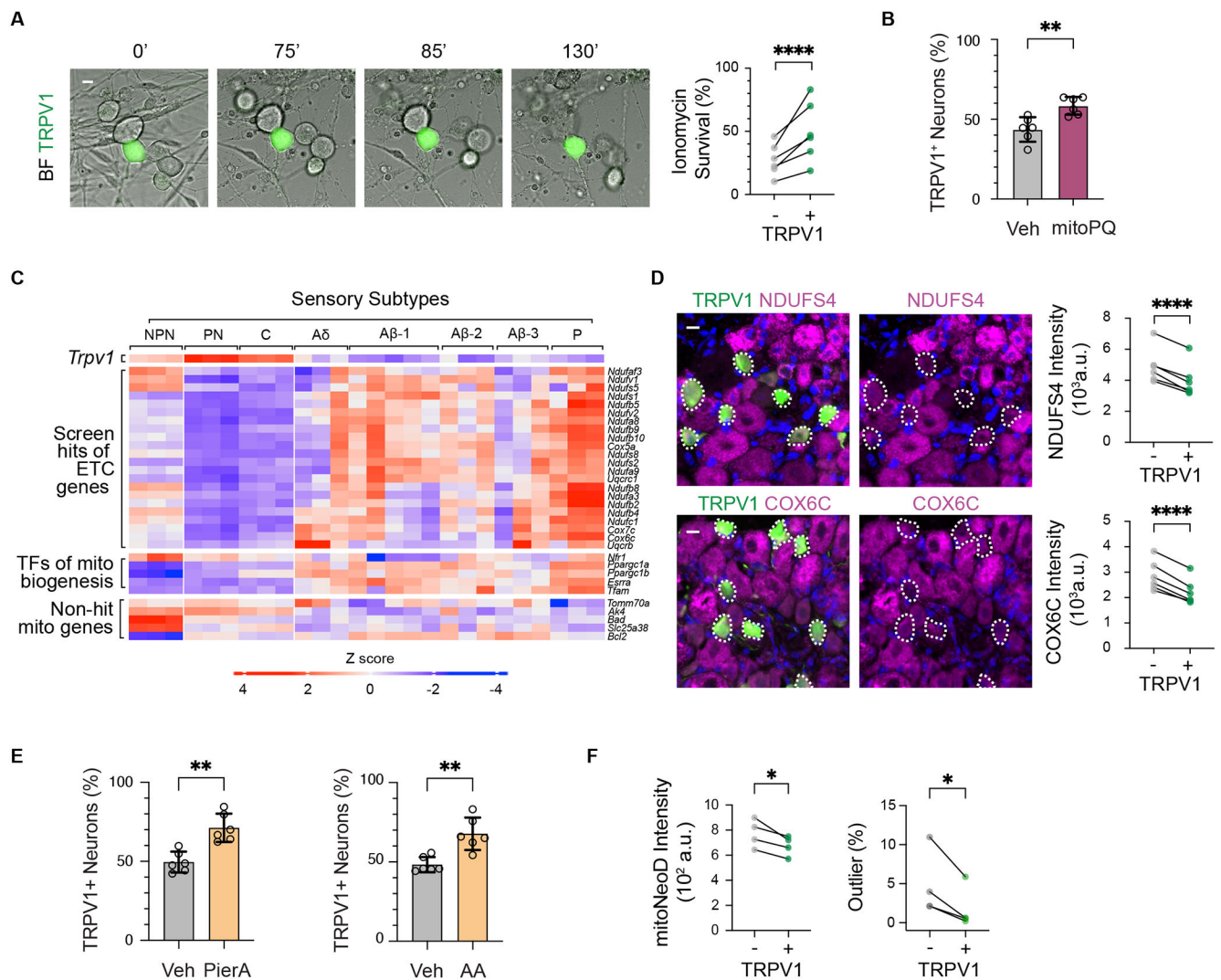


Figure 7. Nociceptors are more resilient to excitotoxicity.

(A) Representative images of DRG neurons taken from *Tg(Tipv1-EGFP)MA208Gsat/Mmcd* mice undergoing necrosis after ionomycin addition. Survival of TRPV1(GFP)⁺ or TRPV1(GFP)⁻ after ionomycin treatment were quantified from microscopy videos. Scale bar: 10 μ m.

(B, E) Relative survival of TRPV1⁺ vs TRPV1⁻ neurons against mitoPQ (B) or high dosages of PierA or AA (E) were revealed by shifting percentages of TRPV1⁺ neurons.

