

DETERMINING FUNCTION OF THE IKAP PROTEIN IN THE PERIPHERAL
NERVOUS SYSTEM FOR TARGETED THERAPEUTIC
INTERVENTION IN FAMILIAL DYSAUTONOMIA

by

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DEDICATION

To the kind, creative, and fearless.

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ABSTRACT

Familial Dysautonomia (FD) is a recessive genetic disorder that leads to devastation of the peripheral nervous system and is the result of incomplete neurodevelopment and progressive neurodegeneration. The disorder is also marked by a continual loss of retinal ganglion cells that leads to blindness. Even with early identification and treatment, the disorder is ultimately fatal. FD is caused by mutation in the *IKBKAP* gene that leads to cell-type specific loss of the IKAP protein, also known as ELP1. IKAP functions as a part of the six-unit Elongator complex. The role of Elongator is unresolved, although data has accumulated that support Elongator function in tRNA modification and efficient translation of proteins and that its absence leads to cell stress and neurological impairment. We have a mouse model of FD in which mouse *Ikbkap* is deleted from the peripheral nervous system, and it recapitulates the death of autonomic and TrkA⁺ sensory neurons observed in FD patients. As we can culture TrkA⁺ neurons *in vitro*, while also studying this neuronal population *in vivo*, we have a system to investigate our goals of (1) determining cellular processes that go awry in absence of Ikap and (2) targeting these cell types and events to prevent their progressive death. We have determined that mitochondrial and cytoskeletal function are disrupted in *Ikbkap*^{-/-}, TrkA⁺ neurons and show activation of stress signaling. Interestingly, disrupted mitochondrial function is an emerging hallmark common to most neurodegenerative diseases. We have identified that the compound, BGP-15, is able to restore aspects of mitochondrial function and stress signaling *in vitro* and can restore neuronal survival of TrkA⁺ neurons lacking Ikap *in vitro* and *in vivo*. BGP-15 also improves actin cytoskeletal function and target innervation. Additionally, we have determined that introduction of the C-terminal half of human IKAP is sufficient to increase neuronal survival *in vitro*. This smaller protein fragment is compatible with viral delivery to retinal ganglion cells and could be utilized for gene therapy, potentially preventing this neuronal death that lead to blindness. Our goals now are to further explore stress pathways common to many neurodegenerative disorders and optimize rescue strategies *in vivo*.

CHAPTER ONE

INTRODUCTION

Familial Dysautonomia

Familial Dysautonomia (FD) is a recessive genetic disorder first characterized by Riley and Day in 1949 where they described children with autonomic dysfunction, marked by a lack of tears, vomiting crises, hypertension, and tachycardia (1). FD is type III of a group of hereditary and sensory autonomic neuropathies (HSANs), which are all characterized by varying degrees of disrupted autonomic function and reduced pain sensation (1). FD is both a developmental disorder with a lack of complete neurodevelopment, thus impairment is present at birth, and a progressive disorder with continual loss of sensory and autonomic neurons into adulthood (2-4). FD is also accompanied by loss of retinal ganglion cells (RGCs) that leads to later onset blindness (5, 6). Identification of the mutation causing FD, a point mutation in the *IKBKAP* gene, was more recently described and accounts for over 99% of FD cases (7-10) affecting those of Ashkenazi Jewish descent, where the carrier frequency is 1:27 to 1:32 (1, 4, 10). FD is ultimately fatal, although 50% of patients now live into their 40s (4).

IKBKAP, or the I κ B kinase (IKK) complex-associated protein, encodes the IKAP protein, also known as ELP1 (7, 8). The major *IKBKAP* mutation is a T to C change in base pair 6 of intron 20 (IVS20+6T > C)—a splice site mutation that leads to variable expression of the IKAP protein (1, 9, 10). The majority of FD patients are homozygous for the mutation. However, three missense mutations have also been reported in patients

heterozygous for the major mutation, though this is extremely rare (9, 10). IKAP is expressed in all tissues, and interestingly, some cell types are able to splice the RNA normally and produce wildtype protein even in the presence of the mutation (9, 10). Neurons are most deficient in wildtype mRNA and protein suggesting they are least capable of accommodating the mutation, splicing correctly, and producing wildtype *IKBKAP* mRNA (1, 9, 10). There is evidence that the mutant *IKBKAP* transcript is targeted for nonsense-mediated decay (9). Though it is unresolved why neurons are unable to splice in the presence of the mutation, while other cell types prove capable, the drastic loss of IKAP in neurons leads to widespread devastation of peripheral neurons and RGCs.

IKAP in Cell Stress

IKAP/ELP1 is a 150 kDa scaffolding protein that functions as part of the six-unit Elongator complex, consisting of ELP1-ELP6, a highly conserved complex with diverse reported roles in the cell. Elongator was first described to aid in transcript elongation, attributed to the acetylase activity of subunit ELP3 and its modification of histones (10-12). The complex was later revealed to have a largely cytoplasmic function, based on its predominant localization in the cytosol and its requirement for modification of uridines in the wobble position of the tRNA anti-codon loop (synthesis of 5-methoxycarbonylmethyl and 5- carbamoylmethyl groups), which is required for efficient translation (10, 13-15). Additional roles of Elongator include regulating vesicle transport and exocytosis (16, 17), microtubule and actin dynamics (18-21), and cell migration (22). Interestingly,

overexpression of specific tRNAs has been shown to correct both the disrupted transcription and exocytosis in Elongator-deficient cells, suggesting these functions, and potentially others, are the indirect consequences of impaired translation (23).

Though the potential roles of IKAP/ELP1 and Elongator are widespread and lead to diverse impairments within the cell, data are accumulating that reveal that in absence of IKAP/ELP1 and/or ELP3 neurons die via common stress pathways (24-26). IKAP was shown *in vitro* to bind c-Jun N-terminal kinase (JNK) and further potentiate JNK/MEKK-1 stress signaling (19, 27). Loss of Ikap/Elp1 in a mouse model of FD was demonstrated to result in neuronal death via apoptosis by p53 and caspase-3 signaling (24).

Additionally, deletion of Elp3 leads to endoplasmic reticulum (ER) stress in mouse cortical neurons (28), and its absence disrupts mitochondrial function in yeast (29). A theme is emerging suggesting IKAP/ELP1 and ELP3 aid in mitochondrial function (29, 30). For example, ELP3 has been shown to localize in mitochondria in various systems including Hela cells, mouse brain, and *Toxoplasma* (31, 32). Clinical reports of FD patients, corroborated in a mouse model of FD, describe a pattern of optic neuropathy very similar to the pattern of loss of RGCs that occurs in disorders marked by mitochondrial dysfunction, where the more metabolically active RGCs are first impaired when compared to the less active RGCs (29, 30). Additionally, in one instance, before a patient was recognized to have FD, a muscle biopsy revealed atrophy, and, after isolating mitochondria, determined there were defects in oxidative phosphorylation and abnormal function of enzyme complexes I, III, and IV of the respiratory chain. No mutations in either nuclear or mtDNA were found (Lefcort, personal communication). Clinical reports

also indicate that FD patients have high rates of rhabdomyolysis (33), a condition marked by rapid breakdown of skeletal muscle. Lastly, FD patients exhibit reduced weight gain and growth, even when caloric intake is increased with feeding tubes (34). These reports suggest that ER and/or mitochondrial stress occur along with the neuronal death observed in FD models and patients.

Current Pharmacological Approaches

Though carrier screening and prenatal diagnosis have dramatically decreased the number of new occurrences of FD each year, new cases arise from parents with unknown Ashkenazi Jewish descent. The disease is ultimately fatal, but patients are now living into their 40s with treatments that targets the symptoms and imbalances occurring upon loss of sensory and autonomic function (10, 34). These include pharmacological and non-pharmacological interventions to minimize various debilitations which include, but are not limited to, vomiting crises, hyper- and hypotension, tachycardia, gastrointestinal dysfunction, seizures, and respiratory infections (10, 34). A few select compounds that target the direct molecular and cellular consequences of the major FD mutation are in development (15, 34-39). Kinetin, a plant cytokine, has been demonstrated to increase inclusion of exon 20, which is aberrantly excluded upon the major FD splice site mutation, in human FD fibroblast and lymphoblast cells. Kinetin is a topical treatment that has been shown to decrease oxidative damages (37, 38), but when added in culture, Hims and colleagues revealed that the compound increases levels of wildtype *IKBKAP* mRNA and IKAP protein levels, potentially from modulating regulatory splicing factors

that target a specific subset of exons (37). Daily kinetin treatment in eight FD patients led to significantly increased wildtype mRNA *IKBKAP* (38). Tocotrienol, a member of the vitamin E family, has been shown to increase levels of wildtype IKAP protein by increasing both mutant and wildtype *IKBKAP* transcripts in FD patient fibroblasts and in a neuroblastoma-derived cell line (38). However, a recent clinical trial in FD patients indicated less significant results (39). Transcription of both wildtype and mutant *IKBKAP* was increased in patient blood samples, but no significant improvements in clinical parameters such as blood pressure, vibration threshold test, nerve conduction, and heart rate variability were found (39). An alternate compound, phosphatidylserine, an FDA-approved supplement, has also been shown to increase IKAP protein levels by increasing transcription of *IKBKAP* in human FD fibroblasts. Gene analysis revealed many genes involved with cell cycle regulation were upregulated after treatment, and FD cells that were arrested in G1 had a cell cycle expression profile more like control cells (15). Phosphatidylserine treatment in a transgenic mouse model of FD demonstrated increased levels of the wildtype gene and IKAP protein. The phospholipid increased expression of genes involved in transcription regulation and decreased expression of genes encoding mitochondrial proteins (36). Lastly, a recent study discovered that a small compound, rectifier of aberrant splicing (RECTAS), corrected the exclusion of exon 20 of *IKBKAP* in human FD fibroblasts and increased IKAP protein levels. RECTAS also corrected the reduced levels of modified wobble position uridines of tRNAs in FD fibroblasts, normally decreased in absence of IKAP (35). Though these compounds exhibit therapeutic potential and assistance to cells *in vitro*, to date only kinetin has been

demonstrated to induce benefit in several patients. Alternate approaches to drug development that target disrupted cellular and molecular processes beyond the inefficiencies in translation are unexplored in FD. With the many cellular impairments previously reported and those that are not yet characterized in absence of IKAP and Elongator, there is great potential to target these events in FD to improve function and health of cells, especially of neurons.

Mouse FD Model

We have previously developed a mouse model of FD using a *Wnt-1* driver of *cre* expression to delete *Ikbkap* from neural crest cells (*Wnt1-Cre;Ikbkap^{-/-}*) (24). Ikap is therefore deleted from peripheral nervous system (PNS) progenitors, through neurogenesis, and in post-mitotic neurons (24). These mice show reduced numbers of sensory and autonomic neurons beginning *in utero*, which is a major hallmark of FD (24). Other groups have reported that *Ikbkap* null mice are embryonic lethal (40), along with mice modified to have a deletion of exon 20 of the *Ikbkap* gene, patterning the erroneous exclusion of exon 20 in FD patients (41). The human *IKBKAP* gene having the major FD mutation has also been successfully inserted into mice, however only one line with variable expression of this mutation has proved to recapitulate the FD phenotype (36, 42, 43). As we have successfully deleted *Ikbkap* from the peripheral nervous system, and our *Wnt1-Cre;Ikbkap* pups survive *in utero* and die just shortly after birth, we believe our mouse model is an excellent system to study, in addition to impairments in PNS development, the progressive neuronal death that occurs in absence of Ikap, especially of sensory (dorsal root ganglia) neurons which are devastated by the disease (2).

These mice, which we use here to explore compounds to prevent neuronal death, have been previously characterized by George and colleagues where they demonstrated that deletion of *Ikap* in neural crest cells leads to loss of sensory and autonomic neurons, revealing a pattern of neuronal loss that is observed in FD patients (2, 3, 24). Specifically, neural crest cells correctly migrate and coalesce in absence of *Ikap* to form the sensory neurons of the dorsal root ganglia (DRG), but neurogenesis leading to correct levels of TrkA⁺ sensory neurons of the DRG, which mediate pain and temperature transmission, is impaired. Additionally, George et al. determined that the TrkA⁺ sensory neurons that are born then die via p53, caspase-3-mediated apoptosis. As FD is marked by a lack of pain and temperature sensation, this mouse model provides excellent opportunity to explore the loss of this specific neuronal population occurring in the disorder (24).

Specifically, as DRG are accessible and can be dissected from mutant and control embryos shortly before birth, and as differentiated TrkA⁺ neurons can be cultured and selected for from the heterogeneous DRG by incubation with their specific growth factor, NGF, we have an opportune system in which to explore and potentially target and prevent death of these neurons. For example, *in vitro*, mutant TrkA⁺ DRG neurons from the *Wnt1-Cre;Ikbkap*^{-/-} FD mouse model being dying in absence of *Ikap* within 24 hours, while neurons from littermate controls can survive for weeks in the same conditions (44). With such rapid death occurring *in vitro*, there is opportunity to both intervene to test compounds for their ability to prevent this neuronal death, even after only 24-48 hours of incubation with a compound, and opportunity to also explore any disrupted molecular

and cellular events that are occurring during this time-frame where sensory DRG neurons die via apoptosis (24).

Goals

Thus in brief, FD results in incomplete neurodevelopment of the peripheral nervous system and progressive death of the neurons that form (2, 3, 9, 30). Additionally, loss of the protein IKAP leads to many disrupted cellular events including impaired cell cycle regulation, cytoskeletal dynamics, and translation (13-15, 17, 18, 21). Data support that cell stress pathways are activated in absence of IKAP and Elongator, as evidenced by *Elp1* and *Elp3* deficient mouse models and by trends in FD patients (24, 29, 33). These stress pathways include potentially both ER and mitochondrial stress (29, 33).

Therapeutics have mainly targeted symptoms of FD, while only one compound reveals potential in elevating wildtype *IKBKAP* transcripts (38). Therefore, with the *Wnt1-Cre;Ikbkap*^{-/-} model in hand, our goals are two-fold: (1) to determine cellular events that go awry and/or which stress signaling pathways are activated in absence of Ikap, and (2) to determine whether we can target these disrupted cellular events and stress pathways to restore function and survival of mutant FD neurons, with hope of identifying targeted therapeutic strategies for FD patients.

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CHAPTER TWO

BGP-15 PREVENTS THE DEATH OF NEURONS
IN A MOUSE MODEL OF FAMILIAL
DYSAUTONOMIA

Contribution of Authors and Co-Authors

Manuscript in Chapter 2

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Contributions: Performed experimental design and its execution and analysis. Generated figures and wrote, reviewed, edited, and submitted manuscript.

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Contributions: Assisted with *in vivo* survival analysis: injected mice with BGP-15 and/or saline and then sectioned, labeled, and imaged tissue in preparation for neuronal counts.

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Contributions: Provided BGP-15 compound, assisted with writing, review, and editing of manuscript.

Co-Author: Frances Lefcort

Contributions: Provided reagents and aided with conceptualization and experimental design, execution, and analysis. Assisted with writing, review, and editing of manuscript.

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BGP-15 prevents the death of neurons in a mouse model of familial dysautonomia

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Hereditary sensory and autonomic neuropathy type III, or familial dysautonomia [FD; Online Mendelian Inheritance in Man (OMIM) 223900], affects the development and long-term viability of neurons in the peripheral nervous system (PNS) and retina. FD is caused by a point mutation in the gene *IKBKAP/ELP1* that results in a tissue-specific reduction of the IKAP/ELP1 protein, a subunit of the Elongator complex. Hallmarks of the disease include vasomotor and cardiovascular instability and diminished pain and temperature sensation caused by reductions in sensory and autonomic neurons. It has been suggested but not demonstrated that mitochondrial function may be abnormal in FD. We previously generated an *Ikbkap/Elp1* conditional-knockout mouse model that recapitulates the selective death of sensory (dorsal root ganglia) and autonomic neurons observed in FD. We now show that in these mice neuronal mitochondria have abnormal membrane potentials, produce elevated levels of reactive oxygen species, are fragmented, and do not aggregate normally at axonal branch points. The small hydroxylamine compound BGP-15 improved mitochondrial function, protecting neurons from dying in vitro and in vivo, and promoted cardiac innervation in vivo. Given that impairment of mitochondrial function is a common pathological component of neurodegenerative diseases such as amyotrophic lateral sclerosis and Alzheimer's, Parkinson's, and Huntington's diseases, our findings identify a therapeutic approach that may have efficacy in multiple degenerative conditions.

familial | dysautonomia | *Ikbkap* | *Elp1* | BGP-15

The autonomic nervous system is essential for homeostasis, and its disruption in familial dysautonomia (FD) can have fatal consequences resulting from cardiovascular instability, respiratory dysfunction, and/or sudden death during sleep (1–3). In addition to developmental decreases in the number of sensory and autonomic neurons, FD patients undergo a progressive loss of peripheral neurons and retinal ganglion cells. The latter loss may ultimately lead to blindness (4, 5). More than 98% of FD cases result from a single base substitution (IVS20+6T > C) in the *IKBKAP/ELP1* gene (3, 6). This mutation is carried by 1 in 27 to 1 in 32 Ashkenazi Jews (3, 6). The protein encoded by the *IKBKAP/ELP1* gene, IKAP/ELP1, is a scaffolding protein for the six-subunit Elongator complex (ELP1–ELP6), which modifies tRNAs during translation (7). Why reduction in the IKAP/ELP1 protein results in neuronal death is unknown, and we have sought to elucidate the cellular and molecular mechanisms that cause progressive neurodegeneration in FD to help identify treatments that improve neuronal function and viability.

