Familial dysautonomia model reveals *Ikbkap* deletion causes apoptosis of Pax3$^+$ progenitors and peripheral neurons

Lynn George[a,b,1], Marta Chaverra[a,1], Lindsey Wolfe[a], Julian Thorne[a], Mattheson Close-Davis[a], Amy Eibs[a], Vickie Riojas[a], Andrea Grindeland[c], Miranda Orr[c], George A. Carlson[c], and Frances Lefcort[a,2]

[a]Department of Cell Biology and Neuroscience, Montana State University, Bozeman, MT 59717; [b]Department of Biological and Physical Sciences, Montana State University Billings, Billings, MT 59101; and [c]McLaughlin Research Institute, Great Falls, MT 59405

Edited by Qiufu Ma, Dana-Farber Cancer Institute, Boston, MA, and accepted by the Editorial Board October 7, 2013 (received for review May 8, 2013)

Familial dysautonomia (FD) is a devastating developmental and progressive peripheral neuropathy caused by a mutation in the gene inhibitor of kappa B kinase complex-associated protein (*IKBKAP*). To identify the cellular and molecular mechanisms that cause FD, we generated mice in which *Ikbkap* expression is ablated in the peripheral nervous system and identify the steps in peripheral nervous system development that are *Ikbkap*-dependent. We show that *Ikbkap* is not required for trunk neural crest migration or pathfinding, nor for the formation of dorsal root or sympathetic ganglia, or the adrenal medulla. Instead, *Ikbkap* is essential for the second wave of neurogenesis during which the majority of tropomyosin-related kinase A (TrkA$^+$) nociceptors and thermoreceptors arise. In its absence, approximately half the normal complement of TrkA$^+$ neurons are lost, which we show is partly due to p53-mediated premature differentiation and death of mitotically-active progenitors that express the paired-box gene *Pax3* and give rise to the majority of TrkA$^+$ neurons. By the end of sensory development, the number of TrkC neurons is significantly increased, which may result from an increase in Runx3$^+$ cells. Furthermore, our data demonstrate that TrkA$^+$ (but not TrkC$^+$) sensory and sympathetic neurons undergo exacerbated Caspase 3-mediated programmed cell death in the absence of *Ikbkap* and that this death is not due to a reduction in nerve growth factor synthesis. In summary, these data suggest that FD does not result from a failure in trunk neural crest migration, but rather from a critical function for *Ikbkap* in TrkA progenitors and TrkA$^+$ neurons.

Hereditary sensory and autonomic neuropathies (HSANs) are a group of five phenotypically diverse but overlapping disorders of the peripheral nervous system (PNS) that result from mutations in 12 distinct genes (1). HSAN type 3, or familial dysautonomia (FD) (also called Riley–Day syndrome), results from an intronic mutation (IVS20 + 6T > C; 99.5% of patients) in a gene called inhibitor of kappa B kinase complex-associated protein or *IKBKAP*, causing mis-splicing and subsequent tissue-specific reductions in IKAP protein (2, 3). FD is marked by tachycardia, blood pressure lability, autonomic vomiting "crises," decreased pain and temperature sensation, and commonly death during early adulthood (4). The function of IKAP in the nervous system is unclear, nor is it understood why deletions in this broadly expressed gene primarily devastate the PNS. The earliest pathology study, performed on a 2-y-old child with FD, showed that ~90% of cells in the dorsal root and sympathetic ganglia (SG) were missing (5). To identify IKAP's function in the developing PNS, we first need to establish the steps in which it is essential.

The vertebrate PNS derives primarily from the neural crest, a multipotent, heterogeneous cell population that delaminates from the neural tube and migrates throughout the embryo (6). Those neural crest cells that stop laterally to the neural tube give rise to the chain of sensory dorsal root ganglia (DRG), whereas those that migrate further ventrally give rise to the vertebral chain of SG. Within the DRG, neural crest cells generate heterogeneous neuronal subpopulations including nociceptors, thermoreceptors, mechanoreceptors and proprioceptors. With the completion of neural crest migration, multiple steps ensue that are essential for normal PNS development, including proliferation of discrete sets of neuronal progenitor cells that derive from different waves of migrating neural crest cells, neuronal differentiation, axonogenesis, target innervation, and circuit formation. FD could theoretically result from failure in any or several of these key developmental processes.