(C) Normalized expression level (rlog) among different sensory subgroups were plotted as Z scores for genes encoding TRPV1, ETC components identified by the CRISPRi screen, transcription factors of ETC biogenesis, and mitochondrial genes that were not screen hits as controls. Heatmap was generated using a published dataset, where sensory subtypes sequenced were non-peptidergic nociceptor (NPN), peptidergic nociceptor (NP), C-LTMR (C), A δ -LTMR (A δ), A β -(RA, SA1, or Field)-LTMR (A β -1-3), proprioceptor (P).⁵⁷

(D) Representative images of DRG sections from TRPV1-GFP mice (Figure S1) immunolabeled with antibodies that target ETC components (NDUFS4 or COX6C) and

GFP. Labeling intensities of ETC components in TRPV1(GFP)⁺ were quantified and compared TRPV1(GFP)⁻ neurons. Scale bars: 10 μ m.

(F) Basal ROS level of TRPV1⁺ or TRPV1⁻ neurons was measured by mitoNeoD intensity (left) and percentage of highly stressed outliers were calculated (right).

(A,D,F) Data are mean per animal and lines connect the same animal. N = 6,6,4 animals. (A,D) Two-way ANOVA (F) Paired t-test was performed on the mean intensity. Outliers were identified with the ROUT method (Q=1%), and represented as a percentage of total number of cells. Data of each animal see Figure S5. **(B,E)** Data are mean per animal with SD. N = 6 animals, calculated from at least 3 technical replicates per animal. Unpaired 2-tailed t-test. *P<0.05; **P<0.01; **** P<0.0001.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit anti-TRPV1 (1:400, IHC)	Alomone Labs	ACC-030
chicken anti-GFP (1:1000, IHC)	Abcam	AB13970
rabbit anti-Ndufs4 (1:100, IHC)	Millipore Sigma	HPA003884
rabbit anti-Cox6 (1:100, IHC)	Millipore Sigma	HPA014295
rabbit anti-Ndufc1 (1:500, WB)	Invitrogen	PA5-68240
mouse anti-beta actin (1:2000, WB)	Sigma-Aldrich	AC-15
Bacterial and virus strains		
<i>E. coli</i> : XL1-Blue Competent Cells	UC Berkeley QB3 MacroLab	N/A
<i>E. coli</i> : Rosetta2 DE3 pLysS Competent Cells	UC Berkeley QB3 MacroLab	N/A
<i>E. coli</i> : MegaX DH10B T1R Electrocomp Cells	Invitrogen	C640003
Chemicals, peptides, and recombinant proteins		
RPMI 1640 Medium, HEPES	Thermo Fisher Scientific	22400071
RPMI 1640 Medium, no glucose	Thermo Fisher Scientific	11-879-020
Fetal Bovine Serum	PEAK	PS-FB1
Bovine Calf Serum	HyClone	SH30072.03
Fetal Bovine Serum, Dialyzed	Sigma-Aldrich	F0392
Natural Mouse Laminin	Invitrogen	23017015
Papain	Worthington Biochemical	LS003126
Collagenase, Type 2	Worthington Biochemical	LS004176
Dispase II	Sigma-Aldrich	D4693
Deoxyribonuclease I, ≥85 %, ≥400 Kunitz units/mg (DNase I)	Sigma-Aldrich	DN25
L-15 Leibovitz media with L-glutamine	Cytiva	SH30525.01
Horse Serum Refiltered-Heat-inactivated	UCSF Media Productions	CCFAW001
Ham's F-12 Nutrient Mix	Sigma-Aldrich	51651C-1000ML
Nerve Growth Factor-7S from murine submaxillary gland	Millipore Sigma	N0513
Human Recombinant BDNF	STEMCELL Technologies	78005
5-Fluoro-2'-deoxyuridine (FdU)	Millipore Sigma	F0503
Uridine	Millipore Sigma	U3003
Capsaicin, ≥95%, from Capsicum sp.	Sigma-Aldrich	M2028
Piericidin A	Cayman Chemical Company	15379; CAS 2738-64-9
Antimycin a from Streptomyces sp.	Sigma-Aldrich	A8674; CAS 1397-94-0
Iodoacetic acid (IAA)	Sigma-Aldrich	I4386 CAS 64-69-7
Sodium Pyruvate	Sigma-Aldrich	792500
Hydrogen peroxide solution, 30 % (w/w) in H ₂ O, contains stabilizer	Sigma-Aldrich	H1009
M40403 (Imisopasem Manganese)	Cayman Chemical Company	31112; CAS 218791-21-0
S1QEL1.1	Sigma-Aldrich	SML1948; CAS 897613-29-5
S3QEL 2	Sigma-Aldrich	SML1554; CAS 890888-12-7

