**Cul3** regulates cytoskeleton protein homeostasis and cell migration during a critical window of brain development

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De novo loss of function mutations in the ubiquitin ligase-encoding gene **Cullin3** (**CUL3**) lead to autism spectrum disorder (**ASD**). In mouse, constitutive **Cul3** haploinsufficiency leads to motor coordination deficits as well as **ASD**-relevant social and cognitive impairments. However, induction of **Cul3** haploinsufficiency later in life does not lead to ASD-relevant behaviors, pointing to an important role of **Cul3** during a critical developmental window. Here we show that **Cul3** is essential to regulate neuronal migration and, therefore, constitutive **Cul3** heterozygous mutant mice display cortical lamination abnormalities. At the molecular level, we found that Cul3 controls neuronal migration by tightly regulating the amount of Plastin3 (**Pls3**), a previously unrecognized player of neural migration. Furthermore, we found that Pls3 cell-autonomously regulates cell migration by regulating actin cytoskeleton organization, and its levels are inversely proportional to neural migration speed. Finally, we provide evidence that cellular phenotypes associated with autism-linked gene haploinsufficiency can be rescued by transcriptional activation of the intact allele in vitro, offering a proof of concept for a potential therapeutic approach for **ASDs**.

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The past decade has seen a major effort to elucidate the genetic underpinnings of autism spectrum disorders (ASDs). Whole exome sequencing of large patient cohorts and their unaffected family members has identified hundreds of ASD-risk loci. However, the molecular and cellular functions of the majority of the identified genes remain poorly understood. One of the identified high-risk ASD genes encodes the E3 ubiquitin ligase Cullin3 (Cul3).

Cul3 is a conserved protein of the Cullin family, comprising eight members, which contain a conserved cullin homology domain, named after its ability to select cellular proteins for degradation. Cul3-associated genetic variants are most often de novo missense or loss of function (loF) mutations, dispersed throughout the entire gene and affecting distinct protein domains. In addition to the ASD core symptoms, patients with Cul3 de novo loF mutations can present with several comorbidities including varying levels of intellectual disability (ID), attention deficit hyperactivity disorder (ADHD), sleep disturbances, motor deficits, epileptic seizures, and facial dysmorphisms. The only known exception is the deletion of Cul3 exon 9 by a specific dominant splice site variant causing a severe form of pseudo-hy poaldosteronism type II (PHAII), featuring hypertension, hyperkalemia, and metabolic acidosis but not ASD.

The well-understood process of CUL3-mediated protein ubiquitination and degradation allows target recognition and speciﬁc proteasomal degradation. CUL3-dependent proteasomal degradation pathway is associated with the deletion of Cul3 protein in the brain, to approximately 50% of wild-type levels, indicating motor defects, such as increased sway and stance length, and mild gait abnormalities, such as limb clasping and mild gait abnormalities, such as increased sway and stance length (Fig. 1b, c and Supplementary Data 2). However, the second phase of the assay, mutant mice show no preference for a stranger mouse (M2) over a familiar animal (M1), a preference displayed by control animals (Fig. 1f). We thus concluded that haploinsufficiency of the Cul3 gene is associated with reduced interest in social novelty. As social recognition is mainly achieved via olfaction in rodents, we assessed the ability of mutant animals to distinguish and familiarize themselves with non-social and social odors. In the odor discrimination and habituation test (ODDH)26, both wild-type and Cul3+/− animals successfully recognize newly and already presented odors (Supplementary Data 2). However, mutant mice spend signiﬁcantly more time exploring odor-embedded cotton swabs and are hyper-reactive to the presentation of social odors (Fig. 1g and Supplementary Data 1). Thus, despite mutant animals spending signiﬁcantly more time sniffing social odors than controls, Cul3+/− mice are able to distinguish between two different social odors, indicating that the reduced social novelty interest is not directly related to odor discrimination issues. Finally, we employed a well-established memory test27 to assess how Cul3 haploinsufﬁciency affects learning. Contextual fear conditioning revealed normal fear acquisition and memory retention in Cul3+/− mice. However, mutant animals exhibit a reduced ability to extinguish the aversive memory after extinction training, pointing towards abnormal cognition (Fig. 1h).

In summary, our analysis indicates that Cul3 haploinsufﬁciency leads to abnormalities in several behavioral paradigms, potentially associated with dysfunction of different brain areas and/or dysfunctional brain connectivity.

