Karyopherin α-3 is a key protein in the pathogenesis of spinocerebellar ataxia type 3 controlling the nuclear localization of ataxin-3

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Spinocerebellar ataxia type 3 (SCA3) is a neurodegenerative disorder caused by a CAG expansion in the ATXN3 gene leading to a polyglutamine expansion in the ataxin-3 protein. The nuclear presentation and aggregation of expanded ataxin-3 are critical steps in disease pathogenesis. To identify novel therapeutic targets, we investigated the nucleocytoplasmic transport system by screening a collection of importins and exportins that potentially modulate this nuclear localization. Using cell, Drosophila, and mouse models, we focused on three transport proteins, namely, CRM1, IPO13, KPNA3, and their respective Drosophila orthologs Emb, Cdm, and Kap-α3. While overexpression of CRM1/Emb demonstrated positive effects in Drosophila, KPNA3/Kap-α3 emerged as the most promising target, as knockdown via multiple RNAi lines demonstrated its ability to shuttle both truncated and full-length expanded ataxin-3, rescue neurodegeneration, restore photoreceptor formation, and reduce aggregation. Furthermore, KPNA3 knockout in SCA3 mice resulted in an amelioration of molecular and behavioral disturbances such as total activity, anxiety, and gait. Since KPNA3 is known to function as an import protein and recognize nuclear localization signals (NLSs), this work unites ataxin-3 structure to the nuclear pore machinery and provides a link between karyopherins, NLS signals, and polyglutamine disease, as well as demonstrates that KPNA3 is a key player in the pathogenesis of SCA3.

Significance

Ataxin-3 is the affected protein in the neurodegenerative disorder spinocerebellar ataxia type 3 (SCA3). Nuclear ataxin-3 has been linked to disease progression and formation of aggregates. Our present findings implicate karyopherin alpha 3 (KPNA3) in the in vitro transport of ataxin-3 and in the SCA3-related phenotypes in Drosophila and mouse models. We have demonstrated that altering transport proteins has an effect on both pathogenic mechanisms (e.g., the intracellular localization and the formation of aggregates) and key features of ataxin-3 toxicity such as anxiety, total activity, and gait abnormalities. A better appreciation of this cellular mechanism can enhance our understanding of polyglutamine diseases and the role of nuclear/cytoplasmic compartments in toxicity and clearance of ataxin-3.


This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1716071115/-/DCSupplemental.

Published online February 23, 2018.
are also intimately involved in the signaling pathways important to ataxin-3, such as activation of Erk, changes in transport of nuclear factor-κB (14), activating-transcription factor 2, and the transcription factor CREB (15, 16). The androgen receptor is dependent on karyopherins for nuclear import (17), and huntingtin possesses CRM1 export signals as well as karyopherins’ β1/β2 (KPNB1/TNPO1) NLSs (18, 19). Transport proteins have also garnered attention in relation to amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration, where importins play a role in the observed nuclear protein depletion and cytoplasmic aggregation. This gave us evidence to postulate that transport proteins could also be involved in the nucleocytoplasmic trafficking and intracellular localization of ataxin-3, thereby influencing the toxicity and aggregation of ataxin-3 and, thus, the pathogenesis of SCA3.

We now report that KPNA3 (also known as importin alpha-4; GeneID 3839) is a key player in the intracellular localization of ataxin-3. We demonstrated in vitro and in vivo that KPNA3 controls the localization and aggregation of ataxin-3 as well as its toxicity. Down-regulation/knockout of KPNA3 in Drosophila and mouse models alleviated the neurological phenotype induced by expanded ataxin-3. Our results provide support for the concept that transport proteins are involved in the disease progression of SCA3 and indicate KPNA3 as a highly promising therapeutic target for the treatment of SCA3.