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DkTx	Bohlen et al., ²⁸	N/A
GSK101	Cayman Chemical Company	17289; CAS 942206-85-1
Recombinant <i>Staphylococcus aureus</i> Hla	This study	N/A
Ionomycin, ≥98% (HPLC), From Streptomyces globatus, Lyophilized Oil	Sigma-Aldrich	I9657; CAS 56092-81-0
mitoPQ	Sigma-Aldrich	SML3152; CAS 1821370-28-8
Fura-2 AM	Thermo Fisher Scientific	F1201
Pluronic F-127	Invitrogen	P3000MP
MitoNeoD	MedKoo Bioscience, Inc.	563761; CAS 2375088-89-2
Z-VAD-FMK	R&D Systems	FMK001
ALLN	Millipore Sigma	208719
Necrostatin-1	Tocris	2324
GSK872	Cayman Chemical Company	23300
U0126	Tocris	1144
E64D	Tocris	4545
AEBSF	Cayman Chemical Company	14321
Cyclosporine A	Tocris	1101
Bafilomycin	Tocris	1334
TROLOX	Tocris	6002
IM-93	Cayman Chemical Company	28794
Deferoxamine Mesylate (DFO)	Millipore Sigma	252750
Necrosulfonamide (NSA)	Cayman Chemical Company	20844
CP-456773	Millipore Sigma	PZ0280
Lipoxstatin-1	Cayman Chemical Company	17730
Ferrostatin-1	Cayman Chemical Company	17729
ABT-888	Cayman Chemical Company	11505
Rotenone	Sigma-Aldrich	R8875
Phenformin	Cayman Chemical Company	14997
mitoSOX	Thermo Scientific	M36008
mitoTEMPO	Sigma-Aldrich	SML0737
Critical commercial assays		
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	G7570
AlamarBlue™ Cell Viability Reagent	Thermo Scientific	DAL1025
CyQUANT™ Direct Cell Proliferation Assay	Thermo Scientific	C35011
CyQUANT™ LDH Cytotoxicity Assay Kit	Thermo Scientific	C20300
NucleoSpin Blood XL kit	Macherey-Nagel	740950
Deposited data		
CRISPRi screen and RNAseq	This study	GEO: GSE302928
Experimental models: Cell lines		