Insight into the mechanisms that cause FD have been complicated by data that implicate functions for IKAP in both the nucleus and the cytoplasm. In yeast, the IKAP homolog, Elp1, serves as a scaffold protein within the multisubunit Elongator complex that binds RNA polymerase II and facilitates transcription via histone acetylation (7–9). Although studies indicate that Elongator also functions in the cytoplasm to acetylate α-tubulin (10, 11), recent findings suggest that Elongator may regulate tubulin acetylation indirectly through tRNA modification (12–15). Independent of its role in the Elongator complex, cytosolic IKAP has also been shown to regulate actin cytoskeletal organization and cell migration, which has prompted the suggestion that FD results from a failure in neural crest cell migration (16–19). Mice that are completely null for *Ikbkap* die early in embryogenesis [by embryonic day (E) 10.5] with failure in neurulation and vasculogenesis, precluding their usefulness for analyzing the aspects of PNS development that are most impacted in FD (20, 21). Although a mouse hypomorphic *Ikbkap* model was recently generated that recapitulates many of the phenotypic

Significance

Familial dysautonomia (FD) is a devastating developmental peripheral autonomic and sensory neuropathy caused by a mutation in the gene inhibitor of kappa B kinase complex-associated protein (*IKBKAP*). It is marked by tachycardia, blood pressure lability, autonomic vomiting "crises," and decreased pain and temperature sensation. FD is progressive, and affected individuals commonly die during early adulthood. To identify the cellular and molecular mechanisms that cause FD, we generated a mouse model for the disease in which *Ikbkap* expression is ablated in the neural crest lineage. This study is a mechanistic analysis of the cellular events that go awry in the developing peripheral nervous system in FD and identifies essential functions of IKAP protein in the peripheral nervous system.

Author contributions: L.G., M.C., M.O., G.A.C., and F.L. designed research; L.G., M.C., L.W., J.T., M.C.-D., A.E., V.R., and A.G. performed research; L.G. and G.A.C. contributed new reagents/analytic tools; L.G., M.C., L.W., G.A.C., and F.L. analyzed data; and L.G. and F.L. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. Q.M. is a guest editor invited by the Editorial Board.

[1]L.G. and M.C. contributed equally to this study.

[2]To whom correspondence should be addressed. E-mail: lefcort@montana.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1308596110/-/DCSupplemental.

hallmarks of FD (22), the data presented here comprise a mechanistic analysis of the molecular and cellular events that go awry during sensory neurogenesis in the absence of *Ikbkap*.

Results

Generation of a Neural Crest-Specific Mouse Model for FD. To determine the steps in PNS development that fail in FD, we used a *Wnt1-Cre* transgene to generate mice in which *Ikbkap* is selectively deleted in the neural crest lineage and in discrete regions of the CNS (Fig. 1 *A* and *B*) (23). Mice homozygous for a floxed allele of *Ikbkap* were crossed to mice heterozygous for both the floxed *Ikbkap* allele and for a *Wnt1-Cre* transgene (Fig. S1). *Ikbkap* conditional knockout (CKO) embryos, which expressed *Wnt1-Cre* and were homozygous for the $Ikbkap^{LoxP}$ allele (*Wnt1-Cre;*$Ikbkap^{LoxP/LoxP}$), were generated at the expected 3-to-1 Mendelian ratio (255 expressing *Ikbkap* to 83 CKO; $P > 0.95$), as determined by PCR-based genotyping (Fig. S1 and *SI Materials and Methods*). Decreased expression of IKAP protein in CKO embryos was confirmed via Western blot and immunostaining for IKAP in the DRG (Fig. 1 *C–E*). Although CKO pups were born alive, they died within 24 h of birth. This early death was not attributable to an inability to suckle as they were often found with milk in their stomachs. Gross analysis of pups at E18.5, 1 d before birth, revealed an overtly normal development, although the *Ikbkap* CKO pups were smaller than their littermates and had a noticeably altered facial morphology (Fig. S2 *A* and *B*). Children with FD can exhibit retrognathism of the mandible and a resulting reduced inferior facial angle (24). Because the $Wnt1^{+}$ cranial crest orchestrates and contributes to much of the cranial facial morphology (23), we measured the inferior facial angle and the position of the mandible relative to the most anterior point on the face (25) (Fig. S2 *C* and *D*). Both of these indices demonstrate that CKO pups exhibit significant retrognathism of the mandible compared with their control littermates.

To determine whether ablating *Ikbkap* in the *Wnt1* lineage was sufficient to recapitulate the neuronal hallmarks of FD, we quantified neuronal numbers in the sympathetic and parasympathetic ganglia and DRG using a battery of markers in E17.5 embryos (Fig. 2 and Table S1). We found that the number of tyrosine hydroxylase $(TH)^{+}$ neurons in the superior cervical ganglion (SCG) was reduced by nearly 70% in *Ikbkap* CKO embryos compared with controls (Fig. 2 *A–C*). The total number of DRG neurons in CKO mice was also reduced by one-third compared with controls (Fig. 2 *D* and *E* and Table S1). To determine whether programmed cell death contributed to this reduction in neuronal number, we quantified the number of cleaved-Caspase 3^{+} cells in both the SCG [wild type (WT), 6.8 ± 2.20; CKO, 13.3 ± 3.70; $P < 0.001$] and DRG (Table S1) at E17.5 and found a significantly increased number of apoptotic cells in both ganglia types in CKO embryos relative to controls.