Results

Nuclear Import of Ataxin-3 Is Mediated by the Classical Importin α/β Pathway via KPNA3 in HEK Cells. Although ataxin-3 is predominantly localized in the cytoplasm of cells, it forms protein aggregates in the nucleus of neurons in SCA3 patients (20, 21). Since karyopherins are known to shuttle protein cargo across the nuclear/cytoplasmic membrane, we explored the role of these proteins in the nuclear transport of ataxin-3. Our previous analysis revealed a clear link between the intracellular localization of ataxin-3 and its propensity to aggregate (10). Therefore, using aggregation as a readout, we used FACS to isolate a population of cells equally expressing ataxin-3 148Q (stably transfected) and overexpressed transport proteins to analyze aggregate formation (Fig. 1A). Our screen of 12 transport proteins (Table S1) yielded KPNA3 and IPO13 as modulators of aggregation, as overexpression of the protein altered the total amount of aggregated ataxin-3 over a 96-h posttransfection window (Fig. 1A and Table S1). CRM1 overexpression also showed an increase in aggregation which did not reach statistical significance. KPNA3 and IPO13 modulated total aggregation of all ataxin-3 samples. KPNA3 overexpression showed the strongest decrease. Relative representative filter trap images are shown from triplicates performed on the same filter. (A) Transport protein overexpression alters levels of aggregated expanded ataxin-3. KPNA3 overexpression increases total aggregated ataxin-3 by filter trap. IPO13 overexpression shows the strongest decrease. Representative scatter plot dot images are shown from triplicates performed on the same filter. n = 11 for IPO13; n = 3 for all other measurements. Each experiment was conducted in triplicate and in dilutions. (B) Microscopy was used for a visual confirmation of altered localization. The cell coexpressing KPNA3 (arrowhead) shows increased nuclear distribution of ataxin-3 compared with the neighboring cell without KPNA3. No significant changes were observed with CRM1 and IPO13. GFP signal is ataxin-3 148Q, red channel transport protein of interest. (Scale bar: 20 μm.) (C) and (D) Transport proteins were increased on a transcriptional and translational level: mRNA (C) and protein (D) in cells transfected with a cDNA control, KPNA3, or IPO13 plasmid. Transcriptional levels of endogenous ataxin-3 are not altered upon transport protein overexpression. KPNA3 and IPO13 antibodies are per Table S2. (E) Total aggregation was verified by visual counting of HEK 293 cells transiently transfected with GFP-tagged ataxin-3. Ten fields were counted under blinded conditions. Number of cells with aggregates was calculated as percentage of total transfected cells. Values are displayed for n = 6. (F) Cell fractionation was performed according to Koch et al. (53). The formic acid (FA) fraction shows a significant increase of insoluble aggregates of expanded ataxin-3 upon KPNA3 overexpression. n = 3. Lanes shown are from the same gel. (G) In vitro nuclear import assay confirms change in localization of nonexpanded ataxin-3 upon addition of KPNA3 and suggests a role for KPNB1 as partner protein. Nuclear import of ataxin-3 is largely blocked when using the loss-of-function RanQ69L mutant (58). n = 4 or 5. (H) Visualization of nuclear import assay quantified in G. Values are displayed as mean ± SEM for A, C, and E-G. *P < 0.05; **P < 0.01; ***P < 0.005 [Student’s two-sided (A and E) or one-sided (F) t test relative to control, with Welch’s correction in G]. Ataxin-3 was detected with 1H9 antibody in A and F. Red, relative.
significant. The filter trap results were confirmed by visual counting (Fig. 1E), and overexpressed levels of KPNA3 and IPO13 were confirmed by quantitative RT-PCR (qRT-PCR) and Western blot (Fig. 1 C and D). Furthermore, we fractionated aggregated ataxin-3 to understand which population of aggregates was increased by KPNA3 overexpression. We were able to see a significant increase in the SDS-insoluble forms (formic acid fraction; Fig. 1F), which could be more damaging for the cell, as they are harder to clear than their more soluble counterparts (22). While aggregates served as a surrogate measure for nuclear translocation, we wanted to verify that an increase in aggregation was also correlated to a visual increase in nuclear ataxin-3.

To this point, we transfected HEK293 cells with the same constructs of ataxin-3 and transport protein to visualize protein localization by microscopy. Cells that were overexpressing KPNA3 showed more nuclear ataxin-3 than cells without additional KPNA3 (Fig. 1B), while cells overexpressing IPO13 or CRM1 [a protein previously implicated in ataxin-3 shuttling (23)] showed no difference in protein localization. We postulated that KPNA3 must thus be involved in an interaction with ataxin-3 which facilitates its transport. Our analysis using the STRING interaction prediction algorithm suggested that the interaction between the two proteins occurs through Ran (Ras-related nuclear protein) and KPBN1, a known interaction partner of KPNA3 which forms a classical importin-αβ beta-Ran complex (24). Thus, we performed an analysis to see whether altering levels of KPBN1 or Ran would alter the observed nuclear import of ataxin-3 by KPNA3. We did, in fact, see that either a disturbance in KPBN1 or the Ran gradient through the use of a Ran mutant decreased the ability of KPNA3 to import ataxin-3 into the nucleus (Fig. 1 G and H). The enhancement of nuclear translocation with KPBN1 further suggested that an interaction with KPNA3 could proceed through this trimeric complex (24) (Figs. 1H and 6).

**Kap-α3, the Drosophila Ortholog of Human KPNA3, Controls the Intracellular Localization of Ataxin-3 in Vivo.** Although cell models serve an important purpose in initial research for polyglutamine diseases, they have a number of limitations. Thus, we complemented our studies using Drosophila models that recapitulate many aspects of neurodegenerative diseases (25), SCA3-relevant phenotypes, such as progressive neurodegeneration, behavioral abnormalities, and early death, can all be well observed. We reproduced these features in several Drosophila SCA3 models to study the effect of Kap-α3, the Drosophila ortholog of human KPNA3 and KPNA4, on ataxin-3-induced toxicity (Tables S3 and S4). We used the same concepts as in the Drosophila analysis by validating our results in different lines across various backgrounds. First, we used a lio-GAL4, UAS-ataxin-3-Q70 line (designated SCA3) to drive expression of full-length ataxin-3 in salivary glands (SGs) of developing larvae. SG cells are small and allowed us to visualize the effect of Kap-α3 overexpression (designated Kap-α3 OE) or RNAi-mediated knockdown (designated Kap-α3 KD) on ataxin-3 localization. To validate our findings, we used three available RNAi lines targeting different parts of the gene that reduced the Kap-α3 mRNA level to 75% (v36104), 50% (v106249), and 45% (f10268) compared with control animals (Fig. S1). We crossed the SCA3 lines to either Kap-α2 or control lines (designated SCA3+Kap-α3 OE/KD and SCA3+Control), extracted SGs from the resulting larvae, and performed immunostaining against ataxin-3. As expected, overexpression of Kap-α3 (UASp-Kap-α3) increased nuclear localization of expanded ataxin-3, whereas knockdown of this protein increased cytoplasmic localization of ataxin-3, thereby confirming our results in vitro results in vivo (Fig. 2B). This was independently confirmed on a second full-length ataxin-3 line.