Accumulating evidence indicates that cells from FD patients and from mouse models with deletions in the Elongator subunits *Ikbkap/Elp1* or *Elp3* experience intracellular stress (4, 5, 8–10) resulting from the direct and/or indirect consequences of impaired translation (7, 11). The C terminus of IKAP/ELP1 has been shown to bind c-Jun N-terminal kinase (JNK) and to regulate JNK cytosolic stress signaling (12). Loss of *Elp3* in mouse cortical neurons triggers endoplasmic reticulum stress (9), and *Elp3* deletion in yeast causes disruptions in mitochondrial function (10). ELP3 has been demonstrated to localize in mitochondria in HeLa cells, mouse brain, and *Toxoplasma* (13, 14).

Recent studies suggest that the loss of *Ikbkap/Elp1* can cause mitochondrial dysfunction. For example, investigations of retinal ganglion cells (RGCs) in mouse models of FD and in human patients indicate that metabolically active, temporal RGCs are more compromised in mutant (FD) retinæ than are the less active nasal RGCs. This pattern is reminiscent of the pattern of RGC loss in optic neuropathies caused by disruption in mitochondrial genes (4, 5, 15). Furthermore, a recent study of FD patients demonstrates that they experience high rates of rhabdomyolysis (16). Finally, we previously have demonstrated that in the mouse model of FD (*Wnt1-Cre;Ikbkap^{-/-}*) used in the present study, TrkA⁺ pain- and temperature-receptive neurons in the dorsal root ganglia (DRG) die in vivo as a result of p53, caspase-3-mediated apoptosis (8).

BGP-15 is a hydroxylamine derivative that has been shown to exert cyto- and neuroprotective effects in mammalian models of injury, stress, and disease (17–30). These improvements in cellular function have been correlated with the activation of several intracellular pathways. For example, the heat-shock response is enhanced by increased levels of the molecular chaperone HSP72 (21, 24, 26, 28, 29), possibly mediated by Rac-1 signaling (31, 32). BGP-15 also decreases phospho-JNK (pJNK) and p38 stress signaling (21, 23) and increases AKT and IGFR1 protective signaling (23, 25). Additionally, in models of stress and injury, BGP-15 has been shown to decrease cell stress by restoring normal mitochondrial function. These studies have identified multiple mechanisms that may mediate BGP-15's positive effects on mitochondria. These include reducing NAD⁺ depletion and overactive PARP, improving antioxidant status, decreasing reactive oxidant species

Significance

Familial dysautonomia (FD) is a fatal genetic disorder that disrupts development of the peripheral nervous system (PNS) and causes progressive degeneration of the PNS and retina, ultimately leading to blindness. The underlying cellular mechanisms responsible for neuronal death in FD have been elusive. Using a mouse model that recapitulates impaired PNS development, we report here that developing peripheral neurons die in FD as the result of disruptions in mitochondrial and actin function. Cell death can be prevented in vivo by the small molecule BGP-15. Given that disrupted mitochondrial function appears to be a hallmark of neurodegenerative diseases, future studies on the efficacy of BGP-15 for mitigating neuronal loss in other disorders is warranted.

Author contributions: S.B.O. and F.L. designed research; S.B.O. and M.L.R. performed research; M.J.B. and F.L. contributed new reagents/analytic tools; S.B.O. analyzed data; and S.B.O., M.J.B., and F.L. wrote the paper.

Conflict of interest statement: A patent has been filed by Montana State University (MSU) and N-Gene, with F.L. and N-Gene named as inventors. F.L. has assigned the technology to MSU, and the technology has been licensed to N-Gene by MSU. F.L. has no interest or equity in N-Gene and was not involved in MSU's license negotiations. M.J.B. is on the Board of Directors of N-Gene, Inc. and has equity in the company.

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(ROS), stabilizing mitochondrial membrane potential, increasing mitochondrial content, and blocking AIF translocation to the nucleus (17–19, 22, 23, 26–28, 30). Given the beneficial effects of BGP-15 in reducing intracellular stress via multiple potential pathways, including repairing mitochondrial function, the goals of this study were (i) to test the hypothesis that *Ikbkap*^{−/−} neurons die because of impaired mitochondrial function, and, having demonstrated this causation, (ii) to investigate the therapeutic potential of BGP-15 in restoring mitochondrial function and neuronal survival in a mouse model of FD.

Results

Mitochondrial Function Is Disrupted in *Ikbkap*^{−/−} Neurons and Is Repaired by BGP-15. DRG were dissected from and *Wnt1-Cre;Ikbkap*^{LoxP/LoxP} (hereafter, “mutant”) and littermate *Ikbkap*^{+/LoxP} (hereafter, “control”) mice (8), dissociated, and cultured in the presence of NGF to select for the small-diameter, TrkA⁺ pain and temperature receptors. We first examined the inner mitochondrial membrane potential, because this membrane is essential for proper mitochondrial bioenergetics and cell survival (33). Based on the degree of accumulation of positively charged MitoTracker Red CMXRos, we found not only that mitochondria from mutant neurons were depolarized compared with those in their littermate controls, but also that their membrane potentials could be fully restored to control levels by BGP-15 (Fig. 1*A* and *B*). To determine whether the reduced accumulation of MitoTracker Red in mutant neurons was caused simply by a reduction in mitochondrial number or size, we incubated neurons with both MitoTracker Red and MitoTracker Green. Because the latter accumulates independently of membrane potential and serves as a measure of mitochondrial mass, this staining allowed us to normalize the measurements of mitochondrial membrane potential to mitochondrial mass. The results of this analysis validated our initial findings that mitochondria of mutant neurons were depolarized but were fully restored to control levels in the presence of BGP-15 (Fig. S1). We next analyzed the mitochondrial morphology of mutant neurons, which might be indicative of dysfunctional mitochondrial dynamics (e.g., in fission, fusion, transport, and/or mitophagy) (34). CellLight Mitochondria-GFP was added to both BGP-15-treated and untreated embryonic control and mutant DRG neurons to visualize mitochondria (Fig. 1*C*). A blinded scoring method was used to determine the shape of mitochondrial networks (Fig. 1*D*). This analysis was followed by ImageJ’s particle analyzer function to measure the number, perimeter, and area of the mitochondrial particles as calculated from converted binary images of the Mitochondria-GFP fluorescence (Fig. 1*C* and *E–G*). We found that mitochondria from mutant DRG neurons were severely fragmented compared with the mitochondria of neurons from their control littermates. BGP-15 improved mitochondrial integrity by significantly reducing fragmentation and partially restoring morphology to control levels. At a concentration of 50 μ M, BGP-15 was toxic to cultured DRG neurons. A 30- μ M concentration induced a slight improvement in the overall morphology of mutant neurons (see elongation score, Fig. 1*D*), but a decline in the morphology of control neurons. A 10- μ M concentration of BGP-15 appeared to be optimal for improving mitochondrial function (Fig. 1*C–G*).

BGP-15 Reduces Elevated ROS in *Ikbkap*^{−/−} Neurons. The discovery of significant impairments in mitochondrial function of mutant neurons, in particular the collapsed mitochondrial membrane potential, prompted us to measure levels of ROS (35). BGP-15 has been shown to reduce ROS in stressed mammalian cells and rodent models (17–19, 30). Using the probe CellROX Deep Red, which fluoresces only when oxidized by ROS, we discovered that ROS were significantly increased in mutant neurons. We observed a strong CellROX fluorescent signal in somas of mutant DRG neurons, a marked increase from the low levels of fluorescence observed in neurons from littermate control embryos (Fig. 2).

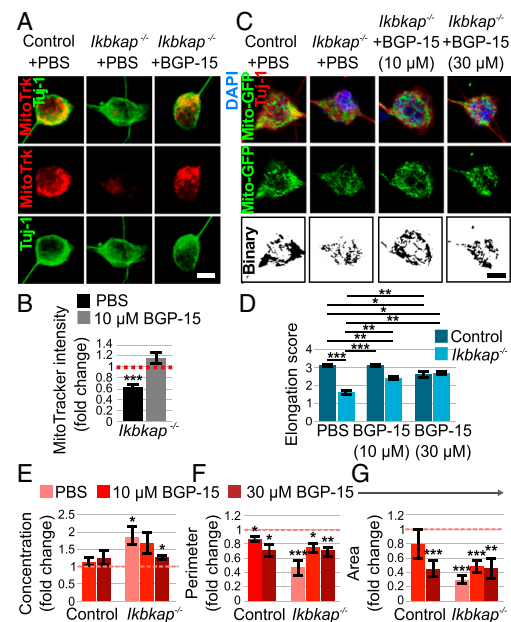


Fig. 1. Mitochondrial membrane potential and morphology are disrupted in *Ikbkap*^{−/−} neurons but are ameliorated by incubation with BGP-15. (A) Representative images of MitoTracker CMXRos retention (red) in mitochondria of Tuj-1⁺ (green) E17.5 TrkA⁺ DRG neurons show decreased MitoTracker intensity compared with control neurons. (B) Average intensity measurements of MitoTracker signal [region of interest (ROI), soma]. Data are presented as fold change where *Ikbkap*^{−/−} intensities were normalized to PBS-treated control intensity (dashed line). BGP-15 (10 μ M) restores MitoTracker intensity to control levels. *n* = 4 experiments from ~100 TrkA⁺ neurons, isolated from a total of nine control and eight mutant embryos. (C) Representative images of Mitochondria-BacMam GFP (green), Tuj-1 (red), and DAPI⁺ (blue) E17.5 TrkA⁺ DRG neurons reveal fragmented *Ikbkap*^{−/−} mitochondrial networks and the partial restoration of morphology with 10 and 30 μ M BGP-15. The bottom row shows the mitochondria-GFP signal converted to binary images used for quantification. (D) The elongation score is a measure of mitochondrial morphology based on comparison with reference images (0 = complete fragmentation; 4 = elongated mitochondria). (E–G) Binary images indicate fragmented *Ikbkap*^{−/−} networks and improvement by BGP-15. Data are presented as fold change with all measurements normalized to PBS-treated control measurements (dashed line). Graphs show the average number (E), the average perimeter (F), and the average area [per unit area of the defined ROI (soma)] (G) of mitochondrial particles. *n* = 590 TrkA⁺ neurons from 14 control and 11 mutant embryos, collected over six experiments. (Scale bars, 10 μ m.) Errors bars indicate SEM. **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.

Incubation with 10- μ M BGP-15 reduced the ROS levels in mutant neurons to those seen in littermate control neurons (Fig. 2).

BGP-15 Normalizes Impaired Actin Dynamics in *Ikbkap*^{−/−} Neurons. It has been postulated that neurons die in FD because of an inability to innervate correctly their targets from which they receive essential survival stimuli in the form of neurotrophins (36, 37). We show here that incubation in NGF is insufficient to rescue the small-diameter TrkA⁺, *Ikbkap*^{−/−} neurons. However, the failure to extend or maintain axons to mediate normal transport during target innervation could also ultimately cause neuronal death. In support of this thesis, IKAP/ELP1 has been shown to be necessary for normal cytoskeletal dynamics (36, 38, 39) and for the retrograde transport of NGF (40), whereas ELP3, the

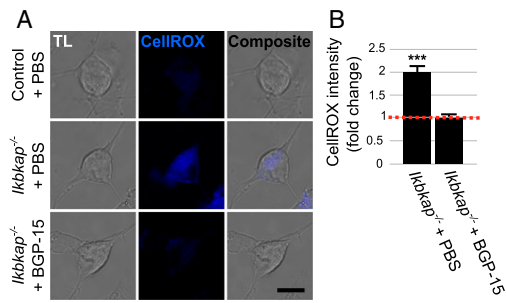


Fig. 2. ROS are increased in *Ikbkap*^{-/-} neurons but are reduced to control levels upon incubation with BGP-15. (A) *Ikbkap*^{-/-} DRG neurons have increased ROS compared with E17.5 DRG neurons from control littermates, indicated by fluorescence of CellROX. TL, transmitted light. (Scale bar, 10 μ m.) (B) Average intensity values of CellROX (ROI, soma). Data are presented as fold change with each set (a replicate of all conditions) normalized to the intensity of controls (dashed line). Incubation with 10 μ M BGP-15 reduces ROS of *Ikbkap*^{-/-} neurons to control levels. $n = 6$ sets over four experiments, representing 11 control and 11 mutant embryos and ~40 cells of each genotype and treatment. Error bars indicate SEM; *** $P \leq 0.001$.

acetyl-transferase subunit of the Elongator complex, has been proposed to regulate actin dynamics (13, 41). For a better understanding of why *Ikbkap*^{-/-} neurons die, we investigated their behavior in vitro, examining their overall morphology, axon outgrowth, and growth cone dynamics. Using live time-lapse confocal imaging, we found that, as compared with control littermate neurons, mutant neurons had stunted axons marked by an atypical, highly branched morphology that included increased extensions off the primary axon and growth cones that extended profuse and abnormally elongated filopodia (Fig. S2 and Movies S1 and S2). Axonal extensions over identical time intervals indicated that mutant growth cones were less motile than controls, with little net gain in growth cone forward position compared with control axons (Fig. 3F and Movies S1 and S2).

To investigate the organization of the cytoskeleton, we immunolabeled actin and microtubules in fixed cultures of DRG neurons (Fig. 3A and C). There were three major differences between *Ikbkap*^{-/-} and control neurons: (i) mutant axons extended for shorter distances (Fig. 3B); (ii) their growth cones sent out an increased number of actin-filled filopodia (Fig. 3C-E); and (iii) their axonal branches were composed primarily of actin with only infrequent microtubule invasion (Fig. 3A and G). Axonal branching is a key component of normal axon outgrowth during development (42). Microtubule invasion is a prerequisite for branch stabilization but is dependent on the local aggregation of mitochondria at branch points (42). To explore branch composition further, we analyzed the distribution of mitochondria at axonal branch points. In control DRG neurons, CellLight Mitochondria-GFP revealed an aggregation of mitochondria at branch points and branches that were composed of both actin and tubulin (Fig. 3G). In contrast, *Ikbkap*^{-/-} axonal branches contained fewer microtubules. Mitochondria were also much less abundant at branch points and were more fragmented than in controls (Fig. 3G). To determine whether BGP-15 could correct the axon outgrowth impairments, we measured axon extension and branching along with filopodia number in control and mutant axons. Although BGP-15 did not promote axon extension or reduce axonal branching significantly (Fig. 3B and Fig. S2C), it was very effective in reducing both the number and length of the excessive actin-rich filopodial extensions from the growth cones (Fig. 3C-E). Together, these data indicate that, although BGP-15 did not rescue

axonal microtubule dynamics, it significantly restored normal actin dynamics in *Ikbkap*^{-/-} neurons.

BGP-15 Prevents the Death of *Ikbkap*^{-/-} Sensory Neurons in Vitro and in Vivo. *Ikbkap*^{-/-} TrkA⁺ neurons begin dying within 24 h of being placed in culture, even in the presence of their preferred

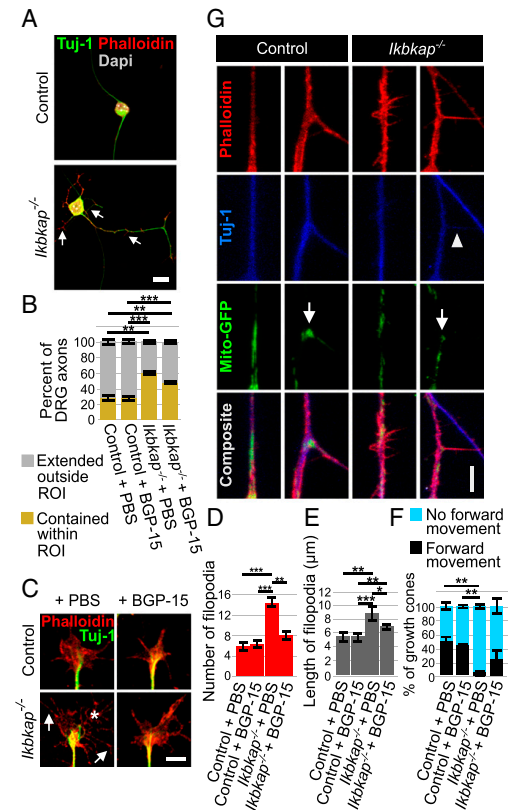


Fig. 3. BGP-15 restores normal filopodia morphology and number to *Ikbkap*^{-/-} growth cones. (A and B) *Ikbkap*^{-/-} neurons are stunted, extending less in culture. (A) Representative images of E15.5 control and *Ikbkap*^{-/-} neurons with Tuj-1 (green) and phalloidin (red) labeling. Arrows mark characteristic branching pattern of *Ikbkap*^{-/-} neurons. (Scale bar, 10 μ m.) (B) Percent of axons that extend outside and within the ROI (field of view). BGP-15 (10 μ M) is unable to restore axon length. $n = 12$ wells from three experiments. (C) Representative images of E15.5 growth cones stained with Tuj-1 (green) and phalloidin (red) show the irregular morphology of *Ikbkap*^{-/-} growth cones; filopodia are more numerous and longer than those of controls (arrows) and often branch (*). (Scale bar, 5 μ m.) (D and E) Quantification of fixed growth cones indicating the average number (D) and length (E) of filopodia upon treatment. Both filopodia number and length are reduced to control levels with 10 μ M BGP-15. $n = 15$ –20 neurons of each genotype and treatment over three experiments. (F) Percent of both control and *Ikbkap*^{-/-} growth cones upon treatment that are stationary or show a gain in position. $n = 15$ –20 growth cones of each genotype and treatment over three experiments. (G) Tuj-1 (blue), phalloidin (red), and mitochondria-BacMam GFP (green) labeling confirms altered cytoskeletal morphology (microtubules, arrowhead) and mitochondria (arrows) in *Ikbkap*^{-/-} axons, branches, and growth cones. (Scale bar, 5 μ m.) Error bars indicate SEM. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

