Mutation in Archain 1, a Subunit of COPI Coatomer Complex, Causes Diluted Coat Color and Purkinje Cell Degeneration

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Abstract

Intracellular trafficking is critical for delivering molecules and organelles to their proper destinations to carry out normal cellular functions. Disruption of intracellular trafficking has been implicated in the pathogenesis of various neurodegenerative disorders. In addition, a number of genes involved in vesicle/organelle trafficking are also essential for pigmentation, and loss of those genes is often associated with mouse coat-color dilution and human hypopigmentary disorders. Hence, we postulated that screening for mouse mutants with both neurological defects and coat-color dilution will help identify additional factors associated with intracellular trafficking in neuronal cells. In this study, we characterized a mouse mutant with a unique N-ethyl-N-nitrosourea (ENU)–induced mutation, named nur17. nur17 mutant mice exhibit both coat-color dilution and ataxia due to Purkinje cell degeneration in the cerebellum. By positional cloning, we identified that the nur17 mouse carries a T-to-C missense mutation in archain 1 (Arcn1) gene which encodes the δ subunit of the coat protein I (COPI) complex required for intracellular trafficking. Consistent with this function, we found that intracellular trafficking is disrupted in nur17 melanocytes. Moreover, the nur17 mutation leads to common characteristics of neurodegenerative disorders such as abnormal protein accumulation, ER stress, and neurofibrillary tangles. Our study documents for the first time the physiological consequences of the impairment of the ARCN1 function in the whole animal and demonstrates a direct association between ARCN1 and neurodegeneration.

Introduction

Intracellular trafficking is critical for delivering molecules and organelles to their proper destinations to perform normal cellular functions [reviewed in [1]]. Proteins and lipids are transported to the target cellular compartments through vesicular trafficking pathways, and organelles are trafficked along microtubules and actin cytoskeleton. Impairment of intracellular trafficking has been implicated in the pathogenesis of various neurodegenerative disorders, such as Alzheimer’s disease [reviewed in [2,3]], Huntington disease [4–6] and Parkinson’s disease [7], indicating the importance of intracellular trafficking for proper function and maintenance of neuronal cells. However, factors that are involved in this process and their associations with the mechanisms causing neurodegeneration are not yet fully understood.

The molecular mechanisms of intracellular trafficking are shared by different cell types and organ systems. This is evident from the fact that multiple organ systems are affected in a number of human diseases caused by defects in intracellular trafficking. For example, Hermansky-Pudlak Syndrome (HPS), a collection of heterogeneous genetic disorders caused by defects in intracellular vesicle trafficking, is characterized by oculocutaneous albinism and defective platelet storage [8], suggesting that the affected protein participates in organelle trafficking in both melanocytes and neuronal cells [9]. The Myo5a mouse mutant, dilute, also exhibits coat color dilution and severe ataxic movement [10]. Studies on dilute mice demonstrated that MYOSA, an actin-based motor protein, participates in organelle trafficking in both melanocytes and neuronal cells [11–13]. Intracellular melanosome trafficking is disrupted in melanocytes of dilute mice, leading to coat color dilution [13]. Moreover, endoplasmic reticulum (ER) transport in cerebellar Purkinje cells (PCs) is disrupted in dilute mutants, which may result in neurological defects [11]. Mutations in Fig4 and Vac14, affecting the conversion of phosphatidylinositol-3-phosphate (PI3P) to the signaling lipid phosphatidylinositol-3,5-bispho-
Author Summary

The fact that mutations in single gene can result in defects in multiple organs suggests the existence of similar fundamental cellular mechanisms shared by different organs. Thus, genes that are important for a certain cellular function in a tissue of interest can be identified by searching for mutations that cause visible abnormalities indicative of such defect in other tissues. Here, we identified a gene essential for vesicle trafficking in neuronal cells by screening for mouse axtaxia mutants with diluted coat color, which often indicates defective intracellular trafficking in skin cells. This mouse mutant, neurological 17 (nur17), was generated by ENU mutagenesis. By positional cloning, we identified a mutation in the archain 1 gene encoding a component of the COPI complex, which is known to be involved in intracellular protein trafficking. The mutation in archain 1 gene results in syndromes commonly observed in neurodegenerative disorders such as abnormal protein accumulation, ER stress, and neurofibrillary tangles. nur17 mice provide a new model to study the association between intracellular trafficking and the mechanisms of neurodegenerative diseases.

sphate (Pl(3,5)P2), also lead to neurodegeneration and dilution pigmentation in mice [14,15]. Pl(3,5)P2 regulates vesicle trafficking in the endosome-lysosome axis in yeast [16], and abnormalities indicative of defects in the regulation of endosomal vesicles are observed in these mutant mice [14,15]. Pigment containing hair follicles are greatly reduced in the skin of Fig4 mutant mice, suggesting that lysosome-melanosome biogenesis may be affected [14]. Another mouse mutant with both pigmentary and neuro- logical defects is the sandy mouse with a mutation in a component of biogenesis of lysosome-related organelles complex 1 (BLOC-1), which regulates trafficking to lysosome-related organelles including melanosomes [17].

Based on the findings that the vesicle/organelle trafficking pathways are shared among multiple organs, we postulated that screening for mouse mutants exhibiting phenotypes indicative of defects in intracellular trafficking in multiple tissues may yield molecules important for this process. Among defects in different organs, coat color dilution is a good indicator for defective intracellular trafficking because the defect can be readily observed in the whole animal. A number of mouse mutants that show coat color dilution have been isolated and defective vesicle and organelle trafficking pathways are the major causes of coat color dilution in these mice [8,18]. For example, all the sixteen mouse mutants with dilute coat color that were identified as models to study HPS carry mutations in genes that are involved in vesicle trafficking to melanosomes [8,19,20]. Mouse models of Griscelli syndrome also exhibit dilute coat color and bear mutations in components of the RAB27A-MLPH-MYO5A complex that are necessary for proper intracellular trafficking of melanosomes [10,21,22].