We next determined whether neuronal deficits in *Ikbkap* CKO mice were specific to particular subpopulations of DRG neurons, because a hallmark of FD is decreased pain and temperature sensation. Pain- and temperature-receptive neurons express the neurotrophin receptor TrkA, whereas proprioceptors and many mechanoreceptors express TrkC (26, 27). Our data demonstrate that the TrkA subpopulation was reduced by one half at E17.5 (Fig. 2 *F* and *G* and Table S1). Nociceptors synthesize and release the neuropeptide substance P and autopsy studies on FD patients show a severe reduction in substance P staining in the spinal cord dorsal horn (28). To further determine the fate of the nociceptive subpopulation of DRG neurons in the absence of *Ikbkap*, substance P expression was examined and found to be virtually absent in the *Ikbkap* CKO DRG (Table S1 and Fig. 2 *H* and *I*). TH is transiently expressed in the embryonic DRG, possibly by future $VGLUT2^{+}$ thermal pain receptors, and in adult cutaneous low-threshold mechanoreceptors (29–32). This DRG subpopulation was also nearly nonexistent in the mutant DRG (Fig. S3). In contrast, not only was there no reduction in TrkC numbers, there was actually a small, but significant, increase in this subpopulation (Fig. 2 *J* and *K* and Table S1). FD is characterized by a decrease in deep tendon reflexes and a reduction in muscle spindles in FD patients has been reported (33). Because the nervous system progressively degenerates in FD, our data showing that proprioceptors arise and differentiate normally indicate that their eventual demise could potentially be thwarted with the appropriate therapeutics.

The substantia gelatinosa is reduced in FD patient pathology studies (28). Consistent with this phenotype, the central projections of $TrkA^{+}$ DRG neurons were considerably reduced in the dorsal horn of the spinal cord in mutant embryos (Fig. S4). $TrkA^{+}$ fibers did innervate the skin in the CKO at E17.5, albeit they were less prevalent than in their control littermates (Fig. S4 *C* and *D*). Parasympathetic cell bodies were reduced by 31% in the CKO submandibular gland (SMG) compared with controls (Fig. S5). Interestingly, we discovered that whereas in the control SMG, parasympathetic neuronal cell bodies were heavily innervated by TH^{+} sympathetic terminals (Fig. S5 *A–C* and *H–J*), TH^{+} axons were rarely present in mutant SMG (Fig. S5 *E* and *F*). This absence in TH innervation of the salivary glands may explain the impaired swallowing experienced by children with FD. Together, these data demonstrate that deletion of *Ikbkap* in the neural crest lineage is sufficient to recapitulate the major hallmarks of FD: severe reduction in DRG sensory neurons that detect pain and temperature and in sympathetic and parasympathetic neurons that are critical for homeostasis.

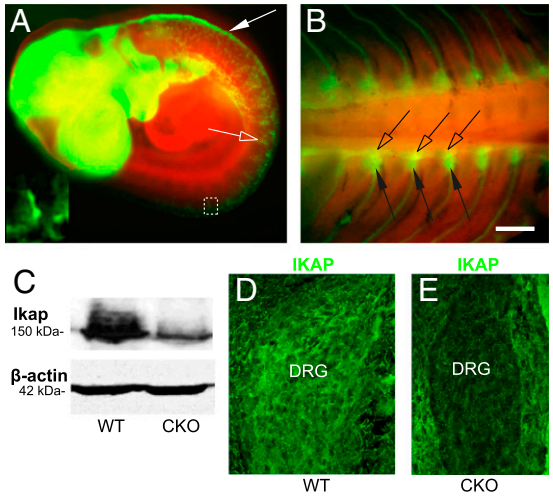

Fig. 1. *Ikbkap* CKO mice. (*A* and *B*) $ROSA^{mT\text{-}mG}$; *Wnt1-Cre* embryos. Red cells convert to green following Cre recombination. (*A*) E9.5. Cre is active in the dorsal neural tube (arrow) and neural crest cells (open arrow and *Inset*). (*B*) E14.5. Cre is active in the SG (open arrows) and DRG (arrows). (*C*) CKO neural tubes exhibit reduced expression of IKAP, but normal levels of β-actin via Western blot. Because *Wnt1* is only expressed in the dorsal neural tube, some residual IKAP is still present in the CKO neural tube. (*D* and *E*) E11.5. IKAP protein is not expressed in CKO DRG but is still expressed in surrounding somite. (Scale bar: *A*, *Inset*, *D*, and *E*, 20 μm; *B*, 400 μm.)