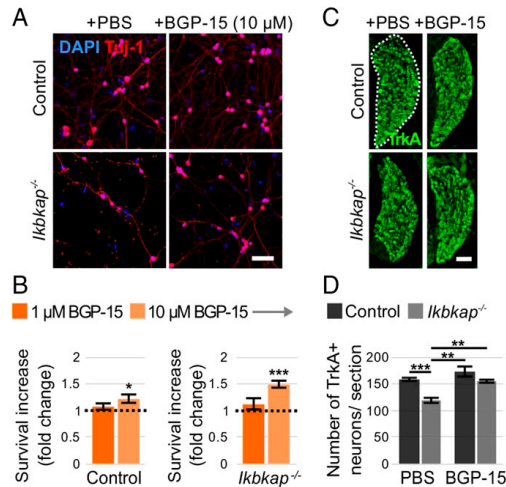


Fig. 4. Death of *Ikbkap*^{-/-} DRG neurons is significantly decreased by BGP-15 both in vitro and in vivo. (A) Representative images of DAPI (blue) and TrkA⁺ (red) control and *Ikbkap*^{-/-} E16.5 TrkA⁺ neurons. (Scale bar, 100 μm.) (B) Average increase in TrkA⁺ neuronal survival upon the addition of 10 μM BGP-15, calculated as fold change in which counts of BGP-15-treated neurons were normalized to counts of PBS-treated neurons (dashed line). *n* = 5 experiments and DRG from a total of 10 control and 10 mutant embryos. (C) Representative images of TrkA⁺ neurons (green) at E17.5 after daily injection of 100 mg/kg BGP-15 or PBS (the dotted line indicates the DRG). (Scale bar, 50 μm.) (D) Average number of TrkA⁺ neurons per section of DRG of the upper lumbar region from E17.5 embryos. *n* = 6 control embryos treated with saline, 5 embryos treated with BGP-15, 4 mutant embryos treated with saline, and 4 mutant embryos treated with BGP-15 embryos, carried out over four separate experiments. Error bars indicate SEM; **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.

growth factor, NGF (Fig. 4A). Neurons from their control littermates can survive for weeks in the same conditions (Fig. 4A). Given the ameliorative effects of BGP-15 on mitochondrial activity and actin dynamics, DRG from *Wnt1-Cre;Ikbkap*^{-/-} embryonic mice were removed, dissociated into single neurons, and cultured in the presence or absence of BGP-15. Although a 1-μM concentration of BGP-15 had little effect, and 50–100 μM was toxic to the cells, 10 μM BGP-15 significantly decreased the death of the TrkA⁺ small-diameter mutant neurons in vitro. The same concentration of BGP-15 on control neurons induced a small but significant increase in survival, perhaps resulting from a reduction in naturally occurring programmed cell death (Fig. 4A) (43). We next tested the efficacy of BGP-15 in vivo by injecting pregnant dams once daily from E12.5 to E16.5 with either PBS or 100 mg/kg of BGP-15 i.p. and counting the number of TrkA⁺ DRG neurons at E17.5. BGP-15 had a striking effect: It significantly reduced the loss of TrkA⁺ neurons in vivo (Fig. 4C and D), restoring their numbers to normal levels (8).

BGP-15 Decreases Elevated pJNK in *Ikbkap*^{-/-} DRG. There is evidence that IKAP/ELP1 participates in JNK stress signaling (12), and pJNK levels have been demonstrated to be reduced by exposure to BGP-15 (21, 23). Therefore, we asked whether pJNK levels were increased in *Ikbkap*^{-/-} DRG neurons, and, if so, whether they could be normalized by treatment with BGP-15 in vivo. Pregnant dams were injected with PBS or BGP-15 from E12.5 to E16.5, and embryos were fixed, sectioned, and stained with antibodies to pJNK. Compared with levels found in control DRG neurons, pJNK expression was significantly

elevated in DRG of mutant embryos. pJNK returned to control levels following daily treatment with BGP-15 (Fig. S3).

BGP-15 Improves *Ikbkap*^{-/-} Cardiac Innervation in Vivo. FD patients have impaired blood pressure regulation and suffer from sudden death resulting from cardiac arrhythmias and asystole (44). These symptoms coincide with a decrease in sympathetic innervation of the heart, particularly its ventral apex (37, 45). Based on our finding that BGP-15 could rectify actin dynamics in *Ikbkap*^{-/-} neurons in vitro, we asked whether treatment with the compound could reverse the defect in cardiac innervation that has previously been reported in *Ikbkap*^{-/-} embryos (37). BGP-15 was injected daily into pregnant dams from E12.5 to E16.5 (100 mg/kg, i.p.), and embryos were harvested at E17.5. As in previous studies, we found that hearts of mutant mice were significantly less well innervated than hearts from littermate controls. Mutants had significant reductions in axonal extension and branching (Fig. S4A). Daily injections of BGP-15 significantly improved cardiac innervation in mutant embryos, increasing both the number and ramification of branches in the heart and restoring innervation of the heart middle region to control levels. Drug treatment partially restored growth of the longest axons that innervate the inferior apical region of mutant hearts (Fig. S4).

Discussion

Why neurons die in both the developing and in the adult FD nervous system has not been resolved, but evidence is accumulating that neurons in both human FD patients and mouse models of FD experience intracellular stress. We report here that mitochondrial function is impaired in neurons lacking *Ikbkap*: Mitochondria are depolarized (Fig. 1A and B and Fig. S1) and fragmented (Fig. 1C–G), and ROS levels are significantly increased (Fig. 2). The small molecule BGP-15 can restore the mitochondrial membrane potential (Fig. 1A and B and Fig. S1), reduce the elevated ROS levels (Fig. 2), and reduce mitochondrial fragmentation (Fig. 1C–G). pJNK levels are also higher in *Ikbkap*^{-/-} DRG neurons than in those of controls, and these elevated levels also were normalized by BGP-15 treatment (Fig. S3). Although BGP-15 exerts diverse actions on cells, our data suggest that, by restoring aspects of mitochondrial function, BGP-15 prevents the death of *Ikbkap*^{-/-} neurons both in vitro and in vivo. Because these mitochondrial disruptions can trigger apoptosis (46) and loss of *Ikbkap*^{-/-} TrkA⁺ DRG neurons is attributed to this method of programmed cell death (PCD) (8), we conclude that BGP-15 acts to prevent the apoptotic death of neurons that lack Ikap/Elp1.

Our data also reveal that axon extension and growth cone morphology and dynamics are perturbed in neurons that lack *Ikbkap*. The impaired actin-mediated functions can be corrected by BGP-15. Axons of *Ikbkap*^{-/-} neurons are stunted and highly branched (Fig. S2), and growth cones extend abnormally long and abundant filopodia (Fig. 3C–E). Perhaps as a consequence of the excess filopodia, their axonal growth cones do not exhibit much, if any, total forward movement (Fig. 3F and Movies S1 and S2). Using the heart as a model to explore innervation in vivo, we found that cardiac innervation is reduced in the absence of *Ikbkap*, especially more inferiorly toward the heart apex (Fig. S4). BGP-15 treatment inhibited the formation of overabundant growth cone actin networks (Fig. 3C–E) and improved cardiac innervation (Fig. S4). Thus, improvements in cytoskeletal networks, especially of actin dynamics, may also enhance the survival of *Ikbkap*^{-/-} neurons treated with BGP-15 in vitro and in vivo (Fig. 4).

These data implicate mitochondrial dysfunction as a major factor mediating the death of *Ikbkap*^{-/-} neurons. First, we show that mitochondria of *Ikbkap*^{-/-} neurons are fragmented and do not distribute correctly in the axon or coalesce at axonal branch points (Figs. 1C–G and 3G), suggesting impaired mitochondrial

dynamics. Disrupted mitochondrial dynamics are detrimental to highly polarized sensory neurons, and such interruptions could contribute to apoptosis and later-onset neurodegeneration (34, 47). This feature is shared with neurons of patients who have Parkinson's, Huntington's, or Alzheimer's disease (34). Second, we show that *Ikbkap*^{-/-} neurons have a significant increase in ROS (Fig. 2), likely resulting from dysfunction in the mitochondrial respiratory chain, which can induce PCD (35). BGP-15 reduces ROS levels to normal in *Ikbkap*^{-/-} neurons, in support of previous work demonstrating that BGP-15 can stabilize complexes of the respiratory chain (18, 28, 30), especially complexes I and III (28, 30). Third, our data indicate a widespread depolarization of mitochondrial networks (Fig. 1 *A* and *B* and Fig. S1). This depolarization may be caused in part by ROS-induced damage leading to a loss of charge across the inner mitochondrial membrane, a point of no return on the road to PCD (33, 35). BGP-15 appeared to prevent the collapse of the membrane potential of *Ikbkap*^{-/-} neurons, thereby rescuing the cells. Whether mitochondrial impairment is the primary cause or simply a critical mediator of cell death is a question being investigated in a number of neurodegenerative diseases (47). Our data suggest that improving mitochondrial health may be beneficial to patients with such problems.

BGP-15-driven improvements in actin function (Fig. 3 and Fig. S4) could also have contributed to the increased survival of *Ikbkap*^{-/-} neurons in vitro and in vivo. There is accumulating evidence that IKAP/ELP1 and the Elongator complex participate in the organization of the cytoskeleton (36–39, 41, 48–52). Specifically, Elongator has been demonstrated to be required for normal neuronal branching (36, 37, 41, 48–51), organization of actin networks (38), and acetylation of α -tubulin (40, 50, 52). Microtubules also have been shown to be altered in growth cones from peripheral neurons lacking IKAP/ELP1 in chick (36). BGP-15 did not induce any significant improvements in *Ikbkap*^{-/-} microtubule networks (i.e., it did not promote the growth of the *Ikbkap*^{-/-} axons). Thus, although we cannot exclude the possibility that BGP-15 affects microtubule defects, we have no evidence for such benefits. Consequently, we focused on the compound's obvious action on actin networks in growth cone filopodia, where we saw significant improvements (Fig. 3 *C–E*). We also observed improved cardiac innervation (Fig. S4). It has been suggested that reduced target innervation in the absence of *Ikbkap* is the primary cause of death of sensory neurons in FD (37). Thus, the BGP-15-induced increase in cardiac innervation could result from the increase in neuronal cellular respiration and/or actin-based growth cone function. This finding would suggest that reduced target innervation is the detrimental consequence of mitochondrial dysfunction and cytoskeletal disruption. These data are supported by the finding that mitochondria are required to initiate and maintain the formation of axonal branches in DRG neurons (42); the ability to extend and retract branches dynamically is integral to the navigation of axons toward their targets. Furthermore, restoration of actin network function could improve neuronal survival, because actin dynamics mediate vesicle transport and are required specifically for the TrkA/NGF endosomal retrograde survival signaling in DRG neurons (53) that is necessary to prevent the default apoptotic response (46). Previous studies have suggested a role of IKAP/ELP1 in neuronal transport (36, 54), and recent work has revealed that the speed of NGF retrograde transport is reduced in neurons lacking *Ikbkap* (40). Additionally, Rac-1, which interacts with actin to regulate growth cone dynamics (55), has been demonstrated in mammalian cells to be a target of BGP-15 treatment (31, 32) and potentially could contribute to the positive effects of BGP-15 on the actin cytoskeleton, because actin has been shown to activate signaling cascades that lead to apoptosis (56). Thus, improvements in cytoskeletal networks following BGP-

15 treatment also could underlie its effects on *Ikbkap*^{-/-} neurons.

Last, we show here that pJNK is increased in *Ikbkap*^{-/-} DRG of mutant embryos (Fig. S3), although the mechanisms mediating this increase will require further study. Because IKAP possesses a JNK association site and has been demonstrated to potentiate JNK signaling in vitro (12), misregulated pJNK could be a direct response to the absence of IKAP. Alternatively, elevated pJNK could result from mitochondrial dysfunction, as suggested by elevated ROS levels (35), and/or from altered Rac and Cdc42 signaling, given the disrupted cytoskeletal networks of *Ikbkap*^{-/-} neurons (56). BGP-15 reduced the elevated pJNK in *Ikbkap*^{-/-} DRG neurons to control levels. The compound has been shown previously to reduce pJNK in many cell types by potentiating the heat-shock response (21), improving mitochondrial function (23), or modulating Rac-1 activity (31, 32).

Clinical data support impairment in mitochondrial function as a contributor to neuronal death in FD patients. For example, in the FD human (and mouse) retina, a progressive loss of RGCs occurs in the more metabolically active temporal half of the retina, reminiscent of the selective loss of temporal RGCs in Leber's hereditary optic neuropathy, a consequence of a mutation in a mitochondrial gene (4, 5, 15, 57). Patients with FD also have been reported to be susceptible to developing rhabdomyolysis (16). These retina and muscle problems both suggest that mitochondrial dysfunction could play a significant role in FD.

IKBAP/ELP1 is one of six subunits of the Elongator complex, and variants in three other Elongator subunits, *ELP2–4*, have also been associated with neurological disorders: *ELP3* with amyotrophic lateral sclerosis (ALS) (49) and *ELP2* and *ELP4* with intellectual impairment and epilepsy (58–60), demonstrating the importance of this complex in the nervous system. Furthermore, ALS is marked by mitochondrial dysfunction (61). Recent work has revealed that in the absence of *Elp3* in mice, cortical neurogenesis is impaired because of the activation of the unfolded protein response, which is triggered by stress in the endoplasmic reticulum (9). That study and ours help elucidate the function of the Elongator complex in neurons and reveal that its absence triggers intracellular stress. Given the requirement for Elongator in translation, the question remains whether this stress is a direct or indirect consequence of impaired translation. Although the complete repertoire of BGP-15's effects on neurons remains to be elucidated, our data demonstrate that it promotes mitochondrial health and cytoskeletal organization, both of which are essential for the function and survival of TrkA⁺ peripheral neurons that extend up to meter-long axons.

In summary, our experiments demonstrate the potential utility of BGP-15 in a neurological disease model. In addition, our data implicate disruption of mitochondrial function as a pathological mechanism contributing to the death of neurons in FD and place FD alongside more prevalent neurodegenerative disorders characterized by mitochondrial dysfunction (34, 47). BGP-15 has been shown to improve metabolic function in rodent models of several human degenerative diseases (18, 22, 23, 26–28) and has been given to more than 400 patients in human clinical trials with no severe adverse drug-related events (62). The data reported here suggest that this drug, or compounds like it, may be effective in slowing or preventing the progressive loss of neurons in FD patients.

Materials and Methods

All research involving animals has been approved by the Institutional Animal Care and Use Committee at Montana State University. All procedures involving mice adhered to the NIH Guide for the Care and Use of Laboratory Animals (63). For additional descriptions of methods, please see *SI Materials and Methods*.

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Supporting Information

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SI Materials and Methods

Primary Culture of Mouse DRG Neurons. DRG were dissected from E15.5, E16.5, or E17.5 *Wnt1-Cre;IkbkapLoxP/LoxP* (mutant) and *Ikbkap+/LoxP* (control) embryos and were cultured in the presence of 25 ng/mL NGF to select for TrkA neurons. Embryos were first genotyped, and DRG from control and littermate mutant pups were dissected, pooled, and stored overnight at 4 °C in Hibernate-E medium (Thermo Fisher) supplemented with 2% B-27 (Gibco) and 2 mM L-glutamine (Gibco). Nunc LabTek II chambered coverglass eight-well slides (Fisher Scientific) were coated on the day of plating with 10 µg/mL poly-D-lysine (Millipore) for 30 min at room temperature) and with 20 µg/mL laminin (Thermo Fisher) for 2 h at 37 °C, 5% CO₂. To dissociate neurons for plating, DRG were incubated with 0.05% trypsin + EDTA (Thermo Fisher) at 37 °C for 16 min. Trypsin was quenched with 10% FBS (Thermo Fisher) in Neurobasal medium (Thermo Fisher), and neurons were spun down at 188 × g for 2 min at 4 °C. The supernatant was decanted, and neurons were resuspended in Neurobasal medium supplemented with 2% B-27, 100 µg/mL penicillin/streptomycin (Mediatech), 2 mM L-glutamine, and 25 ng/mL NGF (Thermo Fisher) to select for TrkA⁺ neurons. Approximately 400 µL of supplemented Neurobasal medium per dissected embryo was added for the resuspension, resulting in an optimal concentration of cells for plating. Neurons were further dissociated with a series of fire-pulled Pasteur pipettes, and the cell solution was passed through a 40-µm Falcon Cell Strainer (Fisher Scientific) to eliminate any remaining clumps. Cells were counted with a hemocytometer and were plated at final concentration of 2.0 × 10⁴ cells per well in a total volume of 300 µL. Cultures were incubated at 37 °C, 5% CO₂, for up to 72 h.