In this study, we screened for mice with both a neurological defect (ataxia) and coat color dilution, and identified a novel mutant, neurological 17 (nur17). We performed positional cloning of the nur17 mutation and identified a single nucleotide substitution in archain 1 (Aren1), a highly conserved gene that encodes the 8 subunit of the coat protein I (COPI) complex [23]. Further characterization of the coat color and neuronal phenotypes indicated that the nur17 mutation perturbs intracellular protein trafficking and ER function in the affected tissues. Our study is the first demonstration of the physiological consequences of the impairment of the ARCN1 function in mammalian tissues in vivo, and provides a direct link between the ARCN1 functions and neurodegeneration.

Results

Coat-color dilution and progressive Purkinje cell degeneration in nur17 mice

The autosomal recessive nur17 mutation was generated by ENU mutagenesis at Mouse Mutagenesis Center for Developmental Defects at Baylor College of Medicine [24,25]. Initial phenotypic characterization showed that nur17 mice exhibit both coat color dilution (Figure 1A) and ataxic movements. The coat color dilution phenotype of nur17 mice was noted to be milder compared with that of dilute-lethal (Myn5a<sup>−/−</sup>) mice [10], and to become more notable with age. The ataxic phenotype, which is also milder in nur17 mice than that of Myn5a<sup>−/−</sup> mice, was observed around the age of 2 months and developed progressively as the mice age. The size of nur17 mice was smaller compared to unaffected control littersmates (+/+ or nur17+/+) (Figure 1A and 1B) [control: n = 5, nur17 mice: n = 7; p<0.01 by student’s t-test].

To further characterize the coat color dilution, we examined the distribution of pigment in hair samples from nur17 and littermate control (+/+, nur17+/+) mice. In the hair of control mice, the pigment was incorporated all the way to the tip in a regularly repeated pattern (Figure 1C, top). In the hair of nur17 mice, we observed increased spacing between pigment bands and an occasional absence of pigment in the midshaft (Figure 1C, bottom). The ratio of hairs with abnormal pigmentation at the base, midshaft and tip was significantly higher in nur17 mice compared to control mice (control: n = 5, nur17 mice: n = 5, 30 hairs from each mouse, Student’s t-test, Figure 1D). These hair phenotypes suggest that the nur17 gene product is required for the proper transport/incorporation of pigment into the hair.

nur17 mice also exhibit ataxic movements beginning around 2 months of age. Because the cerebellum is the center for motor coordination, we examined the morphology of the cerebellum in nur17 mice. Purkinje cells (PCs) were visualized using a PC marker, anti-calbindin [26]. In littermate control (+/+, nur17+/+) mice, PCs were aligned in a single cell layer adjacent to each other (Figure 1E). At 1 month of age before the appearance of ataxic movements, nur17 mice showed normal cerebellar morphology with an intact PC layer despite showing a few signs of degeneration (Figure 1E). However, in 2-month-old nur17 mice, we observed extensive loss of PCs compared to control mice (Figure 1E, right panels). We also found a specific regional pattern for PC degeneration in nur17 mice. By 2 months of age, most of the PCs in a part of lobule VI and lobules VII to X of nur17 mice have degenerated (Figure 1E). In contrast, PCs in lobules I to V remained at this age (Figure 1E).

Positional cloning of the nur17 gene

We genetically mapped the nur17 gene on mouse chromosome 9 using an F2 intercross (nur17 × AKR). All F1 animals (14 females and 13 males) were phenotypically normal and did not show any coat color dilution or ataxic phenotypes. To map the nur17 locus, we employed microsatellite markers and single-nucleotide polymorphisms (SNPs) to distinguish between the alleles of nur17 [mixed background of C57BL/6J, 129S6/SvEvTac and 129S1/SvImJ] and AKR. For initial mapping, we tested genomic DNA from F2 mice including 8 affected mice with 80 microsatellite markers across the whole genome. We observed co-segregation of a marker D9Mit69 with the nur17 phenotypes.
and found no significant linkages with other chromosomal loci. Additional F2 animals were tested to further narrow down the nur17 locus. We collected a total of 822 meioses (411 F2 mice) (Figure 2A). Of note, the coat color dilution and ataxic phenotypes always co-segregated in this mapping cross, indicating that a single gene mutation likely accounts for both phenotypes. After progeny testing of F2 mice carrying critical recombination (Figure 2B), the minimal genetic region of nur17 was determined to be between D9SNP25 and D9Mit69 (Figure 2C). A total of 36 genes are localized within this 1.06 Mb interval. We selected 4 genes, dolichyl-phosphate N-acetylglucosamine phosphotransferase (Dpagt1), vacuolar protein sorting 11 (Vps11), trafficking protein particle complex 4 (Trappc4) and archain 1 (Arcn1), as candidates for nur17, since they are known to be associated with vesicle trafficking. We sequenced the coding regions of these candidate genes in nur17 mice and did not observe any nucleotide change in Dpagt1, Vps11 and Trappc4. However, we identified a single nucleotide change in the Arcn1 gene (Figure 2D). This T to C conversion in the 100th exon of the Arcn1 gene causes an amino acid change from isoleucine (Ile) to threonine (Thr) at amino acid 422 (Figure 2D and 2E). The mutation is in the vicinity of the cargo-binding site of ARCN1 which recognizes arginine-based ER localization signals [27].