***Ikbkap* Is Expressed Throughout Neurogenesis in both Progenitors and Neurons.** Although apoptosis was elevated in the DRG and SCG of CKO mice just before birth, we sought to determine whether disruption in a putative earlier *Ikbkap* function might also contribute to the neuronal deficit observed at birth. To gain insight into the developmental events that might require *Ikbkap*, we used an *Ikbkap:LacZ* reporter mouse and, here, present a developmental analysis *of Ikbkap* expression in mammalian tissue (Fig. 3). Although strongly expressed in the neural tube at the earliest ages examined, E8.5 to E9.0 (Fig. 3*A*), β-galactosidase (β-gal) was not prominently expressed in migrating trunk neural crest cells. By E11.5, the *Ikbkap:LacZ* cassette was clearly active in sympathetic neurons and DRG, in addition to being expressed in motor neurons, dorsal interneurons, and the dorsal ventricular

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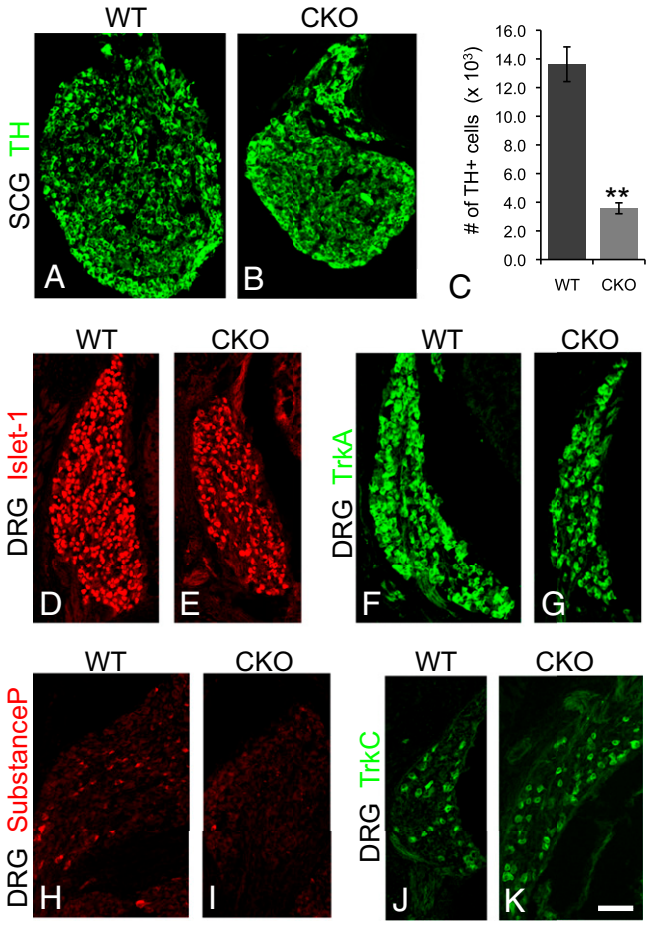

Fig. 2. Reduced numbers of sympathetic and DRG neurons in CKO embryos. (*A–K*) E17.5. Neuron numbers are significantly reduced in the SCG (*A–C*) and DRG (*D* and *E*) (Table S1) in CKO embryos compared with controls. (*F–K*) TrkA$^+$ and substance P$^+$ DRG neurons are depleted in CKO embryos (*G* and *I*, respectively), compared with controls (*F* and *H*), whereas the number of TrkC$^+$ DRG neurons is slightly increased in mutant embryos (*J* and *K*). (Scale bar: 40 μm.) $^{**}P < 0.01$.

zone of the spinal cord (Fig. 3*B*). Neurogenesis in the DRG occurs between E9.5 to E13.5 (34, 35) and comprises two overlapping waves, with the majority of TrkC$^+$ neurons born during the first wave (E9.5 to E10.5) and the majority of TrkA$^+$ neurons born during the second wave (E10.5 to E13.5) (36). During this time frame, the DRG is composed of mitotically active progenitor cells that comprise the DRG dorsal pole and DRG perimeter, with nascent neurons localized in the inner core (37, 38). *Ikbkap:LacZ* reporter embryos demonstrate that *Ikbkap* is expressed in both the progenitor and neuronal zones (Fig. 3*C*) and maintained in neurons through birth (Fig. 3*D*). To confirm IKAP protein expression in progenitor cells, we also immunostained E11.5 embryonic sections with antibodies to IKAP and to Pax3, a marker for progenitor cells that give rise to TrkA$^+$ neurons (38). IKAP protein was strongly expressed in both Pax3$^+$ progenitors and in postmitotic neurons (Fig. 3*E*). Thus, deletion of *Ikbkap* could potentially disrupt the development of either progenitors or postmitotic neurons or both.

Although we did not observe prominent *Ikbkap:LacZ* reporter activity in trunk neural crest, several reports have suggested that impaired neural crest cell (NCC) migration could underlie the neuronal phenotype of FD patients (17–19). To determine whether *Ikbkap* is indeed required at this stage, we examined CKO embryos at E9.5 and E10.5 and found that trunk NCCs migrated along their stereotypical ventral pathways and formed sympathetic ganglia, DRG, and adrenal medulla in their normal locations (Fig. 3 *F–K*). To verify the location and timing of Cre expression, as well as its activity, we analyzed E9 *Wnt-Cre*; *ROSA*$^{mT\text{-}mG}$ embryos and saw robust Cre activity in migrating NCCs (Fig. 1*A*, *Inset*), indicating that *Ikbkap* was likely deleted in NCCs in CKO embryos despite their normal behavior. Together, our data demonstrate that IKAP is not required in mice for trunk NCC migration, pathfinding, nor for the cessation of migration to form PNS derivatives in their stereotyped locations, consistent with our previous findings in chick PNS development (39).