Statistical Analysis. Data are presented as the average ± SEM when a minimum of three experiments were conducted, representing a minimum of embryos from three separate litters. Statistical tests are noted in each experimental description. In some cases, data were preprocessed by normalization to account for experiment-to-experiment variability in plating, staining, or imaging; for that reason all genotype and treatment conditions were always represented in each experimental repetition. Any normalization of data is noted both in the figure legends and in the detailed description of methods below. The sample size, *n*, is also noted in the figure legends. When normalization of data was used (i.e., when an increase or decrease in neuronal survival was compared across experiments), *n* represents the number of experiments; for the in vivo studies, *n* represents the number of embryos considered. Statistical tests detailed in each specific method are found below, but in general, all one-way ANOVA tests assume independence of observations, normal distribution of data, and equality of variances. Student's *t* tests are two-sided and assume normal distribution, random sampling, and continuous and independent observations. These tests use an alpha value of 0.05 and a 95% confidence level. The BootstRatio analysis was used to determine the statistical significance of data normalized to a control treatment, presented as fold change. The test assumes no underlying distribution of data and uses resampling methods, including bootstrap and permutation tests, of fold change ratios to determine significance. These data were considered significant at *P* ≤ 0.05.

Study Design. For the in vitro studies (e.g., the in vitro neuronal survival and mitochondrial health assays), a minimum of three experiments were conducted, with each experiment consisting of

data from littermate control and affected embryos. Studies were concluded after a maximum of five experiments were completed. For in vivo studies (e.g., the in vivo neuronal survival, pJNK, and heart innervation assays), pregnant dams were injected and killed until ~5–10 embryos of each genotype and treatment were collected. Embryos were harvested from a minimum of three dams used over three separate experiments. An experiment represents a set of staining and imaging of tissue from all genotypes and treatments.

As a minimum, each experimental condition being tested (e.g., the addition of a specific concentration of BGP-15) was repeated and statistically analyzed over three experiments. When total neuronal counts of an entire well were evaluated, treatments were carried out over duplicate wells. Any duplicate wells (representing the same genotype and with neurons pulled from a common source of cells pooled from all littermate control or affected embryos) that differed in count by more than 250 neurons were deemed too variable (because of plating technique) and were excluded, and the experiment was repeated. For live-cell imaging, one experiment included measurements of 5–10 cells of each genotype and treatment condition; fixed-cell imaging included data from 20–50 cells of each genotype and treatment condition. All data points were considered. No outliers were excluded.

All experiments were carried out in a controlled laboratory environment. Tissue was collected from mice housed in our fully accredited Animal Resources Center, and observations were conducted using a Leica SP8 confocal microscope for organelle and cytoskeletal-level resolution. For data analysis, the investigator was blinded to the genotype and treatment condition of all collected images after randomization. Specific parameters of each study are detailed below.

Genetically Modified Mice. The *Ikbkap/Elp1* gene was conditionally deleted from the neural crest cell lineage using a *Wnt1-Cre* transgene as previously described (8). Briefly, we crossed homozygous *Ikbkap* conditional-knockout (*IC/C*) mice to hemizygous *Wnt1-Cre* mice (*Cre*⁺) carrying one copy of the conditional *Ikbkap* allele (*I*⁺/*C*). For all analyses, *Cre*⁺; *IC/C* embryos were used as experimental mice, and *Cre*⁺; *I*⁺/*C* littermates were used as controls. All genotyping was performed via PCR. *Wnt1-Cre* mice were purchased from The Jackson Laboratory (stock no. 003829). All procedures involving mice adhered to the NIH *Guide for the Care and Use of Laboratory Animals*.

Immunocytochemistry. After fixation, both adherent cells and sectioned tissue were blocked (1% glycine, 0.1% Triton, and 10% goat serum in 1× PBS) for 1 h at room temperature and then were incubated in primary antibody in blocking solution overnight at 4 °C and in secondary antibody for 1 h at room temperature. Primary antibodies included Tuj-1 (1:1,000; Covance), TrkA (a gift from Louis Reichardt of the University of California, San Francisco) (1:5,000), and anti-GFP (1:2,000; Thermo Fisher). Secondary antibodies and stains included Alexa 488 and 568 (1:2,000; Thermo Fisher), DAPI (1:3,000; Thermo Fisher), and Alexa Fluor 594 phalloidin (1:40; Thermo Fisher). Antibodies for whole-mount heart imaging and diaminobenzidine (DAB) immunohistochemistry included Tuj-1 (1:1,000) and the HRP-linked secondary antibody, goat anti-mouse-HRP (1:500; SouthernBiotech). The DAB-Plus reagent kit (Thermo Fisher) was used for development following the manufacturer's protocol.

Mitochondrial Assays. To measure the mitochondrial membrane potential, DRG cultures were incubated overnight with BGP-15 (10 μ M) or PBS and were incubated the following morning (17–18 h later) with 200 nM MitoTracker Red CMXRos (Thermo Fisher) for 20 min at 37 °C, 5% CO₂. Cultures then were fixed for 10 min with 4% paraformaldehyde at room temperature, stained with Tuj-1 and DAPI, and imaged using a 63 $\times$ objective on a Leica TCS SP8 confocal microscope. Z-stacks were generated using a step size of 0.3 μ m. For normalizing the accumulation of MitoTracker Red CMXRos to the mitochondrial mass, DRG cultures were incubated with 25 nM of both MitoTracker Red and MitoTracker Green for 20 min and were imaged immediately with a 20 $\times$ objective using a 0.3- μ m z-step. Because cultures were imaged live, images were taken alternating between genotypes and treatment conditions. All imaging parameters (laser power, pinhole size, PMT settings, z-step size, and resolution) were held constant for accurate comparison between control and *Ikkap*^{-/-} mitochondria in each experiment. Intensity measurements were taken at 488 and 568 excitations, and the Leica Application Suite Advanced Fluorescence 3.3.0.10134 software was used for taking intensity measurements of somas selected at random throughout the well. For the fixed MitoTracker Red measurements, data represent the average fold change (i.e., the increase or decrease in MitoTracker Red accumulation in *Ikkap*^{-/-} neurons compared with the accumulation in littermate control neurons) from four experiments. The BootstRatio analysis was used to calculate significance of the fold change across experiments. For the ratiometric analyses, MitoTracker Red intensity was normalized to MitoTracker Green intensity for each soma individually. The one-way ANOVA and Student's *t* test were used to determine statistical significance.

To measure mitochondrial morphology, CellLight Mitochondria-GFP BacMam 2.0 (Thermo Fisher) was added according to the manufacturer's protocol to DRG neurons at the time of plating, along with BGP-15 (10 and 30 μ M) or PBS treatment. The length of mitochondria-GFP incubation was optimized at 45 h with an 8- μ L addition of mitochondria-GFP per well (calculated using a delivery of 40 particles per cell). Neurons were fixed, stained, and imaged with a 63 $\times$ objective at the level of the axon, because mitochondrial morphology was too convoluted in a compiled z-stack. Morphology was calculated using both the Analyze Particles function of ImageJ, using binary images converted from the Mitochondria-GFP signal, and a method in which an elongation score was assigned to the overall mitochondria-GFP network based on blinded comparison with preselected reference images. A score of 4 denoted interconnected mitochondrial networks with elongated, tubule-like mitochondrial networks; a score of 0 indicated fragmented and disconnected mitochondrial particles. Mitochondria were scored after randomization of the images by an investigator blinded to the cell type and treatment condition. The significance of the elongation score was calculated using one-way ANOVA and Student's *t* test; the BootstRatio analysis was used to determine the significance of the fold increase from data normalized each experiment relative to PBS-treated control DRG neurons.

To measure mitochondrial ROS, DRG were cultured overnight with BGP-15 (10 μ M) or PBS to which CellRox Deep Red (Thermo Fisher) was added the following day (17–18 h later) to a final concentration of 5 μ M, followed by further incubation for 30 min at 37 °C at 5% CO₂. Cells were washed and fixed for 10 min with 10% formalin at room temperature and were imaged immediately (the probe must be imaged within 2 h according to the manufacturer's protocol). Transmitted light was used to identify neurons, and intensity measurements of somas were calculated using the Leica Application Suite of CellROX Deep Red, which fluoresces only upon oxidation by ROS (emission ~665 nm). Images were taken using a 63 $\times$ objective, and z-stacks were generated using a step size of 0.3 μ m. Representative im-

ages in Fig. 2 show a plane at the level of the axon from z-stack images. Only healthy, fully attached neurons with extended axons were selected and imaged, and images were taken alternating between genotypes and treatments to account for any reduced sensitivity of the probe over time. Each replicate (in which all genotypes and treatments were represented) was normalized to the average intensity measurements of PBS-treated control neurons. The significance of the fold changes between experiments was determined using the BootstRatio analysis.

In Vitro Cytoskeletal Morphology and Dynamics. DRG neurons were dissected from E15.5 embryos and were cultured as described above in the presence of BGP-15 (10 μ M) or PBS. For fixed imaging of the cytoskeleton, cultures were incubated with a solution of 4% paraformaldehyde and 0.2% glutaraldehyde for 10 min at room temperature, treated with 1 mg/mL sodium borohydride for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, and labeled with Tuj-1 and Alexa Fluor 594 phalloidin. Quantification of axon extension was derived from overnight cultures that were fixed at the same time. All neurons in a well were counted, and the percent that extended outside each field of view versus those that were contained within the field of view was noted. Time-lapse imaging was executed at 63 $\times$ magnification using transmitted light over 5 min, with images taken every 5 s. Any displacement of the lamellipodium over the 5 min was characterized as forward movement. To quantify growth cone morphology, the number of filopodia in a defined ROI (a 30- μ m diameter circle centered over the leading edge of the growth cone) was counted, and their length was measured. To count the number of branches extending off the primary axon, quantification was limited to the first 50 μ m of the axon just distal to the soma. All imaging was performed on a TCS SP8 confocal microscope with a 63 $\times$ objective. Quantification was carried out using ImageJ, and statistical significance was determined from the one-way ANOVA and Student's *t* test.

In Vitro Survival Assay. Either 1 $\times$ PBS (vehicle) or BGP-15 (1 or 10 μ M) was added to E16.5 DRG neurons at the time of plating. Neurons were incubated for 40 h at 37 °C, 5% CO₂ and were fixed with 4% paraformaldehyde for 10 min at room temperature. Cells were stained with DAPI and Tuj-1, and wells were imaged on a Leica TCS SP8 confocal microscope. The total number of neurons per well was determined in replicate wells. A total of 10 wells were tallied of each genotype and condition. Survival increase/decrease data represent the average fold change across five experiments, calculated from baseline PBS-treated control counts. Statistical significance was calculated using the BootstRatio analysis.

In Vivo Survival Assays. Pregnant dams were injected with either 1 $\times$ PBS (vehicle) or 100 mg/kg BGP-15 (N-Gene) from E12.5 to E16.5. Embryos were killed at E17.5, genotyped, fixed with 4% paraformaldehyde (2 h at 4 °C), and frozen in O.C.T. (Fisher Scientific) compound after a series of sucrose incubations at 4° (15% sucrose in 1 $\times$ PBS overnight, 30% sucrose in 1 $\times$ PBS overnight, followed by a 1:1 ratio of 30% sucrose to 1 $\times$ PBS for 2 h). Tissue was cryosectioned transversely in 16- μ m sections using a Leica CM 1950 cryostat and was labeled with anti-TrkA antibody. Slides were imaged as a set, representing all the genotypes and treatments (equaling one experiment), on a Leica TCS SP8 confocal microscope. The number of TrkA⁺ neurons was quantified as described in George et al. (8), and after the data were randomized counts were carried out by an investigator blinded to the genotype and treatment condition. Significance was determined using one-way ANOVA and Student's *t* test.

Heart Innervation Analysis. Pregnant dams were injected with either 1 $\times$ PBS (vehicle) or 100 mg/kg BGP-15 from E12.5 to E16.5,

and embryos were collected at E17.5. Hearts were dissected, genotyped, fixed for 2 h at 4 °C in 4% paraformaldehyde, and prepared for whole-mount imaging with DAB immunohistochemistry, which included dehydration in 50% and 80% methanol in 1× PBS (both for 30 min at room temperature) and overnight incubation in a 4:1:1 ratio of methanol:DMSO:H₂O₂ at 4 °C to quench endogenous peroxidase activity. Hearts were stored in 100% methanol at −20 °C. In preparation for staining, hearts were rehydrated and blocked overnight in 4% BSA and 1% Triton X-100 in 1× PBS at 4 °C. Hearts were incubated in primary antibody (Tuj-1 in BSA blocking solution) for 72 h at 4 °C, washed, and incubated overnight in secondary antibody (HRP goat anti-mouse in BSA blocking solution at 4 °C). Hearts were rinsed and developed using the DAB-Plus reagent kit (Thermo Fisher) following the manufacturer's protocol. After development, hearts were postfixed in 4% paraformaldehyde for 20 min at 4 °C and were dehydrated with a series of incubations in methanol (20%, 50%, and 80% in 1× PBS, followed by 100% methanol, all for 10 min at room temperature). Tissue was cleared just before imaging with a 2:1 mixture of benzyl benzoate to benzyl alcohol (5 min at room temperature). Imaging was carried out with a 10× objective and a Zeiss Stemi SV-11 APO stereo microscope with ProgRes Mac Capture Pro software. For quantification, the pulmonary trunk and apex served as the upper and lower boundaries, respectively, in the creation of three ROIs of equal height (dubbed "superior," "middle," and "inferior" ROIs). Significance was calculated using the one-way ANOVA and Student's *t* test.

Activation of JNK. To determine the activation of JNK, pregnant dams were injected with either 1× PBS or 100 mg/kg BGP-15 from E12.5 to E16.5, and tissue was collected, fixed, em-

bedded, and frozen as described previously. Transverse 16-μm sections of the upper lumbar region were stained with pJNK (1:200; Cell Signaling) and colabeled with Tuj-1 (1:1,000; Thermo Fisher) to identify neurons of the DRG. For heat-based antigen retrieval, citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0) was used. Slides were incubated in a water bath in coplin jars with citrate buffer at 90° for 30 min; then the coplin jars were removed from the heat and were cooled on the bench for a further 10 min. Slides were washed with 1× PBS, tissue was blocked for 1 h with normal goat serum, and primary antibodies were added for overnight incubation at 4 °C. Secondary antibodies included Alexa 488 and 568 (1:2,000; Thermo Fisher) and DAPI (1:3,000; Thermo Fisher). Tissue was imaged on a Leica SP8 confocal microscope with a 63× objective. The pJNK signal was quantified via (i) intensity, specifically whether intensity was increased or decreased in relation to the pJNK intensity of PBS-treated control tissue (represented as fold change; the ROI was a 50-μm box in the center of the DRG, and measurements were taken of all 16-μm sections of DRG of the upper lumbar region) and (ii) the percent of pJNK⁺ neurons in a field of view after elimination of background staining. Tuj-1 and DAPI were used to identify neurons, and all neurons in the field of view were counted. Background-level of staining of each experiment was determined from control tissue incubated without primary antibody but taken through all the steps of the antigen retrieval and secondary antibody incubation. Background was reduced using ImageJ's color threshold function. The BootstratRatio was used to determine the significance of any fold change from the baseline control intensity; ANOVA and Student's *t* test were used for to determine the significance of the percent of pJNK⁺ neurons.

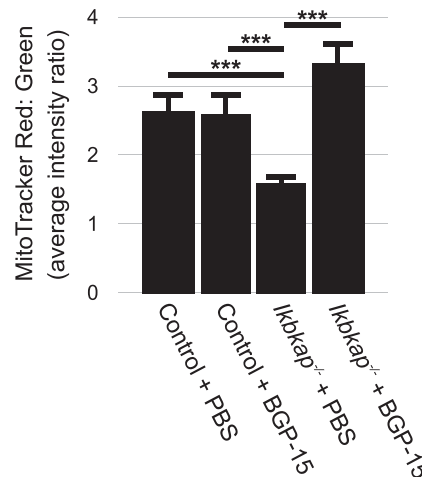


Fig. S1. Ratiometric analysis of the ratio of MitoTracker CMXRos to MitoTracker Green reveals that mitochondrial membrane potential is disrupted in *lkbkap*^{-/-} neurons but is fully restored by incubation with BGP-15. MitoTracker CMXRos, which accumulates within the mitochondrion based on the mitochondrial membrane potential, was normalized to MitoTracker Green, which is retained independently of membrane potential and is a measure of mitochondrial mass. The normalized data indicate that the collapsed membrane potential of *lkbkap*^{-/-} TrkA⁺ neurons is restored to the levels observed in littermate control neurons with 10 μM of BGP-15. *n* = 170 total neurons, ~40 neurons of each genotype and treatment. Error bars indicate the SEM; ****P* ≤ 0.001.