Results

Behavioral defects in Cul3 haploinsufﬁcient animals. To model ASD-linked mutations, we studied a constitutive heterozygous Cul3 knockout (Cul3+/−) mouse (Supplementary Fig. 1a). As predicted, Cul3+/− animals show a signiﬁcant decrease in Cul3 protein in the brain, to approximately 50% of wild-type levels (Supplementary Fig. 1b and Supplementary Data 1). Importantly, Cul3 protein reduction is equal in all brain areas tested (Supplementary Fig. 1b), thus resembling patients with germline mutations.

Although Cul3 haploinsufﬁcient mice have a slightly reduced body weight at birth, their weight is comparable to control animals as adults (Supplementary Fig. 1c), while the brain to body weight ratio is unaffected in mutant newborn and adult mice (Supplementary Fig. 1d). Adult Cul3+/− mice present with hind limb clasping (Fig. 1a) and mild gait abnormalities, such as increased sway and stance length (Fig. 1b, c and Supplementary Fig. 2a), phenotypes which are observed in other ASD mouse models28,29 and indicative of cerebellar dysfunctions. Further, indicating motor defects, Cul3+/− mice undergo when challenged on the accelerating RotaRod (Fig. 1d, d′), a task requiring formation and consolidation of a repetitive motor routine22,23. Mutant mice do not reach the same level of motor performance as their healthy counterparts by the end of the third day of trials, suggesting abnormal motor learning dynamics (Fig. 1d). Additionally, while no general sex differences were observed in Cul3 heterozygous knockout mice, male Cul3 haploinsufﬁcient animals show reduced initial coordination compared to their wild-type littermates (Supplementary Fig. 2b).

Motor defects of Cul3+/− mice, however, do not affect exploratory behavior in the open ﬁeld (Supplementary Fig. 2c), nor on the elevated plus maze, where Cul3+/− animals do not show differences in anxiety-like behaviors (Supplementary Fig. 2d).

Next, we subjected Cul3+/− animals to classical sociability tests. In the three-chamber test, similarly to wild-types, Cul3+/− mice show a preference for a mouse (M1) over an object (Ob.) (Fig. 1e). However, in the second phase of the assay, mutant mice show no preference for a stranger mouse (M2) over a familiar animal (M1), a preference displayed by control animals (Fig. 1f). We thus concluded that haploinsufﬁciency of the Cul3 gene is associated with reduced interest in social novelty. As social recognition is mainly achieved via olfaction in rodents24,25, we assessed the ability of mutant animals to distinguish and familiarize themselves with non-social and social odors. In the odor discrimination and habituation test (ODDH)26, both wild-type and Cul3+/− animals successfully recognize newly and already presented odors (Supplementary Data 2). However, mutant mice spend signiﬁcantly more time exploring odor-embedded cotton swabs and are hyper-reactive to the presentation of social odors (Fig. 1g and Supplementary Data 1). Thus, despite mutant animals spending signiﬁcantly more time sniffing social odors than controls, Cul3+/− mice are able to distinguish between two different social odors, indicating that the reduced social novelty interest is not directly related to odor discrimination issues. Finally, we employed a well-established memory test27 to assess how Cul3 haploinsufﬁciency affects learning. Contextual fear conditioning revealed normal fear acquisition and memory retention in Cul3+/− mice. However, mutant animals exhibit a reduced ability to extinguish the aversive memory after extinction training, pointing towards abnormal cognition (Fig. 1h, h′).

In summary, our analysis indicates that Cul3 haploinsufﬁciency leads to abnormalities in several behavioral paradigms, potentially associated with dysfunction of different brain areas and/or dysfunctional brain connectivity. 
**Fig. 1 Behavioral defects in Cul3 haploinsufficient mice.**