Transgenic expression of wild-type Arcn1 rescues nur17 phenotypes

To confirm that the Arcn1 mutation is responsible for the phenotypes in nur17 mice, we tested whether transgenic (Tg) expression of wild-type Arcn1 can rescue the nur17 phenotypes. We generated a Tg construct (Figure 3A), in which expression of the Arcn1 cDNA is driven by the chicken β-actin promoter and is followed by the rabbit β-globin poly-A sequence (Figure 3A) [28]. We obtained 2 founder mice (2361 and 2368) that carried the transgene and named the mice generated from these 2 founder mice as line 2361 and line 2368 (Figure S1A). Phenotypes of nur17 mice were rescued in line 2361, in which expression of the transgene was confirmed (Figure 3B–3D, Figure S1B). The coat color dilution and lower body weight in nur17 mice were completely rescued in mice expressing the wild-type Arcn1 transgene (Figure 3B and 3C). We also analyzed the ataxic phenotype using rotarod (Figure 3D). While this analysis revealed significantly decreased latency in nur17 mice compared to control mice (p = 0.0046 by student’s t-test), this phenotype was rescued by the expression of the Arcn1 transgene (Figure 3D). In addition, we examined the PC degeneration phenotype in 2 month-old mice and observed no PC degeneration in nur17 mice carrying the transgene (Figure 3D, right). We tested a total of 7 nur17/
nur17/Tg+/- mice and the phenotypes were rescued in all of these mice. In line 2368, the phenotypes of nur17 mice were not rescued (data not shown). However, we found that the transgene is not expressed in this line (Figure S1B). These results demonstrated that the single nucleotide change in Arcn1 is the causal mutation in nur17 mice.

Expression and cellular localization of ARCN1

ARCN1, also known as δ-COP, is a subunit of the coat protein I (COPI) complex [29]. The COPI complex was first isolated from mammalian sources as a cytosolic protein complex and components of Golgi derived vesicles [30,31]. It was originally found to be involved in retrograde trafficking from the cis-Golgi to the rough ER (reviewed in [32]) and in intra-Golgi trafficking (reviewed in [33,34]) mostly based on studies in yeast. Studies using mammalian cells with a mutation or depletion of a COPI subunit have shown that COPI is essential for compartmentalization of secretory compartments, proper Golgi structure, ER-Golgi transport and endosome functions [35–38]. Although the phenotypes of a mutant for the yeast homolog of ARCN1 suggest its involvement in both retro and antero-grade ER-Golgi trafficking [39], the role of this particular COPI subunit (δ-COP) in mammalian cells and the physiological consequences of its impairment in the whole animal have not been examined. In order to understand the function of ARCN1 in mammalian cells, we first examined sub-cellular localization of ARCN1 using anti-ARCN1 antibody (green) in primary cultured mouse melanocytes (Figure 4). ARCN1 was co-localized with Golgi (Figure 4Ai) and ER (Figure 4Aii) markers (red) and showed a punctate staining pattern in the cytoplasm suggesting its localization in the vesicular compartment (Figure 4Ai and Aii). In mouse melanocytes, ARCN1 was co-localized with β-COP (red, Figure 4Aiii), which is a known binding partner of ARCN1 in yeast [29], mainly in ER and Golgi. This result indicated that ARCN1 (δ-COP) is present in a complex with β-COP in mammalian cells. We also noted that there are vesicles positive for only ARCN1 (green, arrowheads) and not β-COP (red) (Figure 4Aiii). This observation was confirmed by co-expressing GFP-tagged ARCN1 and V5-tagged β-COP (COPB1) in Neuro-2a cells (Figure 4B). The ARCN1 signal does not always co-localize with β-COP signal. These results indicate that ARCN1 may also exist unbound to β-COP in mammalian cells, suggesting the possibility that ARCN1 may also have a function independent of β-COP or the COPI complex. Further biochemical studies are required to test these possibilities.

ARCN1 is involved in ER–Golgi and/or intra-Golgi trafficking in melanocytes

In order to examine protein trafficking through ER and Golgi in nur17 cells, we performed pulse-chase analysis in nur17 melanocytes using the melanosomal membrane protein Tyrp1 as an indicator for intracellular trafficking [40,41]. We monitored ER-Golgi transport using endoglycosidase H (endo H), which cleaves N-linked sugar chain moieties on newly synthesized proteins that are localized in the ER or intermediate compartments but not those that have been modified in the Golgi. Without endo H treatment [endo H (-)], newly synthesized Tyrp1 molecules that have been glycosylated during biosynthetic transport through ER and Golgi are represented by bands with higher molecular weight compared to the band in the endo H (+) lane at 0 min. With endo H treatment [endo H (+)], sugar chains on Tyrp1 molecules that have been processed in Golgi and thus become resistant to endo H digestion are represented by bands with higher molecular weight compared to the band in the endo H (-) lane at 0 min. With endo H treatment [endo H (+)], sugar chains on Tyrp1 molecules that have been glycansylated during biosynthetic transport through ER and Golgi are represented by bands with higher molecular weight compared to the band in the endo H (-) lane at 0 min. With endo H treatment [endo H (+)], sugar chains on Tyrp1 molecules that have been glycansylated during biosynthetic transport through ER and Golgi are represented by bands with higher molecular weight compared to the band in the endo H (+) lane at 0 min. In control melanocytes (from +/-, nur17+/ littermates), after 35 minutes of chase, higher molecular weight bands were observed with endo H and without endo H (black arrowheads in Figure 5A), suggesting that some of the Tyrp1 molecules have been trafficked into Golgi.
and undergone processing that confers endo H resistance. In contrast, little or no higher molecular weight bands were observed in the nur17 melanocytes at this timepoint (red arrowheads in Figure 5A). At 45 min, the bulk of Tyrp1 protein was shifted to a higher molecular weight form in control melanocytes, whereas a band of lower molecular weight still remained in nur17 melanocytes (white arrowheads). We quantified this shift by measuring the intensity of radioactive signals in these bands as shown in Figure 5B. The proportion of protein without complex N-linked glycans (lower band) was significantly higher in melanocytes of nur17 mice compared to cells from control mice (P<0.0001, 2 way ANOVA). These results suggest that the efficiency of protein trafficking through ER and Golgi may be affected in nur17 mice. Alternatively, it is possible that these results indicate perturbation of the glycosylation process in the Golgi of nur17 mice. If retrograde transport of oligosaccharide transferases within the Golgi apparatus is defective due to the mutation, deprivation of these enzymes in appropriate Golgi cisternae could occur, causing delay in glycosylation. This scenario would be consistent with the proposed function of COPI in intra-Golgi retrograde transport of resident proteins in the cisternal maturation model of protein transport through Golgi [34].