**CRM1 Overexpression in Drosophila Shows Potential Benefits in SCA3 While IPO13 Impacts Development.** Similar to our in vitro data, Emb (the Drosophila ortholog of CRM1) overexpression and knockdown and Cdm (the Drosophila ortholog of IPO13) knockdown did not affect localization of ataxin-3, while overexpression of Cdm did not produce any viable offspring (likely due to lio-GAL4 expression outside of SGs), suggesting a role for Cdm/IPO13 in embryonic development (Fig. 2 C and D). Next, we wanted to investigate whether these transport proteins can directly regulate neurodegenerative processes. We used the GMR driver in combination with the GAL4 protein to express ataxin-3 and simultaneously modulate the level of Emb/CRM1, Cdm/IPO13, and Kap-α3/KPNA3 in the Drosophila eye. We used well-established SCA3 fly lines with truncated expanded ataxin-3 (MJD.tr-Q78) in different genetic backgrounds to observe the rough eye (26). Emb/CRM1 knockdown in SCA3 flies (SCA3+CRM1 KD) had a severe deleterious effect on the eye morphology, as has been reported (26, 27), while Emb overexpression demonstrated no or a moderate rescue in necrosis and morphology (SCA3+CRM1 OE; Fig. 3C). These features are in agreement with previously described CRM1 functions, as a knockdown of CRM1 is known to disturb the Ran gradient and other vital cellular functions (28), while the up-regulation of CRM1 is thought to stimulate prosurvival pathways (29), which points to its wide-reaching effects throughout the cell. In the Cdm/IPO13 Drosophila lines, we observed a slight improvement with Cdm/IPO13 overexpression lines (SCA3+IPO13 OE) and no effect with Cdm/IPO13 knockdown lines (SCA3+IPO13 KD; Fig. 3D). No effect on the rough-eye phenotype (REP) was observed when modulating these transport proteins without the expanded ataxin-3 protein present (Fig. 3E–H). The next step was to use an inducible neuronal driver (elav-GS) which targets full-length ataxin-3 in a neuron-specific manner to analyze the effect of transport proteins on the longevity of adult flies. Cdm/IPO13 knockdown and overexpression had no effect on prolonged survival and also produced very few offspring (again pointing to a pivotal role in development) (Fig. 2E). In contrast, Emb/CRM1 knockdown had detrimental effects on longevity (as expected), while overexpression of Emb had the largest rescue of survival in the proteins assayed (Fig. 2F). In an analysis of locomotor activity to evaluate the properties of ataxin-3, Emb/CRM1 knockdown had a severe effect on both the induced and uninduced lines, while its up-regulation was able to slightly rescue the locomotor activity (Fig. 2F).

Overall, the Drosophila experiments with Emb and Cdm confirmed previous data on Emb/CRM1 knockdown, giving validity to our further results and showing that Emb/CRM1 overexpression has the potential for positive effects in SCA3 through mechanisms that do not appear to involve ataxin-3 localization. Cdm/IPO13 showed no strong effect on either eye phenotype or longevity, but was concerned with toxicity identified by decrease in offspring when expressed with SG or neuronal drivers.

**Kap-α3 Modulates Neurodegeneration and the Neurological Phenotype Associated with Expanded Ataxin-3 in Vivo.** In the Drosophila experiments, Kap-α3/KPNA3 behaved in accordance with the in vitro experiments without significant side effects. In presence of MJD.tr-Q78, Kap-α3 overexpression with a GMR driver worsened the eye phenotype with collapse of the outer structure, prominent degeneration of the ommatidia, and necrotic lesions seen as black spots. Knockdown of Kap-α3 moderately alleviated the eye phenotype (Fig. 3B). Most importantly, GMR-GAL4-mediated modulation of Kap-α3 without ataxin-3 expression showed no changes to the eye phenotype, confirming that the observed effects are ataxin-3–specific (Fig. 3F). The GMR driver is expressed during development and leads to a strong phenotype which is already observed at emergence. Therefore, to investigate whether later modulation of Kap-α3 may interfere with the MJD.tr-Q78–induced photoreceptor degeneration during adulthood, we turned to the rhodopsin1-GAL4 driver (rh-GAL4), which is expressed only in the late pupal stage. In young rh1 MJD.tr-Q78 flies (8 d), retinal sections did not exhibit specific defects in ataxin-3–expressing regions or when Kap-α3 was simultaneously overexpressed or depleted (Fig. 3 I–K). However, in older flies (20 d), the retinas exhibited striking degenerative phenotypes with disorganized ommatidia and tissue dissociation associated with the appearance of vacuoles. Depletion of Kap-α3 rescued the degenerative phenotype to a large
**Fig. 2. Kap-α3 controls the intracellular localization of full-length ataxin-3 in Drosophila and mitigates the behavioral deficits observed in the SCA3 phenotype.** (A–D) Ataxin-3 immunostaining with H19 anti–ataxin-3 antibody in SGs of Drosophila larvae expressing full-length ataxin-3 with or without overexpression (OE) or knockdown (KD) of Kap-α3/KPN3, Embi/CRM1, or Cdm/IPO13 genes. (A) Control ataxin-3 distribution in SGs. (B) Overexpression of KPN3 relocates the expanded ataxin-3 protein to the nucleus (Left), whereas with KPN3 knockdown, ataxin-3 is preferentially cytosolic (Right). (C) CRM1 modulation does not impact full-length expanded ataxin-3 localization. (D) IPO13 knockdown shows a mild increase in nuclear localization. Overexpression produced no offspring. (E and F) MLS (E) and total activity (F) of flies expressing or not in neurons the full-length ataxin-3 with or without modulation of the expression of Kap-α3/KPN3, Embi/CRM1, or Cdm/IPO13 genes. (E) CRM1 knockdown significantly reduced survival of flies, while CRM1 overexpression had the largest positive effect on survival. IPO13 had no effect on survival, while KPN3 knockdown and overexpression both slightly improved longevity, although not significantly. Differences in longevity are assayed relative to decrease in survival upon induction of ataxin-3. Gray bar demonstrates the decrease in median longevity caused by the induction of ataxin-3 expression. Lighter (overexpression; OE) and darker (knockdown; KD) bars represent the differences with transport proteins. A smaller bar shows a rescue in longevity. Viability was measured on 90–200 flies per genotype. Graphs are shown as nonlinear fit curves of the survival data. (F) Total activity of flies. The loss of total activity seen with ataxin-3–induced expression is rescued with KPN3 knockdown. Total activity was measured by the amount of light beam breaks produced by movement of one fly per tube in a Drosophila Activity Monitor (TriKinetics) over the course of 3 d. Uninduced, n = 8; induced, n = 24, except for KPN3 overexpression: uninduced, n = 20; induced, n = 48, *P < 0.05; **P < 0.01; ***P < 0.005; n.s., not significant (one-way ANOVA). Values are displayed as mean ± SEM. RU486 was used in both survival and activity studies to induce the expression of ataxin-3.