**a** Hind limb claspng in adult *Cul3*+/− mice, not observed in wild-type littermate controls (a top; scoring 0–1 (normal) to 3 (most severe)) (b bottom, n = 25 animals per genotype; ***P < 0.0001; two-tailed Mann–Whitney U-test). **b** *Cul3*+/+ and *Cul3*+/− strides, forepaws (blue), and hind paws (red). **c** Altered gait of *Cul3*+/− mice evidenced by comparison of sway (c top) and stance length (c bottom) (n = 19 mice per genotype; *P = 0.03, ***P = 0.0004; two-tailed t-test). **d, d’** Accelerating RotaRod test revealing defects in motor performance and coordination in *Cul3*+/− mice. Shown: sum of daily latencies of three trials per day on three consecutive days and final rpm on day one—trial 1, i.e.; initial coordination (d′) (n = 30 mice per genotype; *P = 0.021, **P = 0.001, ***P = 0.0005; 2-way ANOVA and Sidak’s multiple comparison test and unpaired two-tailed t-test). **e, f** Heat maps of three-chamber social interaction test (left), quantification of interaction times (middle), and social preference/novelty index (right). Sociability: *Cul3*+/− and control mice spend more time with a stranger mouse (M1) than an object (Ob.) (e); social novelty: *Cul3*+/− mice do not prefer a novel stranger (M2) over the familiar mouse (M1) (f) (n = 24 mice per genotype; *P = 0.01, ***P < 0.0001, n.s. not significant; 1-way ANOVA and Sidak’s multiple comparison test). **g** Both genotypes distinguish and familiarize to non-social and social odors in the olfaction habituation and dishabituation test, yet *Cul3*+/− mutant mice are hyper-reactive to the presentation of social odors (n = 24 mice per genotype; **P = 0.002, ***P = 0.0009, n.s. not significant; 2-way ANOVA and Sidak’s multiple comparison test; details in Supplementary Data 1, 2). **h, h’** Contextual memory retention and extinction scored as percent freezing during a 3 min exposure to the context (h), and fear-acquisition training (h′) (n = 26 mice per genotype; *P = 0.027, n.s., not significant; 2-way ANOVA interaction: F1,100)= 6.18; P = 0.015; Sidak’s multiple comparisons test: Extinction P = 0.027). Sex-matched littermate animals were analyzed. Data presented either as mean ± SEM, as well as scatter plot (a, c, d, e, f, h) or as boxplot showing median value and 25–75th percentile, whiskers show minimum and maximum (d, g, h’). Detailed statistics are provided in Supplementary Data 1.
while \(\text{Cul3}^{+/}\) Cag-CreER mice show normal survival into adulthood (Supplementary Fig. 2e, f). This finding underscores a dose-dependent effect of Cul3 depletion and highlights the necessity of employing construct valid mouse models when investigating human pathophysiology.

Next, we performed a thorough behavioral characterization of the \(\text{Cul3}^{+/}\) Cag-CreER animals treated with tamoxifen, testing all the paradigms in which we observed behavioral abnormalities in the constitutive adult \(\text{Cul3}^{-/}\) line (Fig. 1). We found that induction of Cul3 haploinsufficiency at P30 does not result in any major behavioral defect, except for reduced motor performance on the accelerating RotaRod. More specifically, while we did not observe any increase in hind limb clasping events (Fig. 2d), abnormalities in gait (Supplementary Fig. 2g) or initial coordination on the RotaRod, tamoxifen injected \(\text{Cul3}^{+/}\) Cag-CreER mice significantly underperform on the third day of RotaRod training (Supplementary Fig. 2h and Fig. 2e, e'), indicating that Cul3 function is required for motor learning in adult animals. In contrast, \(\text{Cul3}^{+/}\) Cag-CreER treated with tamoxifen behave comparably to the controls in the three-chamber sociability test,
with normal interest in social novelty (Fig. 2f, g). Further, they react normally to the social odors in an adapted OHDH test (Fig. 2h and Supplementary Data 2) and show comparable cognitive abilities in the CFC paradigm to the controls (Fig. 2i, i’). All of these behaviors were clearly perturbed in animals with germ line Cul3 haploinsufficiency but appear normal when Cul3 is deleted at the juvenile stage. These results indicate that developmental stages are critical for the appearance of Cul3-associated behavioral phenotypes.