Figure 3. Phenotypes of nur17 mice are rescued by wild-type Arcn1. (A) Transgenic construct for generating the transgenic mice expressing Arcn1 gene. Restriction enzyme (PstI) sites to digest DNA for southern blotting were labeled. The black bar represents the region used for the probe for southern blot. (B) Rescue of smaller body weight by transgene in nur17 mice. Smaller size in nur17 mice (middle, n = 13, female) is rescued by the introduction of transgene, Arcn1 (right, n = 6, female) (p = 0.003). There is no significant difference between the weight of rescued mice (right) and the control mice (left, n = 7, female) (p = 0.44). (C) Rescue of coat color dilution. Coat color dilution phenotype in nur17 mice (middle) is rescued by the introduction of transgene, Arcn1 (right). (D) Rescue of the motor coordination defect. Latency to fall on the accelerating rotatod was recorded for control mice (n = 5), nur17 mice (n = 5) and nur17 mice with the Arcn1 transgene (n = 5). The rotarod performance was significantly improved by the transgene (p = 0.015), and was comparable to that in control mice (p = 0.613). (E) Rescue of Purkinje cell degeneration. Purkinje Cell degeneration phenotype in 2-month old nur17 mice (middle) is completely rescued by the introduction of transgene, Arcn1 (right). Purkinje cells are labeled with anti-calbindin (green) and nuclei are stained with DAPI (blue). Scale bar: 1mm.

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ER stress and abnormal protein aggregation in nur17 mice

Based on the possible trafficking defects observed in melanocytes of nur17 mice, we hypothesized that such intracellular trafficking mechanism is also impaired by the nur17 mutation in the PC. Electron microscopic analysis of the cerebellum of nur17 mice showed abnormal accumulation of proteins in the dendrites of PCs (red arrow, Figure 6A) as well as in perinuclear areas (red arrow, Figure 6B, PC nucleus is marked by red star) [42]. Since accumulation of misfolded protein has been associated with defects in vesicle trafficking followed by ER stress [7], we then examined whether ER stress occurs in the degenerating PCs of nur17 mice using an ER stress marker, anti-CCAAT/enhancer-binding protein homologous protein (CHOP) [42]. We observed high levels of CHOP signals in the cell bodies of some PCs in nur17 mice (Figure 6D, white arrowhead), while we never observed CHOP signals in PCs of littermate control (+/+), nur17/+ (n = 3) mice. We examined the distribution of abnormal accumulation of filaments by Gallyas staining, which is known to stain protein aggregates of neurofilaments [43]. Central sagittal sections of the cerebellum from 4 control and 5 nur17 mice at 4 weeks of age were subjected to Gallyas staining (Figure 7B–7D), and the number of positively-stained cells was counted. While we observed no positively-stained cells in control mice (n = 4), an average of 83 ± 28.8 PCs in the central sagittal section of cerebellum were positively stained in nur17 mice (n = 5, p < 0.05, Student’s t-test). High magnification images (Figure 7D) showed that staining is localized to the PC body and dendrites.

Neurofibrillary tangles in the cerebellum of nur17 mice

Through electron microscopic analysis, we also observed abnormal filamentous lesions in the cerebellum of all 30-day-old nur17 mice examined (n = 3) (Figure 7A), but not in the littermate control (+/+), nur17/+ (n = 3) mice. We examined the distribution of abnormal accumulation of filaments by Gallyas staining, which is known to stain protein aggregates of neurofilaments [43]. Central sagittal sections of the cerebellum from 4 control and 5 nur17 mice at 4 weeks of age were subjected to Gallyas staining (Figure 7B–7D), and the number of positively-stained cells was counted. While we observed no positively-stained cells in control mice (n = 4), an average of 83 ± 28.8 PCs in the central sagittal section of cerebellum were positively stained in nur17 mice (n = 5, p < 0.05, Student’s t-test). High magnification images (Figure 7D) showed that staining is localized to the PC body and dendrites.
Although hyperphosphorylated tau is known as the major component of NFTs in Alzheimer's disease, we did not observe signals when immunohistochemistry was performed on the cerebellum of nur17 mice using anti-hyperphosphorylated tau antibodies (data not shown), indicating that NFTs in nur17 mice may not contain hyperphosphorylated tau. It has been suggested that hyperphosphorylation of tau is not obligatory in the formation of neurofibrillary tangles [44], which may be the case in this mutant.