**Knockout of KPN3 Keeps Expanded Ataxin-3 in the Cytoplasm and Prevents Its Aggregation in Mice.** After confirming the importance of its ortholog of KPN3 for the development of an ataxin-3–induced phenotype in Drosophila, we replicated our results in a mouse model. We first obtained KPN3<sup>−/−</sup> MEF cells and transfected them with expanded GFP-tagged ataxin-3. Compared with wild-type MEF cells, KPN3<sup>−/−</sup> MEF cells showed a more prominent cytoplasmic distribution of ataxin-3 (Fig. 4A). Thus, we generated a SCA3/KPN3<sup>−/−</sup> mouse model by crossing CamKII/SCA3 77Q mice (designated SCA3) with KPN3 knockout mice (designated KPN3<sup>−/−</sup>) (30) to evaluate the effect of the loss of KPN3 on SCA3 disease progression. CamKII/SCA3 77Q mice were generated by crossing our inducible SCA3 mouse model (31) with the CamKII promoter mouse line (32), resulting in a neuronal expression of ataxin-3 (33). Mice that express both the transgene and have a KPN3 knockout were designated as SCA3/KPN3<sup>−/−</sup> mice. We confirmed the loss of KPN3 and the presence of the ataxin-3 transgene by Western blot analysis (Fig. 4B). We analyzed the localization of KPN3 in neurons of SCA3 mice and observed no differences with control mice. The next step in this analysis was to test whether the localization and aggregation effects we observed in vitro and in Drosophila were reproducible in our rodent models. We killed animals at 12 mo of age and ran whole-brain lysates through filter trap analysis to visualize the total aggregated protein (Fig. 4C). We observed that an increase in aggregation in our SCA3 model was rescued in the SCA3/KPN3<sup>−/−</sup> mouse model (Fig. 4C). This was in line with an observed trend in the reduction of ataxin-3 upon the knockout of KPN3, possibly due to enhanced clearance of cytoplasmic ataxin-3 (Fig. 4B). We subsequently performed immunohistochemical analysis of ataxin-3 in brains of SCA3/KPN3<sup>−/−</sup> mice.
and in SCA3 animals (Fig. 4D). We observed a shift of ataxin-3 staining from the nucleus to the cytoplasm in neurons of SCA3/ KPNA3<sup>−/−</sup> compared with SCA3 animals, thus providing further evidence that KPNA3 is directly involved in the pathogenic pathways behind aggregate formation and, likely, neurodegeneration. We further identified that SCA3 animals consume less food than their other littermates, but do not weigh less by 12 mo of age. This could point to a metabolic defect which has yet to be elucidated but is also improved in the KPNA3 knockout background (Fig. 4 E and F).

The Knockout of KPNA3 Alleviates the Neurological Phenotype Induced by Expanded Ataxin-3 in Vivo. Since the clinical manifestation of the disease symptoms in SCA3 patients is variable, we explored multiple levels of pathology in our animals to analyze the effect of KPNA3 knockout. First, we analyzed the overall activity of mice using home-cage analyses. At 11 mo of age, SCA3 mice showed an increase in activity during the dark phase which was almost double that of control animals (Fig. 5 A and B). This hyperactivity was correlated to a decrease in anxiety as shown by an increase in the exploration of the center of the home cage (Fig. 5 C and D). KPNA3 knockout rescued these total activity and anxiety deficits (Fig. 5). We then investigated classical neurological symptoms. SCA3 patients suffer from an impaired gait, including a reduced cadence and a reduced stride (34, 35). As cerebellar ataxia is described as a combination of various movement conditions, such as inability to coordinate movement, abnormal movement patterns, and disturbances in the rhythmicity of movement (36), we analyzed the nuanced nature of these abnormalities in our mice. We observed that SCA3 animals had a slower pace and cadence, mimicking the shuffled, slow gait of patients. This phenotype was largely improved by KPNA3 knockout (Fig. 5 F and G). We also observed differences in the base of support and visual distribution of footsteps, but quantification did not reach statistical significance (Fig. 5E). To analyze the pattern of movements, we analyzed the phase dispersion of paw steps, which tracks how the paws are placed in relation to each other. Both the front- and hind-paw phase dispersion was decreased in SCA3 animals, suggesting that they placed their other front or hind paw down sooner than expected in a normal gait, probably to support an unsteady, ataxic walk. This phenotype was statistically significantly rescued with KPNA3 knockout (Fig. 5H).