Cul3 haploinsufficiency is associated with abnormal brain development. Having established that ASD-relevant behaviors are associated with Cul3 functions in the developing brain, we investigated its expression pattern. Temporally, in the mouse brain, Cul3 expression peaks at E14.5 and E16.5 (Supplementary Fig. 3a, b). Spatially, it is predominantly expressed in the cortex and hippocampus (Supplementary Fig. 3c), in glutamatergic and inhibitory neurons (Supplementary Fig. 3d). These data are in line with the Cul3 expression profile in the human brain (Supplementary Fig. 3e–g) and point toward an important role for Cul3 in neuronal cells during early brain development. Thus, to understand whether behavioral defects are accompanied by neuroanatomical changes, we performed crystal violet (Nissl) stainings of adult brain sections obtained from Cul3+/<sup>b</sup> mice, showing both reduced cell density and altered neuronal morphology (Fig. 3a, b). Spatially, it is predominantly expressed in the cortex and hippocampus (Supplementary Fig. 3c), in glutamatergic and inhibitory neurons (Supplementary Fig. 3d). These data are in line with the Cul3 expression profile in the human brain (Supplementary Fig. 3e–g) and point toward an important role for Cul3 in neuronal cells during early brain development. Thus, to understand whether behavioral defects are accompanied by neuroanatomical changes, we performed crystal violet (Nissl) stainings of adult brain sections obtained from Cul3+/<sup>b</sup> mice, showing both reduced cell density and altered neuronal morphology (Fig. 3a, b).

In addition, Cul3/<sup>b</sup> Emx1-Cre mice show severe brain malformations with pronounced cortical and hippocampal atrophy (Fig. 3f and Supplementary Fig. 5c). Similar to what we observed in Cul3+/<sup>b</sup> animals, fluorescence imaging of Cux1 and Ctip2 distribution revealed lamination defects in Cul3<sup>b</sup> Emx1-Cre and Cul3+/<sup>b</sup> Emx1-Cre mice (Fig. 3g–i). Thus, proper Cul3 dosage is essential to guarantee correct brain development in mouse.
Fig. 3 Abnormal lamination of the cortex and migration defects in Cul3 mutant mice. a-d Immunofluorescent stainings for Ctip2 and Cux1 on coronal brain sections revealed laminar thinning in adults (a, b) and newborn (PO) Cul3+/− animals (c, d) (n(adults) = 3 littermates per genotype; n(PO) = 6 littermates per genotype; *P = 0.04; **P < 0.01; 2-way ANOVA and Sidak’s multiple comparison test). e, i Bin-wise comparison of relative cell numbers (in %) revealed a shifted Cux1/Ctip2 layer profile, indicating laminar defects at PO (n = 3 littermates per genotype; *P < 0.02, **P < 0.01, ***P < 0.001; 2-way ANOVA, Sidak’s multiple comparison test). f Nissl-staining of PO coronal, Cul3+/−, Cul3+/+/ Emx1-Cre, and Cul3+/− Emx1-Cre brain sections show severe brain malformations in Cul3+/− Emx1-Cre pups (n = 3 littermates per genotype). g, h Immunofluorescent staining with antibodies against Ctip2 and Cux1 reveal cortical laminar thinning in both Cul3+/− Emx1-Cre and Cul3+/− Emx1-Cre pups at PO (n = 3 littermates per genotype; *P < 0.05, **P < 0.01, ***P < 0.001; 2-way ANOVA, Sidak’s multiple comparison test). j Scheme of the BrdU birthdate labeling experiments. k, m Injection of BrdU at E16.5 and anti-BrdU immunofluorescent (IF) staining and analysis of total BrdU positive (BrdU+) cells in cortical columns at PO shows severely decreased number of BrdU+ cells in Cul3+/− Emx1-Cre brains, but not in the Cul3+/− Emx1-Cre and Cul3+/− cortex (n = 3 littermate pairs per genotype; ***P = 0.0001, n.s. not significant; 1-way ANOVA and Sidak’s multiple comparison test and unpaired two-tailed t-test). l, n Bin-wise analysis of relative numbers of BrdU+ cells showed decreased numbers of BrdU+ cells in upper bins and increased numbers of BrdU+ cells in lower bins in Cul3+/− Emx1-Cre, Cul3+/− Emx1-Cre (c) and Cul3+/− (e) mice (n = 3 littermate pairs per genotype; *P < 0.05, **P < 0.001; 2-way ANOVA and Sidak’s multiple comparison test). Data presented as stacked bar-plots of mean ± SEM in b, d, h, and connected mean ± SEM in e and i. Data presented as mean ± SEM and scatter plot in k, m and connected mean ± SEM in l and n. Scale bars: 50 μm in a, 25 μm in c, g and 200 μm in f, 25 μm in k, m; numbers in c, g, k, m indicate depth in the cortex. Detailed statistics are provided in Supplementary Data 1.
intermediate progenitor cells in the SVZ of the heterozygous knockout (Supplementary Fig. 6b).