**Discussion**

Here we show that a mutation in Arcn1 is responsible for the phenotypes in an ENU-induced mouse mutant, nur17, which was identified by screening for mice with both diluted coat color and ataxia. We identified a missense mutation in Arcn1 in nur17 mice and confirmed that it is responsible for coat color dilution and PC degeneration by transgenic rescue. ARCN1, also known as δ-COP, is a sub-unit of the coat protein I (COPI) complex, which comprises one of the protein coats that mediate vesicle budding from the membrane (reviewed in [32,45–47]). Thus, nur17 mice provide a unique model to study the intracellular trafficking and its association with neuronal function and pigmentation in mammals.

Our results suggest that the single nucleotide substitution causing an amino acid change from isoleucine to threonine results in abnormal function of the ARCN1 protein. Since the mode of inheritance for the nur17 mutation is recessive, the mutation was expected to cause loss of the protein function rather than gain of function. Our transgenic rescue experiment showed that the wild-type allele of Arcn1 completely rescues the phenotypes of nur17.
mice, providing evidence for this notion. We postulate that nur17 causes partial rather than complete loss of ARCN1 function based on the following reasons. First, Arcn1 encodes a protein that is highly conserved from yeast to human, and mutations of Arcn1 (δ-COP) orthologs in other species such as yeast [29], Drosophila (http://flybase.org, Flybase ID: FBal0096846) and C. elegans (http://www.wormbase.org/db, Wormbase RNAi ID: WBRNAi00033328 and WBRNAi00076118, [48]) all result in lethality, suggesting that this molecule is fundamental to cellular integrity and function. Secondly, mutants of other subunits of the COPI complex in Drosophila and zebrafish also result in early embryonic lethality [49,50]. Compared to the phenotypes in other organisms, the phenotypes of nur17 mice are rather mild, supporting the possibility that the nur17 mutation causes partial loss of ARCN1 function. Thirdly, the mutation does not cause RNA instability or improper processing of the protein, which could cause complete loss of function, as the expression and localization of ARCN1 are not altered in the nur17 melanocytes (Figure S3). Alternatively, it is possible that other molecules with similar functions compensate for the loss of ARCN1 function. Thus, the mutation uncovers roles for COPI that would not be discovered in a complete loss of function allele.

The COPI complex was originally found to be involved in retrograde vesicle trafficking from the cis-Golgi to the rough ER (reviewed in [32]). However, studies in yeast suggested that δ-COP may be involved in both anterograde and retrograde ER-Golgi trafficking [39]. Other studies also suggested expanded roles of COPI complex in anterograde ER-Golgi trafficking and trafficking between other compartments such as early endosomes and the secretory pathway [35,38,51]. Consistent with these findings, our results support the possibility that δ-COP has multiple roles in vesicle trafficking. First, the in vitro pulse-chase assay indicated that the nur17 mutation causes either delays in anterograde ER-Golgi protein trafficking or defects in intra-Golgi retrograde transport of resident proteins, suggesting the involvement of δ-COP in these processes in mammalian cells. Secondly, the localization study showed that ARCN1 is not only localized in ER-Golgi, but also distributed in the cytosolic space, which may represent localization of ARCN1 to other vesicular structures such as endosomes or lysosomes. Further analysis of nur17 mice should reveal whether ARCN1 functions in these organelles and whether the mutation affects their functions. Our pulse-chase study suggested a potential link between the Arcn1 mutation and coat color dilution. Tyrp1 is a tyrosinase related protein (TRP) and has been implicated in the biogenesis of melanosomes [52,53]. The retardation of ER-Golgi transport of Tyrp1 from ER to Golgi in nur17 melanocytes or impaired glycosylation of this protein could potentially delay or inhibit melanosome biogenesis resulting in coat color dilution. It is also possible that ER-Golgi trafficking of other proteins required for melanosome synthesis is affected as well. However, impaired ER-Golgi and/or intra-Golgi trafficking may not be the only cause of coat color dilution. As discussed above, the potential site of action of ARCN1 is not restricted to ER-Golgi. Since biogenesis of melanosomes includes multiple aspects of vesicle regulation such as endocytosis and recycling of vesicles (reviewed in [54]), these processes could be also affected in nur17 melanocytes. Further analysis is needed to test these possibilities.

Defects in ER-Golgi or intra-Golgi trafficking similar to those observed in melanocytes of nur17 mice could also be responsible for PC degeneration in the cerebellum. For example, defective ER-Golgi trafficking could be the direct cause of the formation of neurofibrillary tangles in the cerebellum of nur17 mice. (A, B) Ultrastructural analysis showed abnormal filamentous lesions (red arrowhead) in the cerebellum of nur17 mice (A), while such lesions are not observed in control mice (B). The inset in (A) shows the higher magnification of filamentous lesion. (C–E) Gallyas staining for control (C) and nur17 mice (D, E). Arrow denotes the PC that is positive for Gallyas staining. Scale bar for (C, D): 1mm. Scale bar for (E): 0.1mm. doi:10.1371/journal.pgen.1000956.g007
abnormal protein accumulation in the ER, which could in turn result in ER-stress. On the other hand, ER-Golgi trafficking is required for the unfolded protein response (UPR), which is activated by ER-stress [53]. UPR helps to restore the normal ER function by halting protein synthesis and upregulating molecular chaperones involved in protein folding and ER associated degradation (ERAD) to clear the protein accumulation (reviewed in [56]). Therefore, defective ER-Golgi trafficking could be the indirect cause of abnormal protein accumulation as well. Our findings that abnormal protein accumulation and CHOP expression, which is a sign of ER stress, are observed in nur17 mice support both hypotheses. Since ER-stress is known to trigger signal transduction events that could induce cell death (reviewed in [57]), it may cause degeneration of PCs in nur17 mice. However, we did not observe upregulation of another ER stress marker, KDEL, which is the protein sequence found in both BiP/Grp78 and Grp94. This result may indicate that abnormalities in nur17 PCs do not affect the typical ER stress pathway. Alternatively, we may not be able to detect the increase in these proteins, because only small numbers of PCs may be experiencing it at a time, which would be consistent with the low number of CHOP-positive cells we observed in nur17 mice. Nevertheless, our results provide evidence that the defect in the COPI complex leads to neurodegeneration, and suggest that ER abnormalities may be involved in the neurodegenerative mechanism. Recent discovery of Scyl1-like1 (Scyl1) as a gene responsible for motor neuron and PC degeneration in mice also suggests a link between the COPI pathway and neurodegeneration [58,59]. SCYL1 binds to and co-localizes with β-COP and regulates COPI-mediated retrograde ER-Golgi trafficking [59]. Together with our results, these findings support the notion that compromised COPI-mediated vesicle trafficking causes neurodegeneration.