***Ikbkap* Is Required for the Generation of TrkA$^+$ Neurons.** Given our expression analysis demonstrating that *Ikbkap* is expressed throughout sensory neurogenesis and our finding that DRG neuron numbers

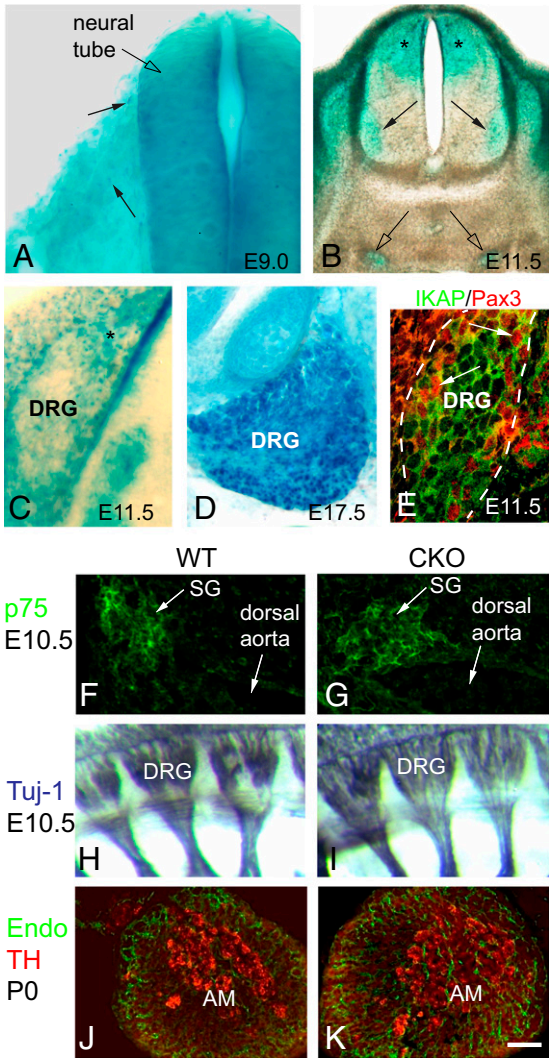

Fig. 3. *Ikbkap* expression in the developing PNS. (*A–D*) *Ikbkap:LacZ* reporter embryos. (*A*) E9.0. β-Gal is robustly expressed in the neural tube but minimally expressed in migrating NCCs. (*B* and *C*) At E11.5, *Ikbkap* is expressed in the dorsal half of the spinal cord including the ventricular zone (asterisks in *B*), in motor neurons (arrows in *B*), in the SG (open arrows in *B*), and in the DRG. (*C*) At E11.5, β-gal is expressed in the DRG dorsal pole (asterisk) and in the core neural zone. (*D*) At E17.5, *Ikbkap* is expressed in mature neurons. (*E*) IKAP protein is expressed in both the dorsal pole and perimeter in Pax3$^+$ TrkA progenitors (arrows) and in neurons in the neural core. (*F–K*) NCC migration and patterning in the CKO is comparable to WT with normal formation of SG (*F* and *G*), DRG (*H* and *I*), and chromaffin cells in the adrenal medulla (*J* and *K*). (Scale bar: *A*, *C–G*, *J*, and *K*, 30 μm; *B*, 100 μm; *H* and *I*, 75 μm.)

were reduced in its absence, we quantified neuronal number through both waves of neurogenesis. At E10.5, there was a significant decrease in the total number of DRG neurons in conjunction with a significant decrease in TrkA$^+$ neurons but not in TrkC$^+$ neurons (Fig. 4 *A–E* and Table S2). Interestingly, this deficit completely recovered within 24 h such that by E11.5, there was no significant difference between control and CKO DRG in either the total number of neurons or in the TrkA$^+$ number (Fig. 4 *A* and *F–I* and Table S2). This recovery is reminiscent of the compensation that occurs in *NT-3* knockout mice (35, 40) and is most parsimoniously explained by premature differentiation of TrkA progenitors into TrkA$^+$ neurons. During normal DRG development, peak neuronal numbers are achieved by E12.5 to E13 (35), corresponding to the end of the second wave of neurogenesis (Fig. 4*A*). However, at E12.5, the DRG of *Ikbkap* CKO embryos contained 30% fewer total neurons and 30% fewer TrkA$^+$ neurons compared with control DRG (Fig. 4*A* and Table S2). Together, these findings indicate that generation of TrkC$^+$ neurons during the first wave of neurogenesis does not require *Ikbkap* because mutant mice have a normal number of TrkC$^+$ neurons from E10.5 to E12.5. The generation of TrkA neurons, however, is critically dependent on *Ikbkap* because in its absence, the full complement of TrkA$^+$ neurons is never obtained. Exacerbating the final deficit in TrkA numbers is the fact that the extent of apoptosis in CKO embryos far exceeds that in controls.