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HEK293T	ATCC	CRL-11268
HEK293T TRPV1	This study	N/A
K562-dCas9-KRAB	Gilbert et al. ²²	N/A
K562-dCas9-KRAB TRPV1	This study	N/A
K562-dCas9-KRAB TRPV1 (NT-1)	This study	N/A
K562-dCas9-KRAB TRPV1 (NT-2)	This study	N/A
K562-dCas9-KRAB TRPV1 (EMC2 KD-1)	This study	N/A
K562-dCas9-KRAB TRPV1 (EMC2 KD-2)	This study	N/A
K562-dCas9-KRAB TRPV1 (FLOT1 KD-1)	This study	N/A
K562-dCas9-KRAB TRPV1 (FLOT1 KD-2)	This study	N/A
K562-dCas9-KRAB TRPV1 (NDUFC1 KD-1)	This study	N/A
K562-dCas9-KRAB TRPV1 (NDUFC1 KD-2)	This study	N/A
K562-dCas9-KRAB (NT-1)	This study	N/A
K562-dCas9-KRAB (NDUFC1 KD-1)	This study	N/A
K562-dCas9-KRAB (NDUFC1 KD-2)	This study	N/A
K562-dCas9-KRAB TRPV1 ev-IRES-GFP	This study	N/A
K562-dCas9-KRAB TRPV1 cytoLbNOX-IRES GFP	This study	N/A
K562-dCas9-KRAB TRPV1 mitoLbNOX-IRES GFP	This study	N/A
K562-dCas9-KRAB TRPV1 (MAFF KD)	This study	N/A
K562-dCas9-KRAB TRPV1 (BACH1 KD)	This study	N/A
MEF	Garg et al. ⁴⁷	N/A
MEF (Micu1 KO)	Garg et al. ⁴⁷	N/A
MEF EV	This study	N/A
MEF TRPV1	This study	N/A
MEF (Micu1 KO) EV	This study	N/A
MEF (Micu1 KO) TRPV1	This study	N/A
Experimental models: Organisms/strains		
Mouse: <i>Tg(Trpv1-EGFP)MA208Gsat/Mmcd</i>	MMRRC	#033029-UCD
Mouse: B6.129S4- <i>Ndufs4</i> ^{m1Rpa/J} (<i>Ndufs4</i> ^{fl/fl})	Quintana et al. ³⁰	JAX: 026963
Mouse: <i>Uqcrg</i> ^{fl/fl}	Martínez-Reyes et al. ³¹	JAX: 038729
Mouse: B6.129- <i>Trpv1</i> ^{tm1(cre)Bbm/J} (<i>Trpv1</i> ^{Cre})	Cavanaugh et al. ³²	JAX: 017769
Mouse: C57BL/6J	JAX	JAX: 000664
Oligonucleotides		
Protospacer for sgRNA cloning of nt00239: GATTGTCACCTTAGATCTGT	This study	N/A
Protospacer for sgRNA cloning: nt00242: GTTGAATTACAGTTCGACGG	This study	N/A
Protospacer for sgRNA cloning: NDUFC1: GGAAAGGGGACGCAGCAAGG	This study	N/A
Protospacer for sgRNA cloning: NDUFC1: GCCCCCGCCAGGCTCCCGAG	This study	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Protospacer for sgRNA cloning: EMC2: GACGGGCCAAGCTGAGGTGG	This study	N/A
Protospacer for sgRNA cloning: EMC2: GCCATCTTCCCAGAACCTAG	This study	N/A
Protospacer for sgRNA cloning: FLOT1: GGCCGTGGCGGATGCAGACT	This study	N/A
Protospacer for sgRNA cloning: FLOT1: GGCAGCAACGGGGTGCGGCA	This study	N/A
Protospacer for sgRNA cloning: MAFF: GGAGCGGAGGGGAGACTGAC	This study	N/A
Protospacer for sgRNA cloning: BACH1: GGGCGGCGGAGGACAATACG	This study	N/A
Recombinant DNA		
pLenti+ TRPV1-IRES-mApple	This study	N/A
pLenti+GFP-TRPV1	This study	N/A
human Genome-wide CRISPRi-v2 library	Horlbeck et al. ²³	Addgene:83969
pU6+ sgRNA Ef1alpha Puro-T2A-GFP	Horlbeck et al. ⁹⁰	Addgene:111596
GW1-PercevalHR	Tantama et al. ³⁷	Addgene:49082
pWPI IRES-GFP	Didier Trono	Addgene:12254
pWPI cyto <i>Lb</i> NOX-IRES-GFP	This study	N/A
pWPI mito <i>Lb</i> NOX-IRES-GFP	This study	N/A
pCS2+MLS-HyPer7	Pak et al. ⁴⁶	Addgene:136470
pLenti+ Pgargc1b-IRES-mApple	This study	N/A
pLenti+ Esrra-IRES-mApple	This study	N/A
pcDNA5_hTRPV4	This study	N/A
pcDNA5_hTRPV4 R269C	This study	N/A
pEXP5-aHL_his	This study	N/A
Software and algorithms		
MetaFluor	Molecular Devices	https://www.moleculardevices.com/products/cellular-imaging-systems/high-content-analysis/metamorph-microscopy
Prism10	GraphPad	https://www.graphpad.com/
FlowJo10	FlowJo	https://www.flowjo.com/
NIS-Elements v5.4	Nikon Instruments Inc.	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
MAGECK-iNC	Tian et al. ⁹¹	https://kammannlab.ucsf.edu/mageck-inc
R and RStudio	Posit	https://posit.co/download/rstudio-desktop/
DESeq2 (R package)	Bioconductor	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
ggplot2 (R package)	CRAN	https://ggplot2.tidyverse.org/
EnhancedVolcano (R package)	Bioconductor	https://bioconductor.org/packages/release/bioc/html/EnhancedVolcano.html

REAGENT or RESOURCE	SOURCE	IDENTIFIER
ComplexHeatmap (R package)	Bioconductor	https://bioconductor.org/packages/release/bioc/html/ComplexHeatmap.html
Gen5	Agilent	https://www.agilent.com/en/product/microplate-instrumentation/microplate-instrumentation-control-analysis-software/imager-reader-control-analysis-software
Fiji – ImageJ	NIH	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3855844/
Other		
Microscopy chamber slides	ibidi	18 well: 81816; 8 well: 80806