In addition to abnormal protein accumulation and possible signs of ER-stress, nur17 mice show neurofilibrillary tangles (NFT) in the cerebellum that are commonly observed in a number of neurodegenerative disorders such as Alzheimer’s Disease, Amyotrophic lateral sclerosis and Down’s Syndrome (reviewed in [60]). Although ER stress and NFT are often observed together in many human neurodegenerative diseases [61–63], the relationship between these conditions is largely unknown. Our findings that a mutation in a component of the COPI complex, which regulates ER-Golgi vesicle trafficking, results in formation of NFT suggests a possible new mechanism underlying NFT formation. Impaired ER-Golgi vesicle trafficking could be the cause for NFT formation in this model. Further analysis of nur17 mice should advance our understanding of the molecular mechanism causing the formation of NFT, which may be shared by a variety of neurodegenerative disorders.

Provided that the Arcn1 gene is likely expressed ubiquitously in all cell types, it is intriguing that gross abnormalities have been detected only in the skin/hair and the cerebellum. It is possible that there is another protein that can compensate for the loss of ARCN1 in other cell types and tissues, although such protein has not been biochemically discovered to date. Alternatively, the affected tissues and cell types may be extraordinarily susceptible to minor changes in efficiency of COPI function. A mutation in another ubiquitously expressed protein known to function in ER-Golgi and intra-Golgi trafficking, TRAPP6A, also leads to a tissue-restricted phenotype, pigment dilution, in mice [64]. Skin cells may be particularly sensitive to defects in these trafficking mechanisms. On the other hand, skin may be a unique tissue where subtle defects in intracellular trafficking can be visibly identified without the integrity of the skin being grossly affected, making it a great in vivo model system to investigate the mechanisms of intracellular trafficking.

Materials and Methods

Animals

All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison.

At Baylor College of Medicine, ENU mutagenesis was performed using C57BL/6J males [25]. nur17 mice were isolated, crossed to 129S6/SvEvTac three times and maintained in a mixed background of C57BL/6J and 129S6/SvEvTac.

After importing nur17 mice to the University of Wisconsin-Madison, we maintained the nur17 mutant allele and obtained the affected animals for characterization by repeating this mating scheme: nur17/nur17 founder females were mated with wild-type 129S1/SvImJ. nur17/+ F1 mice were intercrossed to obtain affected nur17/nur17 mice. nur17/nur17 female mice were again mated with 129S1/SvImJ nur17/+ or +/- littermate mice were used as control mice in the experiments. 129S1/SvImJ and AKR/J mice were obtained from The Jackson Laboratory.

Both 129S6/SvEvTac and 129S1/SvImJ strains are homozygous for the A* allele and display white-bellied agouti coat color. All nur17 and control mice that were phenotyped in this study also displayed agouti coat color.

We replaced the EGFP sequence in the pCX-EGFP vector (kindly provided by Dr. Junichi Miyazaki; [65]) with the full length Arcn1 cDNA and named it pCX-Arcn1. We used pCX-Arcn1 for the transgene construct for the rescue experiment after linearization with BamHI and SalI (New England Biolabs). The construct was micro-injected into pronuclei of FVB/NJ embryos to generate transgenic mice by the transgenic animal facility of the Biotechnology Center at the University of Wisconsin-Madison. We obtained two transgene-positive founders (2361 and 2368) and crossed them to nur17 mice, and then intercrossed to obtain Tg-positive nur17/nur17 mice.

Southern blotting genotyping procedures

We identified Transgene (Tg-positive clones by Southern-blot hybridization using PstI (New England Biolabs) digestion and probes specific to Arcn1. The southern blot differentiated the endogenous Arcn1 and the transgene based on size of bands (1.05kb and 0.45kb for the transgene).

Tail DNA was prepared as described in [66]. ~15μg of DNA was digested with the restriction enzyme PstI (New England Biolabs) and resolved by 0.8% agarose gel electrophoresis. DNA was transferred to Hybond N membrane (Amersham, GE Healthcare) using alkaline capillary transfer, cross-linked with UV (Spectrolink). The membrane was prehybridized with prehybridization buffer (4xSSC, 1% milk powder, 1% SDS, 10× Denharts Reagent) and hybridized with probes labeled with [P32] dCTP (Perkin Elmer) by Rediprime II Random Prime Labeling System. The probe used for hybridization was PCR amplification of mouse brain cDNA using primers, mArcn1-F3 (5’ AAGGACCCAGATTTGGCGG 3’) and mArcn1-R16 (5’ TTA-CAGTTTCTGATTTGTC 3’). Membranes were washed three times with wash buffer I (2xSSC and 0.1% SDS) and twice with wash buffer II (0.5xSSC and 0.1% SDS) for 15 min at 65°C.
for each wash. CL-X Posure Film (Thermo Scientific) was used to detect the hybridization signal.