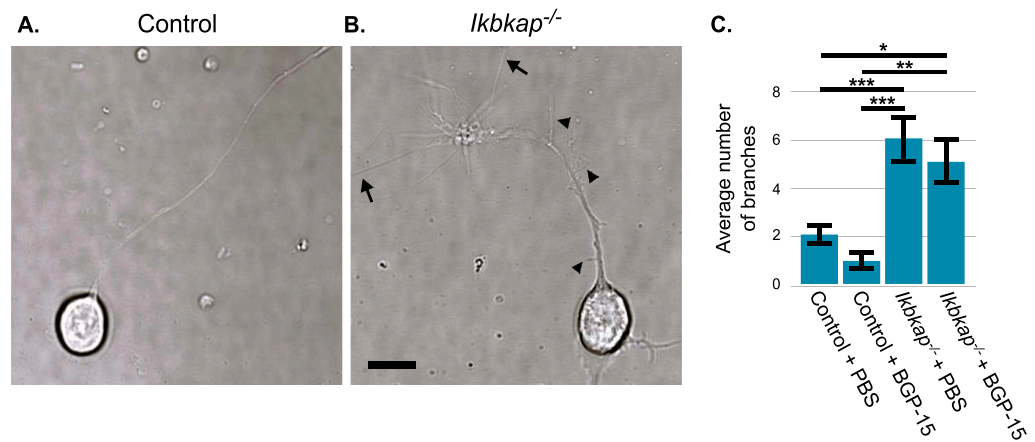


Fig. S2. *Ikbkap*^{-/-} axons are highly branched compared with axons of control littermates. (A and B) Transmitted light, 63x images of a control (A) and a *Ikbkap*^{-/-} TrkA⁺ (B) DRG neuron. *Ikbkap*^{-/-} neurons are more branched (arrowheads). Arrows indicate the abundant and filopodia-like projections of *Ikbkap*^{-/-} growth cones. (Scale bar, 10 μm.) (C) Average number of axonal extensions within an axon length of 50 μm from the soma (includes any extension >2 μm). The addition of 10 μM BGP-15 does not significantly reduce the highly branched *Ikbkap*^{-/-} axons. *n* = 15–20 randomly selected neurons of each genotype and condition that were imaged alternating between genotypes and conditions to account for lapsed time. Results were replicated over three experiments. Error bars indicate SEM; **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.

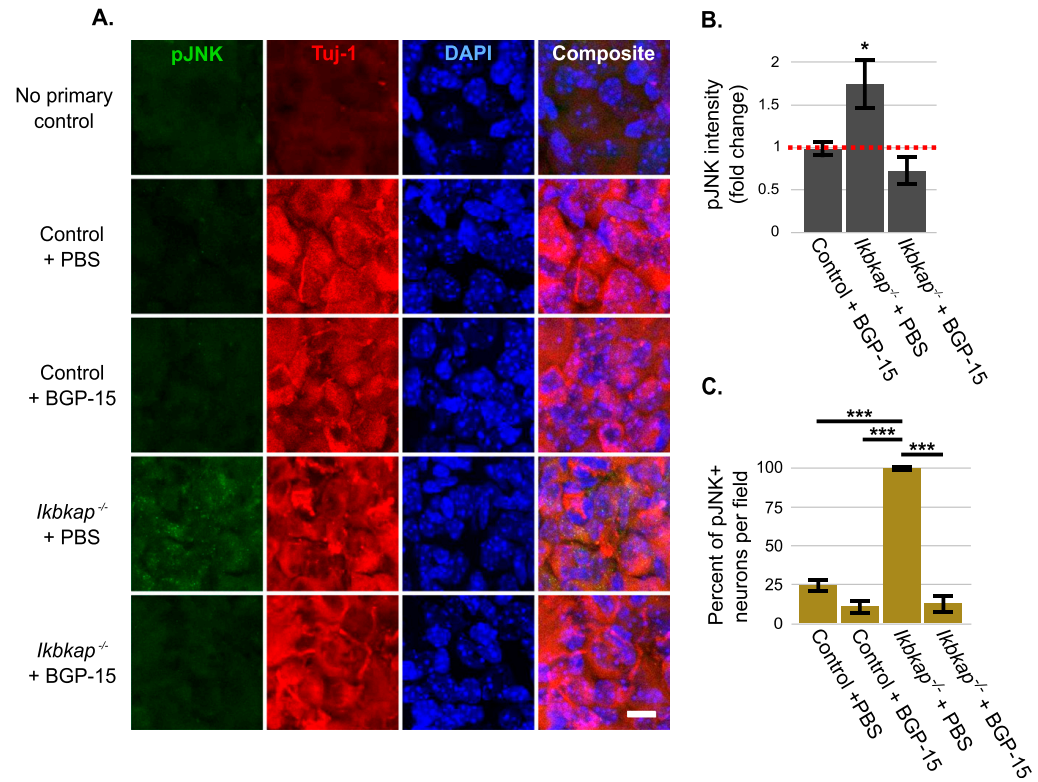


Fig. 53. pJNK is increased in *Ikbkap*^{-/-} DRG of mutant embryos but is reduced with daily injection of BGP-15. (A) Representative cross-sections of DRG from E17.5 control and mutant embryos exposed in utero to either PBS or 100 mg/kg BGP-15 via daily i.p. injection into pregnant dams. DRG neurons of mutant embryos exhibit an increase in pJNK (green), but with BGP-15 treatment levels are reduced to those observed in control DRG. Tuj-1 (red) and DAPI (blue) labeling were used to identify individual neurons of the DRG. The top row represents tissue incubated without primary antibodies, used as a control to confirm the validity of the pJNK staining and to determine the level of background staining for quantification. (Scale bar, 10 μm.) (B and C) In utero exposure to BGP-15 significantly reduces pJNK in *Ikbkap*^{-/-} DRG neurons of mutant embryos to levels found in DRG neurons of control embryos. (Scale bar, 10 μm.) (B) Average intensity measurements of pJNK, represented as the fold change calculated from the baseline intensity of PBS-treated control DRG (dashed line; the ROI is a 50-μm box in the center of the DRG; measurements were taken in all 16-μm sections of the DRG of the upper lumbar region). (C) Percent of pJNK⁺ neurons of the total neurons counted per field (three to five fields were counted in each treatment) after background was reduced (the background, derived from the intensity of the imaged tissue stained with only secondary antibodies, was determined for each experiment). *n* = 4 control + PBS embryos, 4 control + BGP-15 embryos, 4 mutant + PBS embryos, and 4 mutant + BGP-15 embryos, carried out over four experiments. Error bars indicate SEM; **P* ≤ 0.05; ****P* ≤ 0.001.

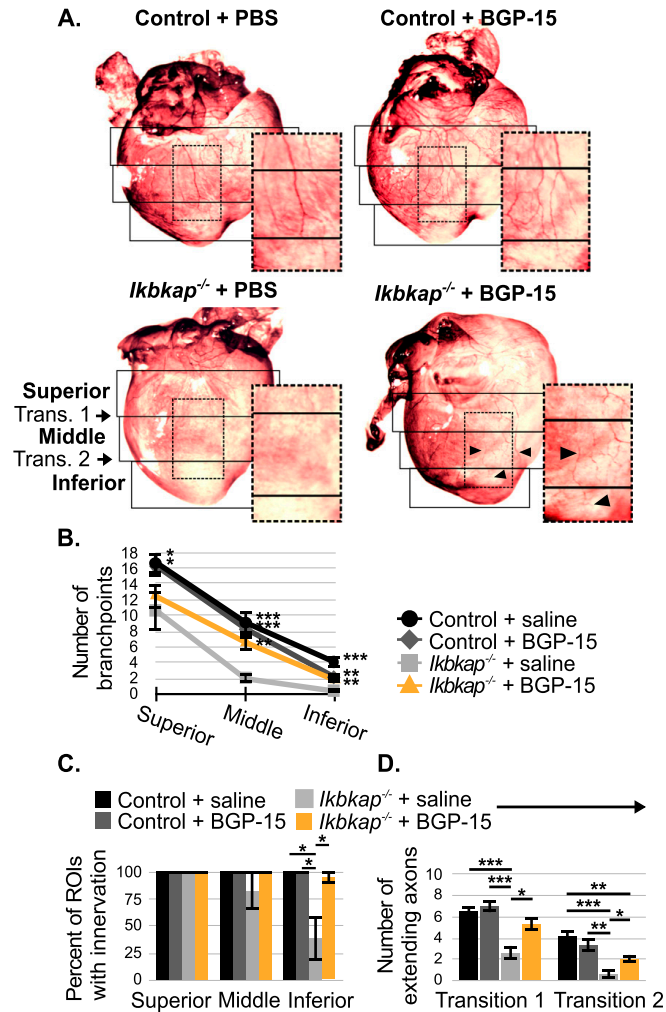
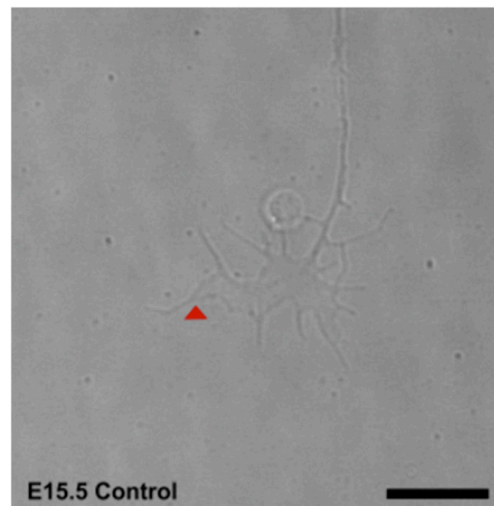
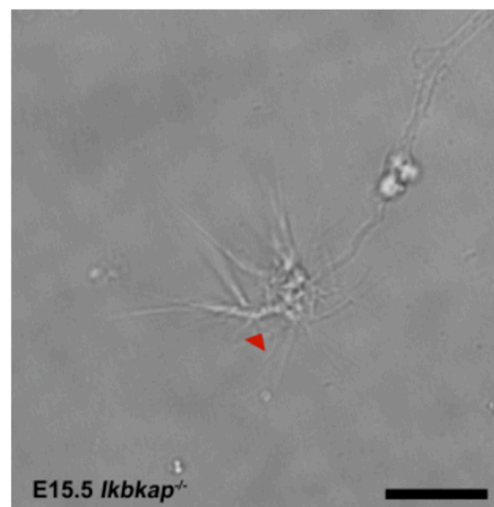


Fig. 54. Innervation is reduced in *Ikbkap*^{-/-} hearts of mutant embryos but improves with daily injection of BGP-15. (A) Representative images of control and *Ikbkap*^{-/-} hearts harvested from E17.5 embryos exposed in utero to either PBS or 100 mg/kg BGP-15 via daily i.p. injection into pregnant dams demonstrate that both the extent and complexity of *Ikbkap*^{-/-} heart innervation is increased with BGP-15 treatment (arrowheads). Boxes mark the three ROIs [superior, middle, and inferior, separated by transition 1 and transition 2 (arrows)] used to quantify the degree and patterning of innervation. Dashed boxes demarcate the areas magnified in the *Insets* (magnification: approximately 2×). (B–D) Quantification of Tuj-1 axons shows the average number of branch points in each ROI (*P* values show statistical significance compared with values of saline-treated mutant hearts) (B), the percent of ROIs with axons present (C), and the average number of axons extending across transition 1 and transition 2 (D). *n* = 10 control + saline, 12 control + BGP-15, 10 mutant + saline, and 11 mutant + BGP-15 hearts, imaged over three experiments. Error bars indicate SEM; **P* ≤ 0.05; ***P* ≤ 0.01; ****P* ≤ 0.001.



Movie S1. Control axons possess motile growth cones. Representative transmitted light, 63 $\times$ time-lapse movie of an E15.5 control growth cone, imaged over 5 min with one frame every 5 s. Control growth cones show a gain in position indicated by movement of their lamellipodia (red arrowheads mark the leading edge of a lamellipodium). Black arrows denote filopodia. (Scale bar, 10 μ m.)

[Movie S1](#)



Movie S2. *Ikbkap*^{-/-} axons possess more branched and less motile growth cones than axons in control littermates. Representative transmitted light, 63 $\times$ time-lapse movie of an E15.5 *Ikbkap*^{-/-} growth cone, imaged over 5 min with one frame every 5 s. Like the growth cones of control DRG neurons, mutant *Ikbkap*^{-/-} growth cones have motile filopodia (black arrows), but the filopodia in the mutants are longer and more abundant than those in control neurons. Mutant growth cones also rarely show a gain in position, as indicated by the lack of movement of their lamellipodia (red arrowheads mark the leading edge of a lamellipodium). (Scale bar, 10 μ m.)

[Movie S2](#)

CHAPTER THREE

NON-MAMMALIAN MODELS OF FAMILIAL DYSAUTONOMIA

Introduction

In addition to studies in mouse, non-mammalian models are utilized to understand the molecular, cellular, and physiological role of the Elongator complex. Loss of function studies on *Caenorhabditis elegans*, chick, zebrafish, *Xenopus*, and *Drosophila* reveal a role of the Elongator complex in both development and neuronal function. These models complement and build on each other's findings and help to elucidate the etiology of FD.

Caenorhabditis Elegans

Studies in *C. elegans* suggest that a critical function of the Elongator complex is in the regulation of microtubules and tRNA modification. Hueston and colleagues demonstrated that *C. elegans* ELP1 (cELP1) associates with microtubules both *in situ* and *in vitro* (1), while Solinger et al. identified the Elongator complex, using a genetic screen, as a potential regulator of acetylation of α -tubulin. Their theory was confirmed in Elongator *elpc-1* and *elpc-3* null mutants which exhibited decreased α -tubulin acetylation and reduced vesicle movement along microtubules. Larval development was also disrupted (2). On another front, Chen and colleagues provided support that, in agreement with studies in yeast (3, 4), cELP-1 and cELP-3 both are required for modification of mcm⁵ and ncm⁵ wobble position uridines of the tRNA anti-codon. Absence of these modifications in *elpc-1* and *elpc-3* deletion mutants was associated with a decreased

translation efficiency. Interestingly, acetylated α -tubulin levels were normal. These mutants also exhibited disrupted learning and developmental defects together with a mutant null *tuc-1*, which is required for thiolation of tRNAs and exacerbates the *elpc-1* and *elpc-3* mutant phenotypes (5). These data extend the conserved function of Elongator in translation to *C. elegans* and illustrate Elongator necessity in normal microtubule function.

Chick

Studies in chick support a role of IKAP/ELP1 in the developing nervous system, particularly in the peripheral nervous system (PNS). Hunnicutt and colleagues demonstrated that decreasing expression of *ikbkap/elp1 in ovo* via RNAi leads to increased levels of cleaved caspase-3+ cells in both the central nervous system (CNS) and PNS, reduced number of cycling progenitors in the spinal cord, and premature neuronal differentiation in dorsal root ganglia (DRG). Neural crest migration was not impaired. These data led the group to theorize that reduced neuronal numbers in FD stem from both loss of progenitors, in addition to premature neuronal differentiation and cell death (6). Abashidze and colleagues also corroborated these findings by showing that neural crest cell migration is normal upon downregulating expression of *ikbkap/elp1* with siRNA interference. They also observed abnormal axon outgrowth and branching *in vitro* and *in vivo* in the developing PNS and impaired peripheral target innervation. Branching and growth cone morphology of DRG neurons were disrupted *in vitro*, which the group attributed to changes in tubulin and Ikap/Elp1 patterning, along with disrupted co-

localization of Ikap/Elp1 with dynein and pJNK at the axon terminals. JNK and NFG signaling were also compromised in these neurons (7). Interestingly, Hunnicut et al. also observed disrupted cytoskeletons in chick upon loss of Ikap/Elp1 with increased branching of motor neurons *in ovo* and of DRG neurons *in vitro* (6). These data emphasize that Ikap/Elp1 of Elongator is essential for neuronal outgrowth, development, and survival.

Zebrafish

Zebrafish models further support Elongator function in axonal biology and nervous system development. Simpson and colleagues observed abnormal branching and reduced axon length of motor neurons of zebrafish embryos *in vivo* upon loss of Elp3 in an *Elp3* specific morpholino (8). Also in zebrafish, Cheng et al. demonstrated that reducing expression of *ikbkap/elp1* with an antisense morpholino leads to either a significant reduction of enteric neurons or complete aganglionosis of the distal intestine in embryos. The group explained how these data suggest a necessity of IKAP/ELP1 in the developing enteric nervous system (ENS) and offers an explanation of the gastrointestinal (GI) dysfunction and co-occurrence of a hirschsprung disease-like phenotype in FD patients (9). Thus, these studies in zebrafish extend the role of Elongator in cytoskeletal function and offers the first model to explain GI impairment in FD.

Xenopus

Xenopus has been used to investigate specifically the developmental requirement of ELP3. Yang and colleagues demonstrated that, in contrast to what was found in chick, xElp3 is expressed in and is required for migrating neural crest cells (NCCs) in *Xenopus* embryos. As they also reported that xElp3 was found to interact with a key regulator of epithelial mesenchymal transition of NCC migration, Snail1, and that this coincided with reduced expression of *N-cadherin*, they described a model in which xElp3 regulates Snail1 and likely leads to the activation of N-cadherin, aiding in the regulation of NCC migration (10). This *Xenopus* study reveals specific developmental necessity of xElp3 of the Elongator complex.

Drosophila

Lastly, *Drosophila* models have helped to elucidate the role of ELP3 of the Elongator complex in development and nervous system function. Elp3 was demonstrated to be imperative for larval development by Walker et al. where null *elp3* mutant larvae were lethal at the pupal stage and showed significant developmental delays. Deletion of Elp3 also led to an upregulation of many stressed-induced gene and development of melanotic nodules, suggesting a role of Elp3 in the *Drosophila* immune response (11). Further supporting a role of Elongator in axonal biology, Simpson et al. used a forward based mutagenesis screen in *Drosophila* and identified *elp3* as key regulator of axonal targeting and synaptic function. *Elp3* null flies possessed disordered targeting and patterning of photoreceptors and their terminals projecting into the medulla. Mutant flies

also had a reduced response to light (8). Using a transgenic fly line where silencing of *elp3* was confined to the in the brain and CNS, Singh et al. described larvae with increased bouton number, axon length, and branching at larval neuromuscular junctions, which coincided with hyperactivity and loss of sleep in adult flies (12). Lastly, Miskiewicz and colleagues also demonstrated a requirement of Elp3 in presynaptic function where they expanded the acetylase activity of Elp3 *in vitro* and *in vivo* to the large structural protein Bruchpilot. Elp3 was predominantly expressed in the cytoplasm of motor neurons and was enriched at *Drosophila* synapses at neuromuscular junctions in *Drosophila* larvae. Acetylation of Bruchpilot by Elp3 was shown to be required for the presynaptic structure and regulated neurotransmitter release. No changes in tubulin acetylation were found (13). These data from *Drosophila* again demonstrate the requirement of Elongator in cytoskeletal and neuronal function.