In summary, we demonstrated in cells, Drosophila, and mice that KPNA3 is a key player in the pathogenesis in SCA3: Its down-regulation keeps ataxin-3 in the cytoplasm alleviates the toxicity of expanded ataxin-3 and thereby phenotypic features in SCA3 models.

Discussion

The nuclear localization of expanded ataxin-3 confers a pathogenicity to the expanded protein whereby the protein hyper-aggregates and clearance of aggregates is deterred. Altering this localization was previously studied via various pathways such as inhibition of calpains or phosphorylation, but our work targets transport proteins (karyopherins) to explore their role in the pathogenic process (10, 11, 23, 37). We suggest that KPNA3 is a key player in the nuclear localization of ataxin-3 and that altering this localization mitigates the disease process (Fig. 6). After screening a subset of transport proteins in HEK cells, we identified three potential candidates (KPNA3, IPO13, and CRM1) for which the in vivo roles in SCA3 and neurodegenerative diseases in general have not been studied. We believe our findings will provide important insights into these proteins in the context of polyglutamine diseases.
Modulating CRM1 Had Reproducible and Promising Effects for SCA3 Pathology. Of all of the transport proteins screened, CRM1 has garnered the most attention in the field of polyglutamine disease. One reason could be the ability to inhibit CRM1 function with leptomycin B, a promising development in the field of cancer therapy. Additionally, knockdown and overexpression have been shown to be a modulator of ataxin-3 toxicity in two screens (26, 27). We were able to replicate the degeneration in the REP of Drosophila ortholog of CRM1 (Emb) was previously shown to be a modifier of ataxin-3 toxicity in two screens (26, 27). The Drosophila ortholog of CRM1 (Emb) was previously shown to be a modifier of ataxin-3 toxicity in two screens (26, 27).

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One reason could be the ability to inhibit CRM1 function with leptomycin B, a promising development in the field of cancer therapy. Additionally, knockdown and overexpression have been shown to be a modulator of ataxin-3 toxicity in two screens (26, 27). We were able to replicate the degeneration in the REP of Drosophila ortholog ofCRM1 (Emb) was previously shown to be a modifier of ataxin-3 toxicity in two screens (26, 27).
hypothetical hypotheses: The first one is that the currently identified NLS present in both constructs is sufficient for KPNA3 binding. It is also plausible that there is another unidentified binding site or that the polyglutamine stretch itself serves as a binding partner for KPNA3. While the exact binding mechanism has to be further investigated, the ability of KPNB1 overexpression to boost nuclear localization of ataxin-3 in vitro suggests that KPNA3 functions as a nuclear importer of ataxin-3, likely in a trimeric complex of KPNA3 and KPNB1 with ataxin-3. We further expanded our analysis by studying the Drosophila eye, a structure well suited to investigate neuronal degeneration. In this model, we observed that overexpression of the Drosophila homolog of KPNA3 increased nuclear localization and studies in SCA6 indicating that knockdown of KPNA3 increased structure breakdown, the amount of necrosis, and bristle disorganization, which fits to the increase in nuclear ataxin-3 upon overexpression. KPNA3 knockdown, on the other hand, was able to rescue the REP, photoreceptor degeneration, and nuclear aggregation of ataxin-3. This connection between cellular localization and neuronal function suggested that altering KPNA3 may be an interesting target to modulate disease progression of SCA3. Our hypothesis is also supported by recent studies of ALS which show that KPNA3 is able to increase TDP-43 nuclear localization and studies in SCA6 indicating that knockdown of Kap-α3 decreased nuclear protein and improved retinal degeneration in flies (39, 40).