**Northern blotting**

The expression of the transgene was tested by a northern blot that differentiates the endogenously expressed *Arenl* (4.4kb) and expressed transgene (2.2kb). Total RNA was extracted from mouse brains by Trizol (Invitrogen). mRNA was purified from total RNA by Oligotex mRNA Midi Kit (Qiagen). mRNA was resolved by formaldehyde agarose electrophoresis and transferred to Hybond N membrane (Amersham, GE Healthcare) using alkaline capillary transfer, crosslinked with UV (Spectrolite). The membrane was prehybridized with Rapid-Hyb Buffer (Amersham) with 37 μg/ml salmon sperm DNA (Sigma) and hybridized with probes labeled Rapid-Hyb Buffer (Amersham) with 37 μg/ml salmon sperm DNA (Sigma). The probe used was the same described above for the Southern-blot; similarly, the membranes were washed and hybridization signal was detected as described above.

**Genetic mapping**

To map the *nur17* gene, we performed a whole genome scan using F2 animals from mating (*nur17 × AKR/J*) F1 mice. We initially used 80 microsatellite markers, which distinguish AKR alleles from both 129S1/SvImJTac and C57BL/6J alleles across the whole genome. All F2 animals were phenotyped by both coat color dilution and ataxia movement. Once the chromosomal locus on chromosome 9 was identified for the *nur17* mutation, additional F2 mice were collected and analyzed using F2 animals from mating (*nur17 × AKR/J*) F1 mice. We used 80 microsatellite markers, which distinguish AKR alleles from both 129S1/SvImJTac and C57BL/6J alleles across the whole genome. All F2 animals were phenotyped by both coat color dilution and ataxia movement. Once the chromosomal locus on chromosome 9 was identified for the *nur17* mutation, additional F2 mice were collected and analyzed.

**Genotyping**

All genotyping was carried out by polymerase chain reaction (PCR). For *nur17* genotyping, PCR primers, mArcn1-F18 (5′ CCTCAAAACTGAGAAATCCGGC 3′) and mArcn1-R19 (5′ TTGGCATCAATCACTGGC 3′), were used for amplification of the wild type (WT) allele and nur17 allele (470bp). Bsd (New England Biolabs) was used to digest the nur17 allele specifically to generate two bands (392bp and 78bp).

**Immunohistochemistry**

Littermate control (+/+, *nur17+/+*) and *nur17* mice were deeply anesthetized with a mixture of ketamine/xylazine and perfused with 4% paraformaldehyde (PFA). The heads were immersed in 10% formaldehyde, washed in 0.1M sodium phosphate buffer at pH 7.4, and embedded in paraffin. Sections were cut at 5 μm thickness. For immunohistochemistry, sections were blocked with 2% donkey serum and were incubated overnight with the primary antibody against Calbindin-D (Swant), Calbindin (Abcam), phosphorylated tau (abcam), hyperphosphorylated tau (Thermo Scientific) and CHOP (Santa Cruz Biotechnology). Sections were rinsed in PBS, and incubated with a 1:200 diluted Alexa 488 conjugated secondary antibody (Invitrogen) and/or Cy3 conjugated secondary antibody (Jackson Immunoresearch) for 45 minutes at room temperature. All sections were imaged on an Eclipse E600 microscope (Nikon) using a SPOT camera (Spot Diagnostics).

**Electron microscopy**

Littermate control (+/+, *nur17+/+*) and *nur17* mice were anesthetized with ketamine/xylazine and perfused with 2% PFA and 2.5% glutaraldehyde in 0.1M phosphate buffer for 11 min. Cerebella were removed from the head and immersed fixed for 30 min. Then, cerebella were sectioned sagittally at 100μm intervals with a VIBRATOME. Sections of the cerebella were osmicated (1% osmium tetroxide) for 1 hr and washed in 0.1M phosphate buffer, dehydrated through an ascending series of ethanol and propylene oxide and embedded in Epon 812. 150μm Dodecenyl succinic anhydride (DDSA), 12g Nadic Methyl Anhydride (NMA) and 1ml 2,4,6-tris(dimethylamino) methylphenol (DMP-30), Electron Microscopy Sciences. Ultrathin sections (70nm) were cut and stained with uranyl acetate and lead citrate. Sections were imaged on a Philips CM120 Scanning Transmission Electron Microscope.

**Gallyas-silver staining**

The Gallyas-silver staining of the littermate control (+/+), *nur17+/+* and *nur17* cerebella was performed as described in [67] in order to examine the existence of neurofibrillary tangles.

**Melanocyte culture**

Mouse melanocytes were isolated from the skin on the back of the Day2 littermate control (+/+), *nur17+/+* and *nur17* mice and cultured. Fresh skin specimens were incubated in foreskin media (Dulbecco’s modified minimal essential medium (DME) (Gibco) supplemented with 2% fetal bovine serum (Sigma-Aldrich), penicillin (100 units/ml, Sigma) and gentamicin (100 μg/ml) (Gibco) at 4°C overnight. The next day, the skin was washed three times with Hanks’ balanced salt solution and excess fat was removed. The samples were cut into small pieces and incubated in 0.25% trypsin solution (Hyclone) supplemented with 3.86 mg/ml trypsin, from porcine pancreas (Sigma-Aldrich) at 4°C overnight. Epidermis was separated from the dermis and epidermal cells were suspended and cultured in Ham’s F10 nutrient medium supplemented with 10% fetal bovine serum, 85 μmol/L 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma-Aldrich), 0.1 mmol/L 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), 2.5 mmol/L cholera toxin (CT) (ICN Biochemicals) and penicillin (100 units/ml) (Gibco).
Mouse Neuro-2a cells (ATCC) were cultured in DMEM with high glucose (Gibco), supplemented with 10% FBS (Sigma). The ARCN1 and COP1B expression constructs were transfected into Neuro2A cells using SuperFect Transfection Reagent (Qagen) following the manufacture’s protocol and cultured for 24 hrs.