Conclusion

Though these data were derived from diverse models, trends of support emerge for Elongator function in nervous system development and survival, tRNA modification, and neuronal-specific roles including microtubule regulation, axonal targeting, and synaptic function. Thus, these data suggest Elongator has conserved and widespread necessity for normal cell function, particularly in the nervous system. Whether IKAP/ELP1 has functions independent from its fellow Elongator subunits remains an important question.

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CHAPTER FOUR

THE IKAP C-TERMINAL-REGION IS SUFFICIENT TO PREVENT THE DEATH OF
NEURONS FROM A MOUSE MODEL OF FAMILIAL DYSAUTONOMIAIntroduction

Familial Dysautonomia (FD) is genetic disorder marked by cell-type specific reduction of the IKAP protein and both developmental and progressive loss of sensory and autonomic neurons (1-3). FD is also characterized by a slow, continual loss of retinal ganglion cells (RGCs), which eventually leads to blindness (4-6). The major FD mutation, which accounts for over 99% of FD cases, is the result of a splice site mutation (1, 2, 7). Neurons in particular and for unknown reasons are unable to compensate for the mutation and splice the mutated RNA normally (2, 3). The mutant transcript is theorized to be targeted by nonsense mediated decay, and thus FD patients suffer from a loss of IKAP protein function (2). Earliest pathological data indicate that a two year old FD patient had undergone an 80% reduction in dorsal root ganglia (DRG) and sympathetic neurons (8, 9). However, the neural networks that are established *in utero* slowly diminish as the neuronal death continues into adulthood, demonstrated by pathological data (8, 9) and worsening gait ataxia, progressive kyphosis, and later onset vision impairment in patients (10). Therefore, our goal is to develop therapeutics to potentially target the progressive loss of neuronal function that contributes to the sensory and autonomic dysfunction and blindness that occurs as patients age.

Loss of the IKAP protein leads to widespread cellular dysfunction, including disrupted transcript elongation (11, 12), vesicle transport (13, 14), cell migration (15), cytoskeletal function (16-19), cell stress signaling (20, 21), and cell cycle progression (22, 23), some or all of which are consequences of disrupted translation as IKAP functions to modify specific tRNAs leading to efficient translation (24-26). Loss of IKAP also results in ER and/or mitochondrial stress (20, 27-29). Thus, IKAP has both reported nuclear and/or cytoplasmic roles. IKAP has been well described to function as a part of the six-unit Elongator complex, though it has also not been resolved whether IKAP can also function independently of the complex (2, 3). Data suggests that Elongator has important neurological necessity, as variants in the other Elongator subunits are associated with ALS, intellectual impairment, and epilepsy (30-33).

Though the roles of IKAP and Elongator are diverse, our goal is to target accessible disrupted events to improve cell function and survival, especially of neurons. We have previously explored compounds that improve neuronal survival by targeting and reducing cell stress pathways that lead to certain death, with success. Thus here, our approach was to determine the therapeutic potential of the IKAP protein itself when its gene is introduced into cells that are null for *Ikbkap*. While it has been previously shown that introduction of the *Ikbkap* gene into null mouse models has prevented embryonic lethality, our study is the first to explore the effects of reintroduction of IKAP and IKAP pieces into *Ikbkap*^{-/-} neurons (34). Understanding the extent of this therapeutic approach has not yet been determined.

Results

Death of *Ikbkap*^{-/-} Neurons is Prevented with Expression of the IKAP Protein

We have conditionally deleted the *Ikbkap* gene from the neural crest lineage via a *Wnt1-cre* driver. TrkA⁺ dorsal root ganglia (DRG) neurons from these *Ikbkap* null *Wnt1-Cre;Ikbkap*^{LoxP/LoxP} (mutant) embryos die *in vivo* (20), recapitulating the loss of sensory and pain transmission that marks FD. In culture, these small diameter TrkA⁺ neurons begin dying as early as 24 hours after plating, even in the presence of their preferred growth factor, NGF. To our knowledge, it has not been demonstrated before in an FD genetic model whether reintroducing the *IKBKAP* gene can restore survival of post mitotic neurons. Thus, we infected E16.5 *Ikbkap*^{-/-} DRG TrkA⁺ neurons with a pLenti virus encoding the full length human *IKBKAP* with a *GFP* tag, driven from a general mammalian expression promoter (CMV), and compared neuronal survival to *Ikbkap*^{-/-} neurons infected with a virus only encoding *GFP* (Fig. 1A, B). After 40 hours in culture, IKAP was expressed broadly in support cells and a smaller percent in neurons (Fig. 1A, B). Even with the robust expression in support cells over neurons, a striking and significant increase in survival was found when compared to survival of mutant neurons expressing only GFP (Fig. 1C).

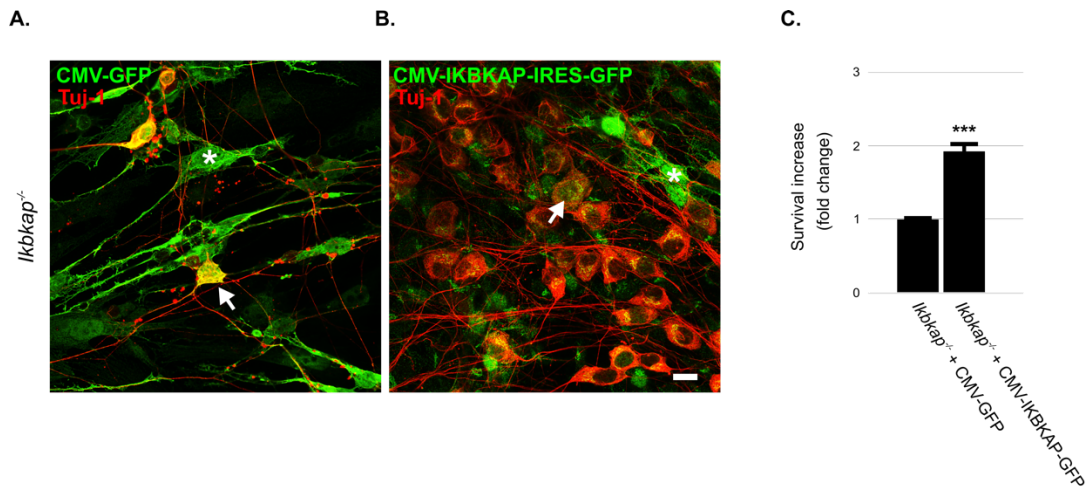


Figure 1. Reintroducing IKAP blocks death of *Ikbkap*^{-/-} TrkA+ dorsal root ganglia (DRG) neurons *in vitro*. 40-hr expression of p-Lenti-CMV-IBBKAP-IRES-GFP in E16.5 cultured *Ikbkap*^{-/-} TrkA+ DRG neurons significantly increases their survival in culture. (A) Representative images of DRG neurons expressing only *GFP* and (B) *IBBKAP* and *GFP*, driven off of the CMV promoter. Both neurons (arrows) and their support cells (asterisks) express the viral constructs, indicated by GFP. Tuj-1 (red) labeling marks neurons used for survival counts. Scale bar = 10 μ m. (C) Quantification of total neuronal counts of each condition is presented as the average fold change, where the average count of *Ikbkap*^{-/-} neurons in wells expressing *IBBKAP* was normalized to the average count of *Ikbkap*^{-/-} neurons in wells expressing only GFP in each experiment. n = 4 experiments.

Death of *Ikbkap*^{-/-} Neurons is Prevented by Expression of the C-terminal Region of IKAP

The human *IBBKAP* gene is large and spans nearly 4 kb (7, 21). Viral delivery systems being employed in gene therapy can often only carry a smaller cargo (35). With our previous success with delivering the full-length *IBBKAP* to both neurons and support cells and increasing survival of *Ikbkap*^{-/-} neurons, we asked whether (1) expressing specific regions of the protein could also increase *Ikbkap*^{-/-} neuronal survival and (2) whether targeting expression to neurons only could similarly induce benefits and/or reduce death. Thus, we sub-cloned specific regions of the *IBBKAP* gene into both plasmid and viral expression vectors, the viral vectors having a neuronal-specific,

synapsin promoter. The gene fragments generated were determined from known protein-protein interacting regions, conserved domains of the IKAP protein, and the site of the major FD mutation (Fig. 2A). In brief, WD40 and TPR domains are highly conserved and span the N and C-terminals respectively (21). Additionally, IKAP was first reported to interact with NIK and IKK of the large interleukin complex, though this result was only observed upon overexpression of IKAP in human cells, and these sites are located more centrally (36). Finally, the major FD mutation in gene *IKBKAP* occurs at approximately amino acid 714 (7, 21) (Fig 2A). To date, pLenti viral vectors expressing a middle region of the IKAP protein, “*IKBKAP2*”, and the C-terminal half of IKAP, “*IKBKAP3*”, were expressed in E16.5 *Ikbkap*^{-/-} DRG, along with the full length gene, driven off of synapsin promoters. Strikingly, *IKBKAP3* significantly increased survival as demonstrated by an increased number of *Ikbkap*^{-/-} TrkA+ DRG neurons after expression when compared to delivery of only *GFP*, equivalent to what was observed with ectopic expression of the full length *IKBKAP*. Thus, targeted neuronal specific expression of both full length IKAP and the C-terminal half of the protein are equally capable of promoting the survival of *Ikbkap*^{-/-} TrkA+ DRG neurons (Fig. 2B).

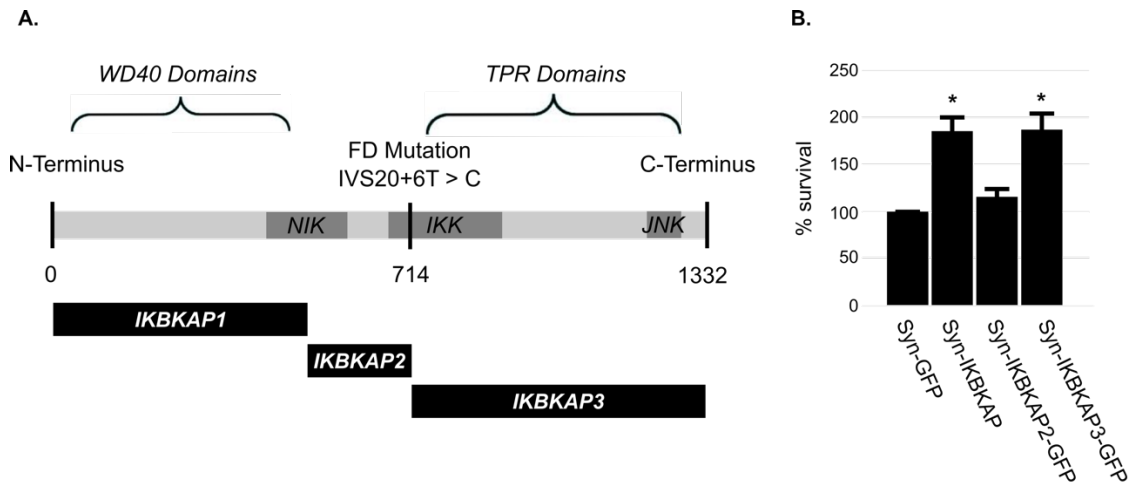


Figure 2. Reintroducing IKAP or C-terminal IKAP prevents death of *Ikbkap*^{-/-} DRG neurons *in vitro*. (A) Diagram showing the 1332 amino acids that constitute the human IKAP protein, marking the specific *IKBKAP* gene lengths encoding 3 regions of the IKAP protein (“*IKBKAP1*”, “*IKBKAP2*”, and “*IKBKAP3*”) that were sub-cloned into both expression plasmids and viruses. Previously described domains and specific protein associated regions of IKAP are indicated (NIK = NF-κB-inducing kinase and IKK = inhibitor of kappa B complex that are both involved in NF-κB inflammatory signaling; JNK = c-Jun N-terminal kinase involved in stress signaling). The N-terminal half possesses WD40 domains, while TRP domains span the C-terminal half of the protein. (B) Counts of *Ikbkap*^{-/-} TrkA⁺ neurons expressing pLenti viruses encoding *IKBKAP*, *IKBKAP2*, and *IKBKAP3*, by Yumi Ueki (co-author, in progress), indicate that expressing the full length *IKBKAP* and *IKBKAP3*, both of which were driven off of the synapsin promoter confining expression to neurons, significantly increases survival, indicated by a significant increase in total neurons per well of E16.5 *Ikbkap*^{-/-} TrkA⁺ DRG neurons after expression of constructs. n = 3 experiments. Error bars = ± SEM. “*” = p-value ≤ 0.05, “**” = p-value ≤ 0.01, “***” = p-value ≤ 0.001.

C-terminal and Full Length IKAP are Predominantly Localized to the Cytoplasm

Since we found that expression of *IKBKAP3* increases neuronal survival to that measured in cells expressing the full length gene, we wanted to explore cellular behavior of the C-terminal half of the IKAP protein, encoded by *IKBKAP3*. It has been demonstrated that IKAP functions as a scaffolding unit of the six-unit Elongator protein complex (37), with data suggesting a major function of the complex is in mediating

translation by modification of tRNA (24, 25). Loss of this function in absence of Elongator may account for some of the previously reported Elongator-null phenotypes, including impairments in RNA polymerase II-mediated transcript elongation and polarized exocytosis (26). It has not been completely excluded, however, that Elongator serves additional roles beyond its function in tRNA modification. Data has accrued that support a role for both IKAP and Elongator in cytoskeletal networks (16, 38). With the benefits to neuronal survival with C-terminal IKAP, we were interested to further characterize its behavior within the cell. Plasmids encoding *c-Myc* tagged *IKBKAP1*, *IKBKAP3*, and *Ikbkap* were expressed in HEK 293 cells, and their localization was determined (Fig. 3). Strikingly, the N and C-terminal regions of the protein had distinct localization: the C-terminal IKAP and full length IKAP were consistently localized to the cytoplasm, while the N-terminal portion of IKAP was both nuclear and cytoplasmic (Fig. 3).

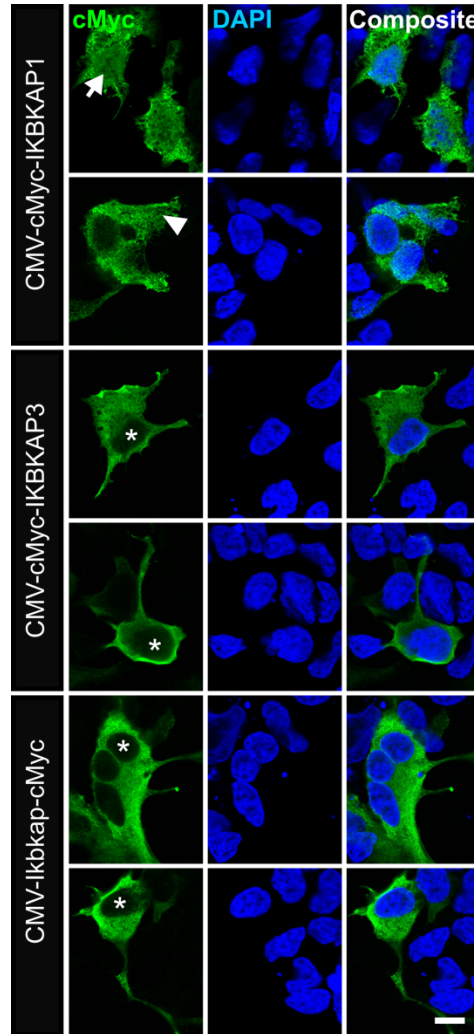


Fig. 3. Expression of *IKBKAP3* and *Ikbkap* is predominantly cytoplasmic, while expression of *IKBKAP1* is localized to both the nucleus and cytoplasm in human embryonic kidney cells (HEK 293). pcDNA3 plasmids encoding *IKBKAP1*, *IKBKAP3*, and *Ikbkap* fused with *c-Myc* tags were expressed in HEK293 cells. Staining for c-Myc allowed for identification of IKAP, encoded by *IKBKAP*, while DAPI allowed for determination of any nuclear localization of IKAP. *IKBKAP1*, encoding the N-terminal portion of IKAP, was found both in the nuclear compartment (arrow) and throughout the cytoplasm (arrowhead). *IKBKAP3*, encoding the C-terminal half of IKAP, and full length *Ikap* were predominantly cytoplasmic and excluded from the nucleus (asterisks). Expression was also more evenly diffuse than that found in *IKBKAP1*. Scale bar = 10 μm . Error bars = $\pm$ SEM. “*” = p-value $\leq$ 0.05, “**” = p-value $\leq$ 0.01, “****” = p-value $\leq$ 0.001. n = 3 experiments.