**Knockout of KPNA3 Rescues the Behavioral Phenotype of Drosophila and Mouse Models of SCA3.** In a previous study, we demonstrated the critical importance of the nuclear localization of expanded ataxin-3 for the manifestation of symptoms in SCA3 (10). We now show that KPNA3 is the key transport protein to shuttle expanded ataxin-3 into the nucleus and to link it to neuronal dysfunction and death. Thus, we hypothesized that downregulation of KPNA3 may be an interesting target to rescue behavioral abnormalities caused by polyQ expansion of ataxin-3 in vivo models. As changes in total activity are well known in animal models of ataxin-3 (41), we used a Drosophila activity assay to analyze the effect of modulating Kap-α3. In our inducible expression model, SCA3 flies showed a decrease in total activity compared with their uninduced control counterparts. This hypactivity was rescued to control levels with a knockdown of Kap-α3, suggesting that its down-regulation is also able to rescue deficits related to the progression of SCA3. To confirm these data in a rodent model, we analyzed SCA3/KPNA3<sup>−/−</sup> mice, studied their total activity, and compared the readout to their SCA3 and CTRL counterparts. It has been reported that SCA3 mouse models eventually develop hypoactivity as seen in Drosophila, but a phase of hyperactivity can be a predecessor to this hypovactive stage (41). Hyperactivity as a sign of neuronal dysfunction has been observed in models for SCA3 (10, 31, 42) as well as in HD (43) and in Parkinson’s disease (44). Our mice did, in fact, show a hypactive phenotype characterized by increased total activity during the dark/active phase. This phenotype progressed throughout the lifespan of the animals and was rescued in the SCA3/KPNA3<sup>−/−</sup> mice. Consistent with our Drosophila data, the total activity of our SCA3 mice was brought back to control levels by knocking out KPNA3. In the same home-cage analysis, we were also able to assay the anxiety phenotype. The degeneration of the cerebellum and its afferent and efferent regions causes a variety of nonmotor behavior concerns for patients, with disturbance of sleep, depression, and anxiety being the most predominant (45, 46). Animal models are able to mimic these disturbances in anxiety, which present themselves as a decrease in open-space avoidance (10, 31, 42, 47). In our SCA3 animals, this decrease in anxiety was rescued by the knockout of KPNA3. This suggests that KPNA3 has an effect on psychiatric symptoms of SCA3 as well as behavioral symptoms such as total activity. This is interesting in relation to the current research which puts KPNA3 at the center of multiple psychiatric disorders.
KPNA3 single-nucleotide polymorphisms were found to be significantly associated with schizophrenia, opiate dependence and alcohol dependence at the genotype level and with major depressive disorder at the allele level (48). Thus, we conclude that the in vitro findings which show KPNA3’s effect on aggregation and localization translate to a rescue of behavior and psychiatric phenotypes in our Drosophila and mouse models of SCA3. These data are significant because they add to the body of knowledge which suggests that localization of the ataxin-3 protein is necessary for disease manifestation and that modulating this localization through transport proteins is a way to control the development of these symptoms.

**KPNA3 Knockout Is Able to Rescue Gait Abnormalities in a Mouse Model of SCA3.** While we were able to see differences in total activity and anxiety with the home-cage analysis, we wanted to know if the benefits of KPNA3 knockout also extend to the gait phenotype observed in patients and animal models. Gait disturbances are some of the strongest features of SCA3, and the initial description of cerebellar ataxia included difficulty with coordinating movement, issues in muscle tone, and abnormal movement patterns (36). These features can be measured in mouse models and present a way to further analyze the effect of KPNA3 knockout on SCA3 progression. For this, we employed the CatWalk gait analysis system, which has been used and validated for the analysis of nerve injury but also of neurodegenerative diseases (49). We were able to demonstrate that SCA3 animals also showed an inability to maintain this normal walking pattern as measured by phase dispersion (the timing of paw placement in relation to a full stride). SCA3 mice preemptively placed their second paw for either support or lack of coordination, which mirrors both joint coordination and walking pattern issues. It is important to note that these results suggest that the ataxic gait observed in patients and in the SCA3 mouse model is influenced by both balance-related impairments and limb control and intralimb coordination. The crude and more nuanced features together paint a fuller picture of the walking disturbances which can be tracked during disease progression and in animal models. Our data add significant knowledge to the field. Not only have we performed a thorough analysis of the gait in SCA3 animals, but we have also been able to demonstrate that these features can be either fully or partially improved with KPNA3 knockout.

**Conclusion**

Overall, we believe this work opens promising insight into the pathogenesis of SCA3. We have demonstrated that altering transport proteins has an effect on both pathogenic mechanisms (e.g., the intracellular localization and the formation of aggregates) and key features of disease manifestation such as anxiety, total activity, and gait. The downstream mechanisms, possibly involving ataxin-3 clearance and degradation, are still to be investigated, and the correlation of KPNA3 with psychiatric disorders warrants further exploration into the interplay between KPNA3 levels and expansion of ataxin-3 within the patient population. We suggest that a better appreciation of the overall cellular mechanism can enhance our understanding of all polyglutamine diseases as well as aid in the development of novel targets for therapy.

**Materials and Methods**

**Cell Culture Analysis.** HEK 293-T, SK-N-AS, and MEF cells were grown at 37 °C and 5% CO2 in supplemented Dulbecco’s modified Eagle medium (Invitrogen). For stable transfection, HEK 293-T cells were transfected with GFP-tagged constructs and FAC-sorted for highly expressing cells. Expression constructs were obtained from Origene (TrueClones; OriGene Technologies).

**Aggregate Analysis and Fractionation.** Filter trap analysis were performed as described (52). Cells were collected 96 h posttransfection, measured by Bradford assay (Bio-Rad Laboratories), and run on a cellulose acetate membrane (Carl Roth) with the filter trap apparatus (SRC 96 D Minifold-1 Dot Blotter; Schleicher & Schuell). Aggregate fractionation was performed as described (53). HEK 293-T cells were transiently transfected with GFP-ataxin-3 and transport protein constructs and incubated for 72 h. After separation, proteins were transferred to a nitrocellulose membrane (Optitran and Protran; Millipore).

**In Vitro Nuclear Import Assay.** U2OS cells were digitonin-permeabilized and incubated with IP buffer plus (IP buffer, pH 7.5, 10 mg/mL BSA, and 2 mM DTT). The import reaction was started by the addition of import mix containing Ran-GDP, fluorescein-labeled ataxin-3, ATP regeneration system (ATP, GTP, creatine phosphate, and creatine kinase), KPNA3, importinß/KPNB1, RanGAP, RanBP1, and EGTA in IP buffer, pH 7.5. The samples were fixed and analyzed by fluorescence microscopy. DAPI fluorescence was used for single-cell discrimination.