To test whether abnormal cell migration causes the lamination defects in heterozygous Cul3 mutant mice we again pulsed E16.5 embryos with BrdU, but this time analyzed the number and position of BrdU+ cells in the cerebral cortex at P0 (Fig. 3j). We found a severe reduction in BrdU+ cells in Cul3+/fl Emx1-Cre P0 animals compared to control samples from Cul3+/+ littersmates (Fig. 3k), consistent with the above-mentioned increase in neural cell apoptosis in Cul3+/fl Emx1-Cre embryos. In addition, we found that a substantially smaller fraction of BrdU+ cells reaches the upper cortical layers and that a significant number of BrdU+ cells remain stranded in lower cortical layers in Cul3+/fl Emx1-Cre animals (Fig. 3i). These results suggest that complete deletion of Cul3 in the forebrain leads to neural cell cycle defects, apoptosis and neuronal migration defects. Importantly, while Cul3+/fl Emx1-Cre samples do not show a reduction in total BrdU+ cells, suggesting normal production and survival of cortical neural cells, Cul3+/fl Emx1-Cre pups present a clear reduction of BrdU+ cells reaching the upper part of the cortex (Fig. 3i). We observed the same defect in the cerebral cortex of constitutive Cul3+/− mice (Fig. 3m, n), indicating that Cul3 haploinsufficiency is associated with a neuronal migration phenotype, thus explaining the observed lamination defects. Accordingly, when assessing the cell density in cortical layers V/VI, we counted a significant increase in the number of cell nuclei in 100 × 100 µm windows in layers V/VI of Cul3+/− animals at P0, indicating that a substantial number of neurons accumulates in those layers (Supplementary Fig. 6c).

To obtain more direct evidence of migration defects in Cul3+/− embryonic cortical tissues, we performed in utero ventricular injections of human Synapsin-GFP-encoding lentivirus at E13.5, prepared acute slices from transduced brains at E17.5, and performed live-cell imaging over the course of 16 h (Fig. 4a). Individual GFP-labeled radially migrating cells were manually tracked and the average migration speed and velocity were calculated for neurons in the Cul3+/− and Cul3+/+ developing cortex. We found that radially migrating neurons in the Cul3+/− cortex travel slower and shorter distances as compared to migrating neurons of their control littermates (Fig. 4b–f).

Next, since we had predominantly quantified the position of glutamatergic neurons (Fig. 3e, i), we tested whether other cell types in the brain might be similarly affected. To this end, we counted the density of interneurons, astrocytes, and microglia in the adult cortex. Interestingly, while the number of interneurons in the cerebral cortex is significantly reduced (Supplementary Fig. 6d), the amount and position, of astrocytes and microglia are unchanged in Cul3 mutant animals (Supplementary Fig. 6e, f). This difference may be explained by the fact that Cul3 expression is highest in excitatory and inhibitory neurons, potentially making them more susceptible to Cul3-dependent defective protein homeostasis (Supplementary Fig. 3d, g).

To better understand how Cul3 mutations affect cell migration, we switched to an in vitro model system. Analysis of neural progenitor cells (NPCs) generated from E13.5 Cul3+/+ and Cul3+/− cortices (Supplementary Fig. 6g) confirmed abnormal neural cell motility in vitro (Fig. 4g–j, Supplementary Fig. 6h). Specifically, we traced the movement of NPCs moving away from neurospheres over the course of several hours and found that Cul3 mutant cells travel shorter distances than control cells (Fig. 4g, h). In addition, the live-imaging analysis revealed that mutant NPCs do not migrate far from the sphere, move less, and have reduced migration speed (Fig. 4i, j and Supplementary Fig. 6h, Supplementary Movies 1, 2). Cul3 haploinsufficiency leads to abnormal neuronal network activity. We reasoned that defects in neural cell migration and cortical lamination could have an important impact on neuronal network activity. Indeed, other ASD-risk genes associated with defects in neuronal migration have been shown to substantially modify neuronal network activity in vivo30. To this end, we evaluated the spontaneous network activity in two-months-old Cul3+/− mice, recording spontaneous postsynaptic currents (sPSC) from pyramidal neurons in layer 2/3 of the cortex in whole-cell configuration. Both spontaneous excitatory and inhibitory postsynaptic currents (sEPSC and sIPSC, respectively) are reduced in mutant animals (Supplementary Fig. 7a–d). In particular, Cul3+/− mice show a reduction in sEPSC amplitude (Supplementary Fig. 7b) and frequency, evidenced by an increased mean inter-event interval (IEI) (Supplementary Fig. 7c) compared to wild-type littermates. Cul3+/− and wild-type mice showed similar sIPSC peak currents, even if we notice a slight shift of the cumulative distribution towards higher amplitudes (Supplementary Fig. 7e), and mean frequency (Supplementary Fig. 7f). Of note, sIPSC distribution is shifted towards lower frequency (Supplementary Fig. 7f). Next, we calculated the ratio of SEPS-to-sIPSC mean amplitudes and found that Cul3 haploinsufficiency results in a reduced E/I ratio (Supplementary Fig. 7g), a circuit dysfunction occurring in a number of other ASD models31–33.