Ectopic Expression of C-terminal IKAP Induces Proliferation of Human Embryonic Kidney Cells

As a direct or indirect consequence of impaired translation, loss of Elongator has been reported to lead to disruptions in the cell cycle (22, 23). Thus, we aimed to further explore whether benefits of the full length and C-terminal IKAP were from changes to the cell cycle. *IKBKAP1*, *IKBKAP3*, *Ikbkap*, and a control plasmid encoding only the *c-Myc* tag were expressed in HEK 293 cells and proliferation of cells was determined with EDU labeling (Fig. 4A). EDU is a thymidine analogue and is incorporated during synthesis. Positive staining for this compound is therefore a measure of cell proliferation. Of the cells expressing the constructs, the percent of EDU positive cells was determined (Fig. 4B). Strikingly, the C-terminal IKAP fragment significantly increased proliferation beyond that of only the c-Myc control plasmid. The full length protein also increased proliferation, though not significantly. N-terminal IKAP did not significantly reduce cell proliferation, but it was significantly less than levels found upon introduction of the C-terminal half of IKAP (Fig. 4B).

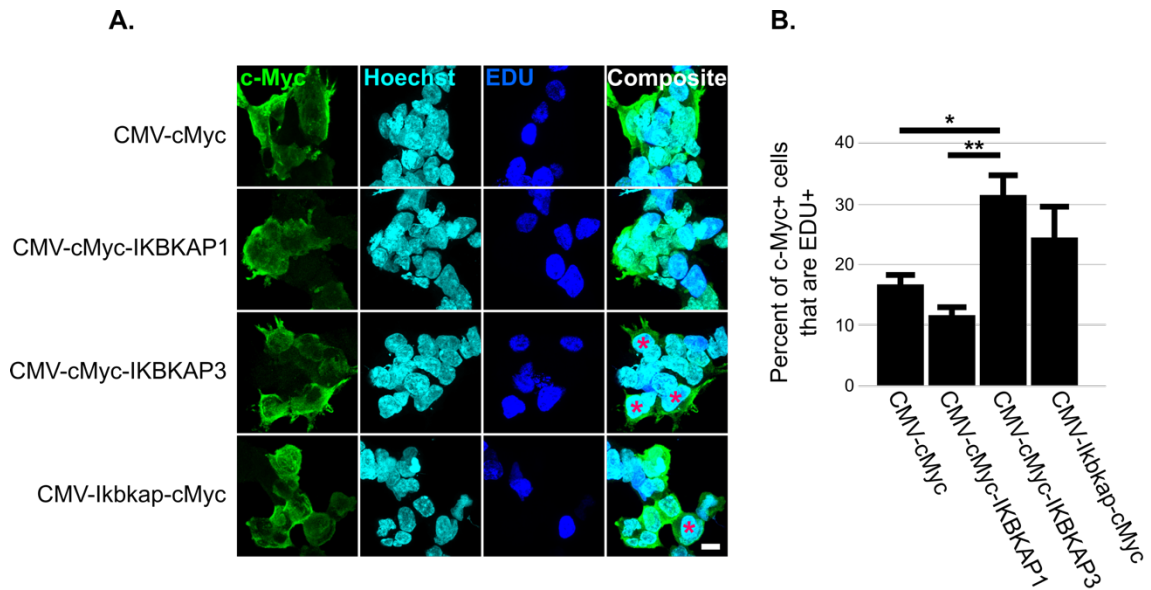


Fig. 4. Expression of *IKBKAP3* promotes proliferation of HEK 293 cells. EDU staining, a measure of cell proliferation, was carried out in HEK 293 cells expressing *c-Myc* tagged *IKBKAP1*, *IKBKAP3*, and *Ikbkap*. (A) Representative images of *c-Myc* (green), Hoechst (nuclear staining, cyan), and EDU (blue) labeled HEK cells that were transfected with pcDNA3 plasmids encoding the *IKBKAP* variants. *c-Myc* only expression was used a control. Many cells expressing *IKBKAP3* and *Ikbkap* were also EDU+ (astericks). Scale bar = 10 μ m. (B) Quantification of EDU and *c-Myc* labeling showing the percent of *c-Myc*+ cells that stained for EDU. While few cells labeled positive for EDU in *IKBKAP1* expressing cells, *IKBKAP3* significantly increased the number of cells that were positive for EDU labeling. *Ikbkap* also increased the occurrence of EDU labeling, though not significantly. For counts, only *c-Myc*+ cells were evaluated, and of those, the percent that were EDU+ was noted. $n = 3$ experiments. Error bars = $\pm$ SEM. “*” = p -value ≤ 0.05 , “**” = p -value ≤ 0.01 , “***” = p -value ≤ 0.001 .

C-Terminal IKAP Induces Proliferation of FD Patient Fibroblast Cells

As *IKBKAP3* was found to induce proliferation of HEK 293 cells, we wondered if this observation would also extend to affected cells from an FD patient. We thus expressed the *IKBKAP* constructs in fibroblasts from an FD patient testing positive for the major FD mutation (IVS20+6T>C) and one testing negative, serving as a control. We first found that mutant FD fibroblasts exhibited decreased proliferation when compared

to the control unaffected cell line (Fig. 5A). However, after quantification of EDU labeling, expression of both the full length and N-terminal IKAP further induced proliferation in affected FD fibroblasts when compared to expression of the c-Myc only control plasmid (Fig. 5B).

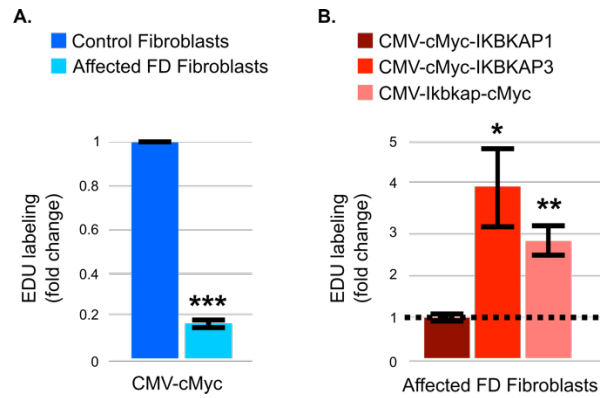


Fig. 5. Expression of both *IKBKAP3* and *Ikbkap* promotes proliferation of fibroblasts from a Familial Dysautonomia (FD) patient. Proliferation of cells was determined with EDU labeling where the percent of *IKBKAP* expressing cells that stained positive for EDU was quantified. (A) EDU labeling is significantly decreased in affected FD fibroblasts when compared to EDU labeling in control fibroblasts with expression of a c-Myc control plasmid. (B) EDU labeling is significantly increased in affected FD fibroblasts upon expression of *IKBKAP3* and *Ikbkap* fibroblasts when compared to expression of *IKBKAP1* and baseline expression of the c-Myc control plasmid (dashed line). Quantification of EDU positive cells is represented as fold change where the percent of EDU labeling found upon expression of each *IKBKAP* construct was normalized to the percent observed with expression of the control c-Myc plasmid. n = 3 experiments. Error bars = $\pm$ SEM. “*” = p-value $\leq$ 0.05, “**” = p-value $\leq$ 0.01, “***” = p-value $\leq$ 0.001.

Discussion

Loss of TrkA⁺ sensory neurons that mediate transmission of pain and temperature sensation is a hallmark of the hereditary and sensory neuropathy type III, Familial Dysautonomia (FD). TrkA⁺ neurons from our *Wnt1-Cre;Ikbkap*^{-/-} mouse model that lack

Ikap begin dying in culture within 24 hours, in contrast to control neurons that survive for weeks in the same conditions. We found that both general and neuronal-specific ectopic expression of the full length *IKBKAP* gene in *Ikbkap*^{-/-} TrkA+ DRG neurons significantly increases their survival *in vitro*. Strikingly, not only did the full length *IKBKAP* increase survival of these neurons, but the fragment of the human *IKBKAP* gene encoding the C-terminal half of the protein also increases numbers comparable to the full length. This fragment, *IKBKAP3*, predominantly localizes to the cytoplasm, like that of the full length, when expressed in HEK cells and induces both HEK 293 cells and FD patient fibroblasts to proliferate in culture.

Studies have accumulated that support a requirement of IKAP in regulating cell cycle progression. In chick, loss of *Ikbkap* results in premature differentiation of neuronal progenitors (39). This is in agreement with our own *Wnt1-Cre;Ikbkap*^{-/-} mouse model where it was revealed that Pax3+ progenitors (which give rise to TrkA+ neurons) prematurely exit the cell cycle and either differentiate or die via apoptosis (20). Both studies suggest that IKAP is required for neurogenesis to maintain progenitor number and identity and that its absence may decrease the progenitor pool leading to loss of neuronal numbers. In FD patient fibroblasts, a microarray analysis revealed that genes involved in the cell cycle progression are mis-regulated with reduced levels of IKAP. Furthermore, cell sorting demonstrated that significantly more FD cells are in G1 and that fewer are in mitosis as compared to control cells (23). Data from a neuroblastoma cell line of neural crest origin with reduced IKAP also indicated genes associated with the cell cycle are mis-regulated when cells are directed to differentiate, particularly genes associated with S

and M phases of the cell cycle (22). Lastly, Elongator mutants in *Arabidopsis* have decreased cell number after germination, which is partially attributed to decreased cell proliferation (40). Though these studies and ours demonstrate various outcomes of dysfunction, they all suggest a role of IKAP and Elongator in regulation or maintenance of the cell cycle.

Our data indicate that both the full length and the C-terminal half of the protein promote cellular proliferation, in contrast to the N-terminal half which does not. Studies have demonstrated significant functions of the C-terminal half of IKAP. For example, the C-terminal half of human IKAP possess a JNK binding site, sufficient to potentiate the JNK stress response (21). The premature differentiation of neural progenitors in chick with reduced levels of Ikap, previously described by Hunnicut and colleagues, can also be rescued with the C-terminal half (39). Additionally, mammalian cell lines with reduced Ikap have impaired migration and adhesion, which interestingly cannot be restored with the truncated N-terminal half of human IKAP but can be rescued by expression of the full length (19). IKAP/ELP1 is a scaffolding unit of the Elongator complex, and it has been demonstrated that the C-terminal region of IKAP/ELP1 is necessary and sufficient for its self-dimerization, which is required for formation of the Elongator complex and Elongator function in tRNA modification (41-44). These studies are in line with our data here that demonstrates functional importance of the C-terminal region of human IKAP, with significant effects observed in survival and proliferation, comparable to the full length and in contrast from the N-terminal region. Though the truncated protein has not been isolated in FD-derived cell lines and therefore may not

have physiological relevance (2), perhaps ectopic expression of the C-terminal region is sufficient to promote improved cellular function.

The C-terminal half of IKAP possesses highly conserved TPR domains and IKK α and IKK β association sites, all of which could contribute to the observed increase in proliferation, potentially independent of the C-terminal half in promoting formation of the Elongator complex altering translation. JNK is known to phosphorylate many downstream targets and includes those involved in proliferation and cell cycle progression, being demonstrated to regulate entry, synthesis, and the G2 to M transition (45-47). Additionally, TPR domains function as scaffolds in the assembly of larger protein complexes and are found in multiple proteins belonging to the anaphase protein complex (APC), which is an essential protein complex regulating progression through the cell cycle by marking proteins for decay (48). The C-terminal half of IKAP also possesses IKK α and IKK β association sites. IKK α and β are major components of the IKK complex, which activate the NF- κ B complex by freeing its release to translocate to the nucleus and alter transcription. Interestingly, NF- κ B can increase transcription of cyclin D₁, which is responsible for progression through the G1 phase of the cell cycle (49), and, as previously described, data suggests that cells with reduced IKAP are halted in G1 (23). Therefore, this C-terminal IKAP length includes known binding domains that alter the cell cycle upon its expression.

The question remains of why post-mitotic neurons respond with increased survival to a region of the *IKBKAP* gene that pushes replicative cells to proliferate further. These effects may result from independent activities of the C-terminal region.

The C-terminal IKAP region could alter JNK and IKK signaling, both of which mediate apoptotic death signaling (50, 51) leading to increased survival of neurons. As these pathways are potentially altered in absence of IKAP, which we previously demonstrated by showing that levels of pJNK are elevated in *Ikbkap*^{-/-} DRG neurons (52), their expression could correct for any detrimental consequences. Conversely, it has been reported that neurons re-activate the cell cycle prior to apoptosis upon insult, including but not limited to, DNA and/or oxidative damage (53). As it has been demonstrated that these *Ikbkap*^{-/-} TrkA⁺ DRG neurons from our FD mouse model die via apoptosis and exhibit increased levels of P53 (20), reactive oxygen species (52), and DNA damage (preliminary data), perhaps the proliferative effects observed with the C-terminal half contribute to the found increase in neuronal survival as the protein acts to alter the cell cycle. Though a complete mechanism is beyond the scope of this study here, C-terminal human IKAP has the potential to increase survival of neurons that exhibit stress and die via apoptosis. A critical next step will be the expression of this smaller cargo to specific neuronal populations that progressively die in FD as a targeted therapy. The full length *IKBKAP* spans nearly 4 kb and is too large for most viral expression systems. However, *IKBKAP3* would be deliverable with, for example, adenovirus, which via intravitreal injection could infect retinal ganglion cells (RGCs) *in vivo* that normally die and cause the adult onset blindness in FD (10). Thus, *IKBKAP3* has therapeutic potential perhaps over that of the full length gene.

In summary, we demonstrate here potential of the C-terminal region of the human *IKBKAP* gene to increase neuronal survival in a mouse genetic model of FD. We also

show that this region, when expressed in HEK 293 and fibroblast cells from an FD patient, can induce proliferation *in vitro*. This region primarily is expressed in the cytoplasm, which supports a role in regulating the JNK or IKK response that could contribute to cell cycle progression and also neuronal survival. Thus, ectopic expression of the C-terminal half of IKBKAP in specific neuronal populations could represent an exciting new approach to minimizing the progressive neuronal loss occurring in FD.

Methods

Genetically Modified Mice

The *Ikbkap/Elp1* gene was conditionally deleted from the neural crest cell lineage using a *Wnt1-Cre* transgene as previously described (20). Briefly, we, crossed homozygous *Ikbkap* CKO (*IC/C*) mice to hemizygous *Wnt1-Cre* mice (*Cre*⁺) carrying one copy of the conditional *Ikbkap* allele (*I*⁺/*C*). For all analyses, *Cre*⁺; *IC/C* embryos were used as experimental and *Cre*[−]; *I*⁺/*C* littermates were used as controls. All genotyping was performed via PCR. *Wnt1-Cre* mice were purchased from The Jackson Laboratory (stock no. 003829). All mice procedures adhered to the “NIH Guide for the Care and Use of Laboratory Animals”.

Primary Culture of Mouse Dorsal Root Ganglia (DRG) Neurons

DRG were dissected from E15.5, E16.5, or E17.5 *Wnt1-Cre;Ikbkap*^{LoxP/LoxP} (mutant) and *Ikbkap*^{+/LoxP} (control) embryos. Embryos were first genotyped, and DRG from control and littermate mutant pups were dissected, pooled, and stored overnight at

4°C in Hibernate-E media (Thermo Fisher), supplemented with 2% B-27 (Gibco) and 2 mM L-glutamine (Gibco). Nunc LabTek II chambered coverglass 8-well slides (Fisher Scientific) were coated the day of plating with 10 µg/mL poly-d-lysine (Millipore, 30 minutes at room temperature) and 20 µg/mL laminin (Thermo Fisher, 2 hours at 37°C, 5% CO₂). To dissociate neurons for plating, DRG were incubated with 0.05% trypsin + EDTA (Thermo Fisher) at 37°C for 16 minutes. Trypsin was quenched with 10% FBS (Thermo Fisher) in Neurobasal (Thermo Fisher), and neurons were spun down at 1000 rpm for 2 minutes at 4°C. The supernatant was decanted, and neurons were re-suspended in Neurobasal media supplemented with 2% B-27, 100 µg/ml penicillin/streptomycin (Mediatech), 2 mM L-glutamine, and 25 ng/mL NGF (gift from Dr. Thomas Large) to select for TrkA⁺ neurons. Approximately 400 µL of supplemented Neurobasal media per embryo dissected was added for the resuspension, which led to an optimal concentration of cells for plating. Neurons were further dissociated with a series of fire-pulled Pasteur pipettes, and the cell solution was passed through a 40 µM Falcon Cell Strainer (Fisher Scientific) to eliminate any remaining clumps. Cells were counted with a hemocytometer and plated at final concentration of 2.0×10^4 cells/ well in a total volume of 300 µL.

Cultures were incubated at 37°C, 5% CO₂ for up to 72 hours.

Culture of Human Embryonic Kidney Cells

HEK 293 cells were cultured in Corning 75 cm² cell culture flasks (Fisher Scientific) at 5% CO₂, 37°C in Advanced DMEM/F12 media (Thermo Fisher), supplemented with 10% FBS (Thermo Fisher), 100 µg/ml penicillin/streptomycin (Mediatech), and 2 mM L-glutamine (Gibco). Cells were split using 2 mLs of 0.05%

trypsin + EDTA (Thermo Fisher), incubated for 5 minutes at 37°C, 5% CO₂, to which cells were quenched with 8 mL of a 10% FBS solution in Advanced DMEM/F12 and split at a ratio of 1:10 into supplemented Advanced DMEM/F12 media. Cells were plated for EDU and localization assays on Nunc LabTek II chambered coverglass 8-well slides (Fisher Scientific), coated the day of plating with 50 µg/mL poly-d-lysine (Millipore, 30 minutes at room temperature).