The transcription factor Runx3 is required for the maintenance of TrkC expression in the DRG (41). Ectopic overexpression of Runx3 causes an increase in the number of TrkC neurons (42), whereas its deletion results in a TrkC$^+$ neuronal deficit (42, 43). Given that there was a slight but significant increase in the number of TrkC$^+$ neurons in *Ikbkap* CKO embryos at E17.5, we asked whether that increase was preceded by an increase in the number of cells expressing Runx3. In fact, we found a significant increase in the number of Runx3$^+$ cells in the CKO DRG at E11.5 compared with littermate controls (Fig. S6 and Table S2).

Second-Wave Pax3$^+$, TrkA Progenitors Require *Ikbkap*. We next sought to determine why *Ikbkap* DRG fail to generate the normal complement of TrkA$^+$ sensory neurons. Given our finding that *Ikbkap* is expressed in DRG progenitors (E11.5; Fig. 3 *C* and *E*), we tracked the proliferation and death of progenitors throughout neurogenesis (Fig. 5 *A–F*). At E10.5, we found a small but significant reduction in the number of mitotically active phosphorylated histone H3$^+$ (pH3) progenitors in CKO DRG ($P = 0.014$) and a 30% decrease at E11.5 relative to controls ($P < 0.0001$) (Fig. 5*A*). Furthermore, analysis of the data demonstrates that the 25% increase in the number of cycling progenitors that normally occurs in the DRG between E10.5 and E11.5 does not occur in the absence of *Ikbkap*. Given that TrkA$^+$ neurons are born last, these data suggest that one function of *Ikbkap* is to maintain the survival and/or proliferation of second-wave DRG progenitors; in its absence, TrkA progenitors prematurely differentiate between E10.5 and E11.5 to cause an abnormal increase in TrkA numbers at E11.5, followed by a steep decline because of the failure of TrkA progenitors to remain in the cell cycle.

We previously demonstrated that the majority of TrkA$^+$ sensory afferents in chick derive from a specific subset of second-wave progenitors that express Pax3 and colonize the DRG dorsal pole and perimeter (38). Analysis of Pax3 expression in mouse demonstrates that these same geographically distinct progenitor zones exist in mammals (Fig. 5*B*). Because it is the TrkA$^+$ pool that is specifically compromised in both FD and in our mouse model, we determined the fate of Pax3$^+$ progenitor cells in *Ikbkap* CKO embryos. Quantification at E11.5 shows that the number of these progenitors was reduced by 25% in the CKO DRG relative to controls ($P < 0.0001$; Fig. 5*C*). To determine whether the reduced number of cycling progenitors and Pax3$^+$ cells indicated a role for *Ikbkap* in progenitor cell survival, we quantified the number of apoptotic cells throughout neurogenesis in the DRG. Although there was no significant difference

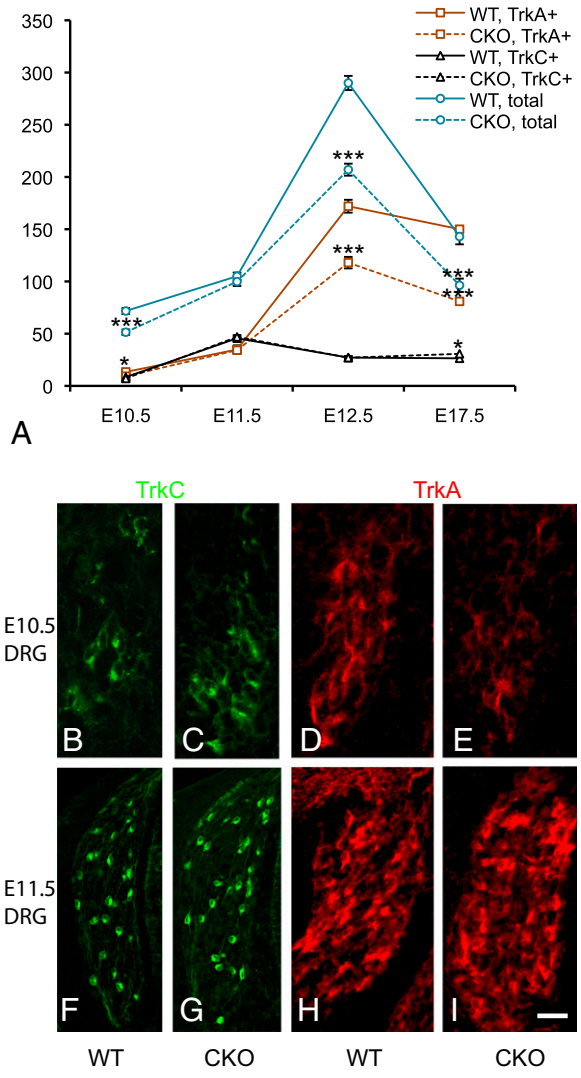

Fig. 4. *Ikbkap* is required for the generation of TrkA$^+$ neurons, but not TrkC neurons, in the DRG. (*A–I*) Quantification of total neurons, TrkC$^+$ neurons, and TrkA$^+$ neurons from E10.5 to E17.5. (*B*, *C*, *F*, and *G*) Immunostaining for TrkC at E10.5 (*B* and *C*) and E11.5 (*F* and *G*) shows no difference between mutant and control. (*D*, *E*, *H*, and *I*) Although TrkA numbers are reduced in the mutant at E10.5 (*D* and *E*), they recover by E11.5 (*H* and *I*). (Scale bar: 40 μm.) *$P < 0.05$; ***$P < 0.001$.