To test whether the observed differences in neuronal network activity are due to morphological defects, we performed Golgi stainings and analyzed the morphology of layer 2/3 pyramidal neurons in adult Cul3+/− mice (Supplementary Fig. 7h–j). However, neither the dendritic length, nor the number of dendrites or spines (Supplementary Fig. 7i), nor dendritic branching (Supplementary Fig. 7j) or spine morphology (Supplementary Fig. 7k) is altered in Cul3 haploinsufficent mice. In line with comparable spine morphology, the G-actin/F-actin ratio was also unchanged in the mutant (Supplementary Fig. 7k). Altogether these results point to a tissue level reduction in network activity and global synaptic transmission, likely linked to the cortical lamination defects in Cul3+/− mice.

Whole-proteome analysis reveals abnormal amounts of cytoskeletal proteins in Cul3 mutant mice. To gain insight into the molecular mechanisms underlying the observed defects, and in view of Cul3’s E3 ubiquitin ligase function, we assessed the impact of Cul3 loss on the global proteome of the developing forebrain in Cul3+/−, Cul3+/fl Emx1-Cre and Cul3+/fl Emx1-Cre mutants. Protein extracts from dissected E16.5 cortices from control and mutant animals were analyzed by quantitative proteomics (Fig. 5a and Supplementary Fig. 8a). Analysis of the total proteome of Cul3+/−, Cul3+/fl Emx1-Cre, and Cul3+/fl Emx1-Cre mutants, as well as corresponding controls, resulted in the identification of 8100 protein groups. For differential protein expression analysis, first, the differences between the two controls, Cul3+/+ and Cul3+/fl, were assessed and as these differences were minor (i.e., four proteins Wdfy1, Abca1, Ep400, and Cwf19l2), Supplementary Fig. 8b) a unique Cul3+/− data set was used for further analysis. Protein groups were then filtered based on fold change and False Discovery Rate (FDR) thresholds. Employing FDR thresholds of 10% we identified 31 up- and 33 down-regulated proteins in the Cul3+/− embryonic cortex (Fig. 5b and Supplementary Data 3), and 38 up- and 22 downregulated proteins in the Cul3+/fl Emx1-Cre embryonic cortex (Fig. 5c and Supplementary Data 4). As expected from the more severe phenotype of Cul3+/fl Emx1-Cre mutant pups, a much larger number of deregulated proteins were identified in conditional homozygous knockout embryos (146 up- and 94 downregulated
proteins, Fig. 5d and Supplementary Data 5). Overall, and in agreement with previous observations\textsuperscript{34–36}, the fold changes were mild, in line with the hypothesis that the ubiquitylated isoform of a protein represents only a small fraction of the total pool of that given protein at any time point\textsuperscript{37}. The small number of deregulated proteins in the forebrain tissue of Cul3 haploinsufficient embryos did not justify GO-term enrichment analysis. Therefore, to get an indication of the classes of proteins affected in Cul3 mutants, we performed GO-term enrichment analysis on the Cul3\textsuperscript{fl/fl} Emx1-Cre data at an FDR of 20%. We found that deregulated proteins were significantly enriched for DNA-directed RNA polymerase II core complex members and...
proteins of the proteasome core complex. In addition, deregulated proteins were functionally linked to CNS and forebrain development, the regulation of cell migration, actin- and microtubule-cytoskeletal organization, cell–cell adhesion, and apoptosis (Fig. 5e and Supplementary Data 6). Given the migration phenotype observed in both heterozygous and conditional homozygous Cul3 mutant animals, we decided to investigate changes in cytoskeletal proteins further and found several of these to be misregulated also in Cul3+/− embryonic tissue (Fig. 5b–d in purple). We annotated these proteins manually, drawing on published literature and focusing on those appearing to follow a dose-dependent response to Cul3 loss, when the mean raw expression levels were fitted to a linear model (Cul3+/fl < Cul3+/fl Emx1-Cre and <Cul3fl/fl Emx1-Cre) (Fig. 5f–h and Supplementary Data 7). Several have a putative or a confirmed function in cytoskeletal organization, and/or regulation of cell migration and differentiation. Some downregulated proteins belong to a family of microtubule-associated proteins linked to abnormal brain development in humans (i.e., Tubb2b, Map2)38,39. However, as Cul3 targets proteins for degradation, we were most interested in the identified up-regulated proteins, as their upregulation likely represents a direct consequence of Cul3 loss. To validate the results obtained by proteomic analysis we quantified by western blot two of the top up-regulated cytoskeletal-associated proteins observed in both Cul3+/− and Cul3fl/fl Emx1-Cre samples, Plastin 3 (Pls3) and Interneuron neuronal intermediate filament protein A (INA) (Supplementary Data 7) as well as the cytoskeleton interacting protein Nischarin (Nisch) altered in Cul3fl/fl Emx1-Cre samples. Indeed, we confirmed that Cul3 deficiency leads to higher amounts of Pls3 and INA in both Cul3+/− and Cul3fl/fl Emx1-Cre E16.5 cortical lysates, as well as elevated levels of Nisch in Cul3fl/fl Emx1-Cre samples (Fig. 5i, j). Furthermore, we found that the upregulation of these proteins is not due to increased gene expression since there were no corresponding increases at the mRNA level (Fig. 5k), but rather to a translational or posttranslational effect. Interestingly, Pls3 was actually downregulated at the mRNA level, likely a compensatory response to the accumulation of the protein, indicating a feedback loop to adjust the protein level of Pls3 and thus pointing towards a potentially important functional role of Pls3 in the brain.