**Immunocytochemistry**

Cells were cultured on coverslips and were fixed by 4% PFA for 10 min at 4°C. Cells were permeabilized using 0.5% Triton-X in PBS for 30 min followed by blocking in 2% normal donkey serum for 30 min. Then the cells were incubated with 1:200 diluted primary antibody against ARCN1 (Novus Biologicals), 58K Golgi protein (58K-9) (Abcam), KDEL (Abcam), β-COP (Sigma), GFP (Synaptic System) and V5 (Invitrogen) in PBS with 0.1% Triton-X for 1 hr at room temperature. Cells were washed in PBS and then incubated with 1:200 diluted Alexa 488 conjugated secondary antibody (Invitrogen) and Cy3 conjugated secondary antibody (Jackson Immunoresearch) for 45 min at room temperature. All immunocytochemistry slides were imaged on a Zeiss 510 confocal laser scanning system and Axio Imager microscope using LSM 510 software (release 4.2) (Carl Zeiss MicroImaging, Inc).

**Pulse-chase labeling**

Melanocytes grown in a 5cm culture dish until 90–100% confluency were starved in DMEM without methionine (Gelgro) supplemented with 10% fetal bovine serum (Sigma-Aldrich), penicillin (100 units/ml) (Sigma) for 2 hours. Each plate of cells was washed with 50 uCi [35S] methionine (EXPRE35S35S protein (Gibco), supplemented with 10% FBS (Sigma), and diluted with 25 ul Tris/HCl, pH 7.5, 0.15 M NaCl, 5 mM EDTA, and 1% NP-40 (TNEN), followed by TNEN containing 0.5 M NaCl, and finally with distilled water. For Endo H digestion, the immunoprecipitates were dissociated by suspending in 0.2% SDS, heated for 5 min at 100°C, and diluted with 25 ul 0.1 M sodium citrate buffer, pH 5.5, Endo H, 50 mM/ul. The reaction mixture was layered over with 50ul toluene and digestion was carried out at 37°C for 18–24 h. Littermate control (+/+, nur17+/+) and nur17−/− mice and the latency to fall in the rotarod analysis.

**Statistical analysis**

Student’s t-test was performed for statistical comparison of the weight of mice, the number of cells positive for Gallyas staining or the CHOP1 staining between littermate control (+/+, nur17+/+) and nur17−/− mice and the latency to fall in the rotarod analysis. Two-way analysis of variance (ANOVA) was performed for the statistical comparison of the proportion of protein without complex N-linked glycans in the pulse-chase experiment. GraphPad Prism software (GraphPad software, Inc.) was used for statistical analysis and to create all graphs reporting numerical values. * p<0.05, ** p<0.01.

**Rotarod analysis**

Mice that were of the genotype of +/- or nur17+/+ TG− (n = 5), nur17+/+ TG− (n = 5) and nur17/nur17 TG+ (n = 5) at 5–8 months of age were subjected to rotarod analysis. Mice were first exposed to a training period at constant speed to familiarize them with the rotarod apparatus (Ugo Basile). For the testing, the rotarod was gradually accelerated (1 rpm/s) from 4 rpm over the course of 3 min. The time was recorded when a mouse fell from the device (latency to fall). After all mice fell from the rotarod, the group was given a rest period and then reloaded on the device. The testing was performed a total of 3 times for each mouse. Values from the three trials for each mouse were averaged to give a single score.

**Supporting Information**

**Figure S1** Genotyping and transgene expression in the transgenic mice. (A) Southern blot for the transgene. The mice from line 2361 and line 2368 with the transgene exhibit two extra bands (1.06kb and 0.45kb), which are marked by arrowheads. (B) Northern blot analysis to test the expression of transgene. The mice from line 2361, control and line 2368 all exhibit the band for endogenous Arcn1 (1.4kb), while only line 2361 exhibits the band from the transgene (2.2kb, arrowhead). Found at: doi:10.1371/journal.pgen.1000956.s001 (1.65 MB TIF)

**Figure S2** Immunofluorescence for KDEL in the cerebellum. Positive staining is observed in both control (left) and nur17 PCs. Nuclei are stained with DAPI (blue). Scale bar: 10 μm. Found at: doi:10.1371/journal.pgen.1000956.s002 (1.65 MB TIF)

**Figure S3** Comparison of the subcellular localization of ARCN1. Primary culture of wild-type control (A,C,E) and nur17 (B,D,F) mouse melanocyte are all labeled with anti-ARCN1 (green) indicating the localization of ARCN1 and anti-Golgi protein (red) (A,B); anti-KDEL (red) (C,D) and anti-SCOP (red) (E,F). Nuclei are stained with DAPI (blue). Merged pictures are shown and high magnification pictures are shown in the right panel. Scale bar for low magnification: 10 μm. Scale bar for high magnification: 5 μm. Found at: doi:10.1371/journal.pgen.1000956.s003 (2.18 MB TIF)

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**Author Contributions**

Conceived and designed the experiments: XX SI VS AI. Performed the experiments: XX RK HH SI MJJ AI. Analyzed the data: XX HH SI VS AI. Contributed reagents/materials/analysis tools: KK MJJ VS AI. Wrote the paper: XX SI MJJ VS AI.
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