Culture of Human Fibroblast Cells

Human fibroblasts were obtained from the Coriell Institute and included the affected GM02342 and unaffected GM04643 testing positive and negative, respectively, for the 2507+6T>C mutation in the IKBKAP gene (IVS20+6T>C). Cells were cultured in Corning 75 cm² cell culture flasks (Fisher Scientific) at 5% CO₂, 37°C in Eagle's Minimum Essential Medium supplemented with 100 µg/ml penicillin/streptomycin (Mediatech), 2 mM L-glutamine (Gibco), and 10% FBS (Thermo Fisher). To split, cells were incubated with 2 mLs of 0.05% trypsin + EDTA (Thermo Fisher) for 9 minutes at 37°C, 5% CO₂, to which cells were quenched with 8 mL of supplemented MEM and split according to Coriell Institute protocol of each cell line (GM02342 at 1:2, and GM04643 at 1:4). Cells were plated for the EDU assay on Nunc LabTek II chambered coverglass 8-well slides (Fisher Scientific), coated the day of plating with 10 µg/mL poly-d-lysine (Millipore, 30 minutes at room temperature).

Immunocytochemistry

After fixation with 4% paraformaldehyde at room temperature, adherent cells were blocked (1% glycine, 0.1% Triton, and 10% goat serum in 1x PBS) for 1 hour, incubated in primary antibody for 3 hours, and incubated in secondary antibody for 1 hour, all at room temperature. Primary antibodies included Tuj-1 (Covance, 1:1000), anti-GFP (Thermo Fisher, 1:2000), anti-c-Myc (1:200, BD Biosciences), anti-GFP (Thermo Fisher, anti-2000), and anti-IKAP (1:100, gift from Dr. Dan Sussman). Secondary antibodies and stains included Alexa 488 and 568 (Thermo Fisher, 1:2000), and DAPI (Thermo Fisher, 1:3000). For labeling of incorporated EDU, the Click-iT Plus EdU Alexa Fluor®647 Imaging Kit (Thermo Fisher) was utilized, following manufacturer's protocol. In brief, cells were rinsed after fixation, washed with 3% BSA in 1x PBS, permeabilized with 0.5% Triton X-100 in 1x PBS for 20 minutes, and incubated with the Click-iT reaction cocktail for 30 minutes, all at room temperature. Wells were then washed and incubated with primary and secondary antibodies, which included the nuclear hoechst stain (1:2000), apart of the Click-iT Imaging Kit.

Expression Plasmids and Viruses

Constructs were cloned by the QB3-Berkely Macrolab using human *IKBKAB* (cDNA from Origene Ref. NM_003640) and the pCDNA3 LIC cloning vector (Addgene Ref. #30124), and included pcDNA3-CMV-cMyc-IKBKAP1, pcDNA3-CMV-cMYC-IKBKA2, pcDNA3-CMV-cMyc-IKBKAP3, pcDNA3-CMV-Ikbkap-cMyc, and pCS2+MT-CMV-cMyc, which was a kind gift from Dr. Roger Bradley (Montana State University). Lentiviruses encoding the full length human *IKBKAP* driven from the CMV

and Synapsin promoters were obtained from AGTI and included pLenti-CMV-IKBKAP, pLenti-CMV-GFP, pLenti-Syn-IKBKAP, and pLenti-Syn-GFP, pLenti-Syn-IKBKAP2-GFP, and pLenti-Syn-IKBKAP3-GFP.

In vitro Survival Assays

For the full length *IKBKAP* rescue experiment, p-Lenti-CMV-IKBKAP-IRES-GFP or pLenti-CMV-GFP (control) were added to E16.5 DRG plated at a 2×10^4 cell per well. For survival increase of *IKBKAP* pieces, either pLenti-Syn-IKBAP2-GFP, pLenti-Syn-IKBKAP3-GFP, pLenti-Syn-Ikbkap, or pLenti-Syn-GFP (control) were added to E16.5 DRG cultures and incubated for 40-45 hours, to which cells were fixed for 10 minutes with 4% paraformaldehyde at room temperature. Wells were stained with Tuj-1 and DAPI for neuronal counts and either anti-GFP (1:2000) or anti-IKAP (1:100) to determine IKAP expression. The total number of neurons per well was determined in replicate wells using a Leica TCS SP8 confocal microscope with a 20x objective. Replicate wells that differed in count by more than 250 were deemed too variable due to variability in plating technique and excluded. Survival increase/decrease and percent survival data represent the average change across a minimum of 3 experiments, calculated from baseline control-treated counts. Statistical significance was calculated using the BootstRatio analysis.

Localization of IKAP in HEK 293 Cell Analysis

HEK 293 were plated on coverglass 8-well slides at 1×10^5 cells per well. When the monolayer had reached 70-80% confluence, cells were transfected with pcDNA3-

CMV-cMyc-IKKBAP1, pcDNA3-CMV-cMyc-IKKBAP3, pcDNA3-CMV-Ikbbkap-cMyc, and pCS2+MT-CMV-cMyc (control, 63% coverage and 99% identity to pcDNA3) using Lipofectamine LTX & PUS Reagent (Thermo Fisher) following the manufacturer's protocol. The transfection was optimized delivering 500 ng of DNA, 1 μ L of PLUS Reagent, and 1.5 μ L of LTX (diluted in Opti-MEM; Thermo Fisher) per well for 4 hours, to which media was decanted and supplemented Advanced DMEM/F12 was added. Cells were incubated at 37°, 5% CO₂ for 24 hours, fixed with 4% paraformaldehyde for 10 minutes, and labeled with anti-c-Myc (1:200) and DAPI (1:3000). Cells were imaged with an SP8 confocal microscope using a 63x objective, and stacks were generated with a step size of 0.3 μ m. Figure 3 shows the plane at the level of the nucleus to show nuclear and cytoplasmic expression. Approximately 10 fields were imaged each experiment, which was repeated 3 times.

Measurement of Proliferation: EDU Analysis

HEK 293 and human fibroblasts were plated on coverglass 8-well slides at 1×10^5 cells per well and transfected with pcDNA *IKKBAP* constructs as described above. Cells were incubated at 37°, 5% CO₂ for 24 hours. After the 24-hour incubation which allowed for adequate expression of *IKKBAP* constructs, half of the cell solution volume was removed, and an equal volume was added of 20 mM EDU in supplemented media for a final concentration of 10 mM EDU, minimizing cell shock. EDU was incubated with HEK cells for 1 hour and fibroblasts for 18 hours. Cells were then fixed and labeled using an anti-c-Myc and the Click-iT Plus EdU Alexa Fluor®647 Imaging Kit as previously described. Cells were imaged with a Leica TCS SP8 confocal microscope using a 63x

objective, and z-stacks were generated using a step size of 2 μm . Approximately 150-250 c-Myc⁺ cells were counted from each transfection experiment over 5-10 regions. Of those, the percent that were also EDU⁺ was marked. n = 4 experiments for the HEK EDU analysis and n = 3 experiments for the fibroblasts analysis. Statistical significance was determined using the one-way ANOVA and Student's t-test.

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CHAPTER FIVE

CONCLUSION

Summary

Our data reveal here significant impairments in mitochondrial function, cytoskeletal dynamics, and cell cycle regulation upon reduced *IKBKAP* expression, and suggest that all may potentially contribute to the increased death of neurons in FD. Specifically, we demonstrate that upon loss of *Ikap* in our FD mouse model, TrkA⁺ neurons of the DRG have a significant mitochondrial impairment, demonstrated by their depolarization and fragmentation and with the elevated levels of ROS. Mitochondria in mutant TrkA⁺ neurons also do not coalesce correctly at branch points, and these neurons have axons that are more branched and stunted and growth cones that are less motile with increased filopodia, when compared to littermate control embryos. Innervation of the heart is decreased in mutant neurons. pJNK activity is also elevated in mutant DRG. In cells derived from FD patients, we observe reduced proliferation. These events occur alongside the death of sensory DRG neurons in our mouse FD model and death of sensory neurons in FD patients (1, 2). Disrupted mitochondrial function, cytoskeletal dynamics, cell cycle regulation, and JNK stress signaling can all lead to death via apoptosis (3-7), which we observe in our FD mouse model as the death pathway that leads to their demise (8).

These pathways that lead to cell death can potentially offer specific molecular and cellular targets for therapeutic development in FD, and we demonstrate this concept here.

BGP-15 and expression of C-terminal IKAP both restore neuronal survival upon loss of Ikap in TrkA+ *Ikbkap*^{-/-} sensory neurons. BGP-15 is able to restore aspects of mitochondrial function, improve actin cytoskeletal function and cardiac innervation, decrease pJNK signaling, and increase survival both *in vitro* and *in vivo* of *Ikbkap*^{-/-} TrkA+ DRG neurons. Expression of C-terminal IKAP and full length Ikap promote FD patient fibroblasts to proliferate. Delivery of the genes encoding these protein fragments can also restore survival *in vitro* of *Ikbkap*^{-/-} TrkA+ DRG neurons. As current pharmacological treatments have targeted primarily the symptoms of FD and the aberrant splicing of FD, we provide data that suggest new potential targets for treatment of the disorder.

Future Studies

These data now uncover opportunity for further investigations into potential targeted therapies. Such targets may include those that affect the stress pathways that are activated in absence of Ikap. These data also suggest future experiments to be executed *in vivo* to increase *Ikbkap*^{-/-} neuronal survival.

First, our data here suggest that a major bioenergetic component contributes to the pathophysiology of FD and indicate that other metabolic pathways could be impaired, which has not yet been demonstrated. Determining the extent of changes to mitochondrial function in absence of Ikap could benefit FD patients, as compounds are in development that target specific mitochondrial disruptions (9). *Thus, (1) to what extent does mitochondrial dysfunction directly contribute to neuronal death in FD, and (2) which*

other mitochondrial impairments could be targeted with therapeutics? It could first be determined whether mitochondrial dysfunction is an independent disruption that occurs in absence of Ikap and Elongator or, with the accumulated support for Elongator function in translation and modification of tRNA (10-12), is a consequence of inefficient translation. The latter could be determined with the expression of modified specific tRNA species, as previously demonstrated in yeast, where specific tRNA delivery corrected altered cell pathways that occur in absence of Elongator (12). This could reveal whether translational impairments are responsible for the mitochondrial dysfunction and also could provide an alternate approach to targeted therapy (12). Second, the mitochondrial impairments we discovered in our FD model could implicate diverse mitochondrial pathways. Though it may be difficult to determine which mitochondrial events are causal and which are the downstream consequences of other mitochondrial disruptions, targeting any of these events could potentially promote mitochondrial and cellular function (9, 13). For example, *Ikbkap*^{-/-} TrkA⁺ neurons are depolarized, perhaps from disruption at the mitochondrial respiratory chain (e.g., of specific enzyme complexes) (14, 15). ROS are also elevated in these neurons, which could be a byproduct of dysfunctional enzyme activity of the respiratory chain or as a result of an impaired antioxidant system, which normally eliminates the highly reactive ROS (16). Mitochondria are also fragmented in TrkA⁺ *Ikbkap*^{-/-} neurons, which could indicate disrupted mitochondrial dynamics from impairment in fission and/or fusion machinery. Thus, there are many changes in mitochondrial function in *Ikbkap*^{-/-} neurons. Further characterizing these events could reveal additional therapeutic targets. For example, several enzymes or compounds exist

that can increase mitochondrial output by targeting upstream sensors of mitochondrial metabolism (e.g., IGF1, mTOR, AMPK, and the sirtuin family of deacetylases) and also downstream events affecting transcription involved in energy homeostasis (9, 13). Compounds that promote or inhibit mitochondrial fusion machinery (e.g., MFN1, MFN2 and OPA1) and fission machinery (e.g., DRP1 and FIS1) are also being tested for improving mitochondrial function (9, 13). Additionally, if mitophagy or mitochondrial biogenesis are also impaired, which has been demonstrated to occur in Alzheimer's and Parkinson's disease for example, drugs that target these events could also help FD patients. Changes in diet and antioxidant supplements are alternative options (13). Mitochondrial events are increasingly demonstrated to be involved in diverse disorders, including major neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's diseases, and ALS (3), and therefore focusing on these impacted pathways could benefit FD patients.

Second, we demonstrate that the small compound BGP-15 can correct aspects of the mitochondrial impairment along with actin function and can restore neuronal survival of *Ikbkap*^{-/-} neurons. *Thus, in what other manners is BGP-15 functioning to promote neuronal survival, and can we target these pathways with other compounds? Could BGP-15 be used in concert with other identified compounds?* BGP-15 has diverse protective mechanisms including stabilization of mitochondrial function (17-23), but additionally and beyond our study here, has been previously reported to reduce levels of PARP-1, poly (ADP-ribose) polymerase-1, an enzyme involved in DNA repair (17-19, 24). In pathological conditions, over-activation of PARP-1 can lead to depletion of a cell's

energy, while also altering mitochondrial function and gene regulation leading to cell death (25). PARP-1 can be triggered from ROS and the consequent DNA damage. In addition to elevated ROS in our model here, our laboratory has recently demonstrated an increase in DNA damage in our *Ikbkap*^{-/-} DRG neurons (unpublished data). Does BGP-15 function to reduce *Ikbkap*^{-/-} death by also reducing levels of PARP-1 in our mutant embryos, and can we use other PARP-1 inhibitors to also restore neuronal function in our model? PARP-1 inhibitors have been demonstrated to provide protection in models of neurodegenerative disease (25, 26). Thus, treatment with a Parp-1 inhibitor or knockdown of the enzyme (i.e., via a lenti or adeno virus encoding shPARP-1 (27)) could provide benefit. Additionally, we also show that BGP-15 reduces levels of pJNK. Inhibitors of pJNK stress signaling have been demonstrated to reduce apoptosis in animal models and humans (28, 29), indicating other possible compounds to be tested in our FD model, though JNK inhibitors may struggle with specificity (29). Lastly, as previously mentioned, phosphatidylserine has been used to increase levels of *IKBKAP* in FD cells (30). This compound can also increase acetylation of α -tubulin, which is reduced in models of FD (31, 32), as well as increase axonal outgrowth in an FD mouse model (32). As we show that BGP-15 did not improve the stunted outgrowth of *Ikbkap*^{-/-} neurons, perhaps phosphatidylserine, and/or other previously mentioned compounds, could be used together with BGP-15 as a therapeutic cocktail.

Third, we show that mitochondrial stress contributes to death of neurons, which leads us to question which other stress pathways are activated in absence of *Ikap* and whether they could also be targeted. Elongator is now well supported to aid in translation

(10, 12, 33), and deletion of Elongator subunit Elp3 in a mouse transgenic model was demonstrated to lead to impaired translation and endoplasmic reticulum (ER) stress (34). As the ER communicates extensively with mitochondria biochemically and also directly through mitochondria-associated ER membranes (MAMs) (3), the ER could also be disrupted upon loss of Ikap. *Thus, does ER stress contribute to neuronal death in FD, and can we target ER stress with therapeutics?* The ER is essential for maintaining calcium homeostasis and is the site of both protein and lipid synthesis (35). Dysfunction in these processes can result in ER stress and lead to cell death, particularly stemming from protein aggregation triggering a constitutently active unfolded protein response (UPR), and is a common emerging theme to major neurodegenerative diseases including Alzheimer's, Huntington's, and Parkinson's diseases and ALS, as well as the prion diseases (3, 36, 37). Targeting and reducing the UPR is currently being investigated for the treatment of neurodegeneration (36, 37). Therefore, identifying involvement and the specific players of ER stress in the death of *Ikbkap*^{-/-} neurons could benefit FD patients.

Fourth, we demonstrated C-terminal IKAP can significantly increase survival of *Ikbkap*^{-/-} neurons *in vitro*. FD, which is marked by loss of sensory and autonomic neurons of the peripheral nervous system, is also characterized by adult onset blindness attributed to the progressive death of retinal ganglion cells (38-40). Targeting this neuronal population is of interest as it is more accessible and could be targeted via intravitreal injection (41). Adeno viruses infect RGCs well (42), however, the gene length, their cargo, is limited when compared to other viral vectors (43). Thus, as we demonstrated that both the full length and C-terminal human IKAP can rescue *Ikbkap*^{-/-} neurons, can

human *IKBKAP3*, which encodes the C-terminal half, be delivered in vivo via adenovirus to prevent further loss of RGCs? Our FD mouse is a perfect model in which to test this question for its potential in FD patients.

Final Remarks

Though few new cases of FD emerge each year, FD patients are currently living into their 40s. Therefore, targeting the cellular and molecular consequences that occur from a loss of the widely-expressed protein IKAP is significant. Additionally, the role of the Elongator complex, to which IKAP/ELP1 belongs, is emerging as an essential complex in neurological function. For example, Elongator subunits Elp2-4 have also been associated with neurological disorders: *ELP2* with intellectual impairment (44, 45), *ELP3* with ALS (46), and *ELP4* with epilepsy (47). Additionally, loss of Elongator subunits *ELP1* and/or *ELP3* result in cell stress (34, 38-40), as supported by these data here. We demonstrate that reducing mitochondrial stress that occurs in absence of Ikap/Elp1 with the compound BGP-15 coincides with an increase in neuronal survival in a transgenic mouse model, most likely attributed to blocking pathways leading to apoptosis (8). Identification of this bioenergetic interruption puts FD in category with other major neurodegenerative disorders marked by mitochondrial dysfunction (3). These data also demonstrate that delivery C-terminal IKAP in *Ikbkap* deficient cells and neurons is sufficient to promote cell proliferation and increase neuronal survival. Thus, here we have determined two avenues of therapeutic intervention that could potentially help FD patients and others with mitochondrial and/or Elongator dysfunction.

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