between CKO and control embryos at E10.5, by E11.5, there was significantly more programmed cell death in the DRG of *Ikbkap* CKO embryos compared with littermate controls (Fig. 5*D*). Our data showing equivalent neuronal numbers in mutant and control embryos at this same time point (E11.5), when the DRG is composed exclusively of mitotically active progenitor cells and of postmitotic neurons (34, 35), suggest that the dying cells were progenitors. In further support of a role for *Ikbkap* in the survival of neuronal progenitors, the distribution of apoptotic cells in the CKO DRG was concentrated in the dorsal pole and perimeter (Fig. 5*E*), zones densely colonized by Pax3$^+$ cells (Fig. 5*B*). Together, these data indicate that *Ikbkap* is required for survival of the mitotically active progenitor population that generates TrkA$^+$ nociceptors and thermoreceptors during the second wave of neurogenesis in the DRG. In addition, quantification of apoptotic cells at E12.5 and E17.5 demonstrates elevated levels of cell death in the CKO DRG throughout the rest of embryonic development (Table S2).

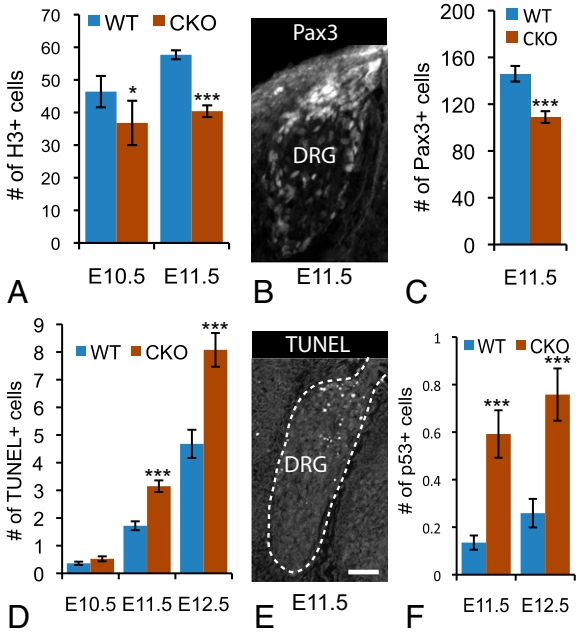

Fig. 5. *Ikbkap* is required for the second wave of neurogenesis in the DRG. (*A*) The number of $H3^+$ progenitors is reduced in CKO embryos at E10.5 and E11.5. (*B* and *C*) E11.5. $Pax3^+$ progenitors colonize the dorsal pole and perimeter of the murine DRG (*B*) and are depleted in *Ikbkap* CKO embryos (*C*). (*D* and *E*) The number of apoptotic cells is consistently higher in mutant embryos compared with controls (*D*), with $TUNEL^+$ cells concentrated in the DRG progenitor zones (*E*). (*F*) Compared with controls, the number of $p53^+$ cells is also dramatically higher in *Ikbkap* CKO embryos. (Scale bar: 40 μm.) $^*P < 0.05$; $^{***}P < 0.001$.

Nerve growth factor (NGF) is a critical target-derived survival factor for $TrkA^+$ sensory and sympathetic neurons (26, 27, 44). To test whether sympathetic neurons die because of a reduction in NGF synthesis, we compared NGF mRNA levels in two sympathetic targets, the heart and the submandibular glands. These experiments show that rather than being reduced, NGF levels were actually elevated in the *Ikbkap* CKO mouse (Fig. S7).

We next sought to determine why *Ikbkap* deletion leads to the apoptosis of mitotically active, $Pax3^+$ progenitors. Because Pax3 is known to destabilize the p53-mediated apoptosis pathway (45, 46), we determined whether p53 levels were altered in CKO embryos. In fact, we found that the number of $p53^+$ cells was increased fourfold in the DRG of CKO versus control embryos from E11.5 to E12.5 (Fig. 5*F* and Fig. S8). We also found a significant increase in $p53^+$ cells in the SG during this same timeframe (Fig. S8).