**Western Blot Analysis.** Filter trap and Western blot membranes were essentially detected as described (21). Membranes was detected by using an Odyssey Fc Imager (LI-COR Biosciences) and quantified by using the Image Studio Software (LI-COR).

**Immunofluorescence and Immunohistochemical Stainings.** Cells were fixed with 4% PFA and then incubated with primary and secondary antibody. Immunohistochemical stainings were performed as described (54). Sections were stained by using the biotin-conjugated secondary antibody, detected by using the VECTASTAIN Elite ABC Kit (Vector Laboratories) and 3,3’-diaminobenzidine (DAB; Sigma). Fluorescence and DAB staining was visualized with an Axioplan 2 imaging microscope (Carl Zeiss Microimaging).
**Drosophila Strains and Housing.** The GMR-GAL4 (FlyBaseID: FBti0002994, Tables S3 and S4), UASp-Kap-alpha3 (FlyBaseID: Fbi019875), Kap-alpha3 (TRI) [JF01311] (SCA3 [JF01311]), UASp-MJD.tr-Q78 (SCA3 [JF00456]) strains were obtained from the Bloomington Stock Center (Indiana University). The lio-GAL4 (FlyBaseID: Fbi0115618) strain was provided by J. M. Dura, Institute of Human Genetics, Montpellier, France. The UAS-ataxin-3-Q70 (S5) and the da-GS (S6) have been described. The sg1 [v2106] line is used as a RNAi negative control (since it targets a protein absent in adult flies). This line, the Kap-alpha3 [v36104], and the v106249 lines were obtained from the Vienna Drosophila Resource Center. The elav-GS line was derived by recombination of the elav-GS-201.2 line (S7) and provided a strong neuronal inducer. Fly stocks were maintained on a standard cornmeal-agar–yeast–sugar–based diet at 18 °C. Standard cross-breeding and other experiments with larvae and adult Drosophila were conducted at 26 °C on the same diet. Prenymph was collected from the day of hatching and sorted by gender and appropriate phenotypic markers. For induction of UAS transgene expression, flies were treated with 100 µg/mL RU486 (in ethanol); uninduced flies were treated with ethanol only.

**Drosophila SG Expression of Ataxin-3.** SGs from third-instar larvae were dissected, fixed in 4% PFA, permeabilized with 0.1% Triton X in PBS, and blocked with 2% BSA in PBS. To detect ataxin-3, tissues were incubated overnight in 1H antibody (1:1,000, Chemicon, Merck Millipore) or anti-HA antibody (1:500; Millipore) at 4 °C. To detect primary antibodies, Alexa Fluor 594 anti-mouse or Alexa Fluor 488 anti-rabbit secondary antibodies (diluted 1:200; Life Technologies) were used. Imaging was done with the Zeiss LSM700 confocal microscope. Genotypes in Fig. 2 A–D are lio-GAL4, ataxin3-7Q0/sg1 [v21206] (SCA3; Control); lio-GAL4, ataxin3-7Q0/Uasp-Kap-alpha3 (SCA3; KPNA3 OE); lio-GAL4, ataxin3-7Q0/Kap-alpha3 (TRI [JF02686] SCA3; KPNA3 KD); lio-GAL4, ataxin3-7Q0/Emb [EY08770] (SCA3; CRM1 OE); lio-GAL4, ataxin3-Q70/Emb [TRI [JF01311] (SCA3; CRM1 KD); lio-GAL4, ataxin3-Q70/Emb [EY08770] (SCA3; CRM1 KD); lio-GAL4, ataxin3-Q70/Cdm [TRI [JF01428] (SCA3; CRM1 KD); lio-GAL4, ataxin3-Q70/Cdm [EY08770] (SCA3; CRM1 KD); lio-GAL4, ataxin3-Q70/Cdm [EY08770] (SCA3; CRM1 KD); lio-GAL4, ataxin3-Q70/Cdm [EY08770] (SCA3; CRM1 KD); lio-GAL4, ataxin3-7Q8/Kap-alpha3 (TRI [JF02686] SCA3; KPNA3 KD); lio-GAL4, ataxin3-7Q8/Uasp-Kap-alpha3 (SCA3; KPNA3 OE);

**Drosophila REP Modification.** For analysis, flies were grown at 25 °C. Male flies were collected 1 d posthatching and imaged on a Leica M205FA microscope. Genotypes in Fig. 3 A–D are GMR-GAL4, MJDtr-7Q0/sg1 [v21206] (SCA3; Control); GMR-GAL4, MJDtr-7Q0/Uasp-Kap-alpha3 (SCA3; KPNA3 OE); GMR-GAL4, MJDtr-7Q0/Kap-alpha3 (TRI [JF02686] SCA3; KPNA3 KD); GMR-GAL4, MJDtr-7Q0/Emb [EY08770] (SCA3; CRM1 OE); GMR-GAL4, MJDtr-7Q0/Emb [TRI [JF01311] (SCA3; CRM1 KD); GMR-GAL4, MJDtr-7Q0/Cdm S1766 (SCA3; IP3013 OE); GMR-GAL4, MJDtr-7Q0/Cdm [TRI [JF01428] (SCA3; IP3013 KD). Genotypes in Fig. 3 E–H are GMR-GAL4, MJDtr-7Q0/sg1 [v21206] (SCA3; Control); GMR-GAL4, MJDtr-7Q0/Emb [EY08770] (SCA3; CRM1 OE); GMR-GAL4, MJDtr-7Q0/Emb [TRI [JF01311] (SCA3; CRM1 KD); GMR-GAL4, MJDtr-7Q0/Cdm S1766 (SCA3; IP3013 OE); GMR-GAL4, MJDtr-7Q0/Cdm [TRI [JF01428] (SCA3; IP3013 KD). Genotypes in Fig. 3 I–N and P are R1-GAL4, MJDtr-7Q0/sg1 [v21206] (SCA3; Control); R1-GAL4, MJDtr-7Q0/Uasp-Kap-alpha3 (SCA3; KPNA3 OE); R1-GAL4, MJDtr-7Q0/Kap-alpha3 (TRI [JF02686] SCA3; KPNA3 KD); R1-GAL4, MJDtr-7Q0/Emb [EY08770] (SCA3; CRM1 OE); R1-GAL4, MJDtr-7Q0/Emb [TRI [JF01311] (SCA3; CRM1 KD); R1-GAL4, MJDtr-7Q0/Emb [EY08770] (SCA3; CRM1 KD); R1-GAL4, MJDtr-7Q0/Emb [TRI [JF01428] (SCA3; IP3013 KD).

**Drosophila Retina Morphology.** Eight- or 20-d-old Drosophila heads were paraffin-embedded, and frontal retinal sections (5 µm) were prepared. For global retinal morphology, sections were stained with hematoxylin and eosin and imaged on a Leica DM2000 LED microscope. For aggregates visualization, sections were immune-labeled with the rabbit anti-HA antibody (1:200; Millipore) and the Alexa Fluor 594 anti-rabbit secondary antibodies (diluted 1:200; Life Technologies). Images were acquired by using a Leica TCS SP5 confocal microscope.

**Drosophila qRT-PCR.** For validation of Kap-alpha3 down-regulation by RNAi, RNA was extracted with TRIzol from 10 flies. After reverse transcription using SuperScript III (Invitrogen), flyma primer verified primers (www.flyrna.org/flyprimerbank) were used. For Fig. S1, genotypes mentioned (Table S7) were used. For the lio-GAL4, ataxin3-7Q0/sg1 [v21206] line was used as a RNAi negative control (since it targets a protein absent in adult flies). The Kap-alpha3 [v36104], and the v106249 lines were obtained from the Vienna Drosophila Resource Center. The elav-GS line was derived by recombination of the elav-GS-201.2 line (S7) and provided a strong neuronal inducer. Fly stocks were maintained on a standard cornmeal-agar–yeast–sugar–based diet at 18 °C. Standard cross-breeding and other experiments with larvae and adult Drosophila were conducted at 26 °C on the same diet. Prenymph was collected from the day of hatching and sorted by gender and appropriate phenotypic markers. For induction of UAS transgene expression, flies were treated with 100 µg/mL RU486 (in ethanol); uninduced flies were treated with ethanol only.

**Mouse Housing and Experiments.** Mice were housed specific pathogen-free in type II cages on a 12-h light–dark cycle with a 50–55% relative air humidity. Mice had ad libitum access to food and water. All research and animal care procedures were approved by the government department and performed according to international guidelines for the use of laboratory animals in the Forschungsverfugungsgebäude (FORS) animal facility in Tuebingen, Germany. Animal welfare was routinely monitored by the caretakers of the animal facility and the veterinary service of the University of Tuebingen.

**Mouse Genotyping.** Transgenic animals were identified by using DNA extracted from ear biopsy (Roche High Pure PCR Template Preparation Kit; Roche) as described in ref. 6 (Table S6).

**Preparation of Mouse Samples.** Mice were euthanized by carbon dioxide and transcardially perfused with PBS. Brain was excised from the cranium, and brain hemispheres were dissected sagittally. Hemispheres were flash-frozen in liquid nitrogen and stored at −80 °C.

**Mouse Behavior Testing.** Animals were tested in increments of 3 mo until 12 mo of age, and a break of at least 48 h was applied between different tests. The experimenter was blinded for the genetic status of the mice during the behavioral tests, and the order of mice was independent of their genetic status. Control and compound mice were represented by heterozygous ataxin-3 mice and single transgenic SCA3 mice. During behavior testing, mouse weight was monitored. No significant difference in weight was observed. Spontaneous activity was measured in a home-cage environment by using an automated tracking system (LabMaster; TSE Systems). Mice were recorded for 22 h, which started before the onset of the dark phase. To measure gait abnormalities and footprint disruptions, the CatWalk gait analysis system was used (Noldus). Mice were put on the CatWalk system and allowed to freely cross the runway without interruption. Three runs were gathered with a speed variation <50%.

**Statistical Analysis.** Data are presented as mean ± SEM. Statistical significance was calculated by using GraphPad Prism (GraphPad Software). Student’s two- and one-sided t tests and two-way ANOVA were used as appropriate. A P value of <0.05 was treated as statistically significant. CatWalk, LabMaster Homecage, and TriKinetics parameters were reanalyzed and analyzed with Windows Excel and GraphPad Prism.

**ACKNOWLEDGMENTS.** We thank Gemma Arlands and Raheleh Heidari for their technical help, Jeannette Huebener-Schmid for her support, Maciej Fijgel for graphics, and Nicholas Casadei for statistical support. The work leading to this invention was supported by European Commission Seventh Framework Programme FP7/2010 under Grant 264508 (TreatPolyQ) and the Federal Ministry of Education and Research (PPPT-MJD Grant 01GM1309B) under the umbrella of E-Rare-2 (ERA [European Research Area]-Net for research programmes on rare diseases).
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