Discussion

In summary, these studies indicate that *Ikbkap* exerts pleiotropic effects in the developing PNS, including a critical function in neurogenesis and neuronal survival. The data reported here demonstrate that although *Ikbkap* is broadly expressed within the embryo, its ablation in the PNS is sufficient to generate the classic hallmarks of FD: devastation of the sympathetic and sensory nervous systems. These data also establish that this loss is not attributable to abrogation of trunk neural crest migration but, rather, to failure of DRG progenitor cells to generate the full complement of pain and temperature receptors, in addition to premature death of sensory and sympathetic progenitors and neurons. We show here that *Ikbkap* is expressed in both DRG progenitors, including $Pax3^+$ TrkA progenitors, and in postmitotic neurons. Our data indicate that in the absence of *Ikbkap*, second-wave progenitors exit the cell cycle prematurely and either differentiate precociously into neurons (including, aberrantly, $Runx3^+$ neurons) or die, leaving fewer progenitors available to generate the complete set of $TrkA^+$ nociceptors and thermoreceptors that would normally be obtained by E13. The fact that $Runx3^+$ cell numbers (a marker of first-wave neurons) increased at the same age when $Pax3^+$ progenitors were reduced (a progenitor of second-wave neurons) implicates the presence of a coordinated feedback system between the two waves of DRG neurogenesis, which was observed in the *Ngn1* and *Ngn2* knockouts (40).

How IKAP functions to maintain the survival of postmitotic neurons remains to be elucidated. Depletion of *IKBKAP* activates several proapoptotic p53-mediated genes in colon cancer cells (43), and we did find a significant elevation in p53 expression in the immature DRG and SG of CKO embryos. Given that the key neurons that undergo apoptosis in FD are NGF-dependent, it was of interest to discover that, rather than being reduced, NGF levels were actually elevated in target tissues of CKO mice. Interestingly, HSAN types 4 and 5 result from mutations in the TrkA gene, *NTRK1*, and NGFβ, respectively. In the absence of *Ikbkap*, neurons could be dying before, during, or after target innervation; if the latter, this could suggest a requirement for IKAP in target-derived retrograde transport of NGF. Disruption in axonal transport has been observed in mutation of Elp1 in *Caenorhabditis elegans* (11) and in HSAN type 1 and type 2 (1). We did find fewer $TrkA^+$ axons in target tissue, but additional studies will be required to determine whether this is attributable to the reduction in $TrkA^+$ neuronal cell bodies and/or a requirement for IKAP in target innervation.

Our data also indicate that IKAP is required for proliferation and survival of $Pax3^+$ progenitors. Given that acetylation of Pax3 regulates its ability to activate downstream targets, including *Hes1* and *Ngn2* (47), IKAP may play a role in Pax3 acetylation, either directly via Elongator-mediated acetylation or indirectly through Elongator-mediated tRNA modification. In support of a direct association between IKAP and Pax3, IKAP contains WD40 domains that have been shown in Gro proteins to interact with Pax and Runx family members (48, 49). Pax3, in turn, also directly associates with p53 and mediates its binding to the ubiquitin ligase Mdm2, triggering its degradation (46). Via this pathway, the genetic ablation of p53 rescues the apoptosis and neural tube defects that characterize Pax3 mutant *Splotch* embryos (46, 50). Another possible link between p53 and IKAP/Elongator is that p53 activity is also critically dependent on acetylation (51). Thus, multiple pathways point toward a role for IKAP in affecting the posttranslational modifications of one or more of these key proteins.

Although it has been posited that IKAP/Elp1 may be required for the Elongator subunit Elp3 to acetylate tubulin (10), we did not find any alteration in tubulin acetylation in our CKO mice. Pax3 mutations also cause Waardenburg syndrome type 1, which results from a failure in development of the cardiac outflow track and may explain the death of our CKO embryos perinatally. In summary, the *Ikbkap* CKO mouse model presented here provides an ideal system for identifying the molecular pathways that could be therapeutically targeted to thwart the developmental pathologies and progressive degeneration that marks FD and related HSANs.

Materials and Methods

Facial Morphology Measurements. The inferior facial angle was defined on a sagittal view (Fig. S2 *A* and *B*) by the crossing of two lines: (*i*) a reference line from the inferior border of the eye to the anterior tip of the nose (solid line in Fig. S2 *A* and *B*); and (*ii*) a line joining the anterior tip of the nose and the anterior border of the chin. The position of the mandible was determined by measuring the distance between two parallel lines along the anterior tip of the nose and the anterior tip of the chin (double ended arrows in Fig. S2 *A* and *B*). These lines were drawn perpendicular to the reference line described above (solid line in Fig. S2 *A* and *B*).

Mice. *Ikbkap* CKO mice were obtained from the International Mouse Consortium. For additional information on mice and other materials and methods, see *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Drs. Sylvia Arber, Louis Reichardt, and Eric Turner for the generous gift of antibodies; Drs. Steven Eiger and Lino Tessarollo for helpful discussion; and Dr. Ed Schmidt for the Rosa-EGFP/tomato mice and guidance. This work was supported by National Institutes of Health (NIH) Grants R01 35714 (to F.L.) and P01 NS041997 (to G.A.C.), NIH National Research Service Award F31 AG031630 (to M.O.), and the Dysautonomia Foundation (F.L.). In memory of Michael Kronick and Barbara Kronick.

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