In contrast to early development but in line with an important regulatory function of Cul3 mostly during early brain development, we found that in adult mutant cortical, hippocampal, and cerebellar tissues, Cul3 deficiency results in very few deregulated proteins (at 10% FDR, none in the cortex, hippocampus: 3 up- and 1 down-, and cerebellum: 2 up- and 1 downregulated proteins; Supplementary Fig. 8c–e and Supplementary Data 8f). However, we found that Pls3 is also elevated in the juvenile and adult brain in constitutive Cul3 mutants (Supplementary Fig. 8f, g) as well as in tamoxifen-induced Cul3 haploinsufficient animals (Supplementary Fig. 8h), further supporting a tight link between Pls3 and Cul3.

Recently, two studies exploring the role of Cul3 in the adult brain suggested a link between Cul3 and two distinct proteins, Smyd336 and elf4F1G135. Although the aims and methodologies employed are different from what used here, we analyzed the protein levels of Smyd3 and elf4F1G1 in constitutive Cul3 haploinsufficient mice, which best resemble the human condition. Importantly, we could not observe changes in either Smyd3 or elf4F1G1 in any of our proteomic data sets (Supplementary Fig. 8i,j). Accordingly, we did not find any change in elf4F1G1 protein abundance in either the cerebral cortex, the hippocampus, or the cerebellum obtained from P14 male Cul3+/− pups (same time point, sex, and antibody as in Dong et al.35) (Supplementary Fig. 8k). In contrast, we found that Pls3 protein level is consistently increased in the Cul3 mutant mouse models employed by our colleagues35,36, supporting our finding that Pls3 is an important substrate of the Cul3 ubiquitin ligase.

Taken together, our analysis points to a central role of Cul3 in the homeostatic regulation of cytoskeletal proteins of which the most significant appears to be Pls3.

Pls3 regulates cell migration speed and actin organization. Proteomic analysis of embryonic cortices highlighted an alteration of cytoskeletal protein levels in Cul3 mutant samples. In particular, Pls3 protein levels are consistently increased in all of our data sets. Therefore, we assessed whether Pls3 does play a direct role in cell migration. First, we employed the murine B16-F1 melanoma cell line, a routinely used cell line model to investigate cell migration of ectodermal cells40,41. To study the effect of Pls3 loss of function we employed a CRISPR-based system and generated three independent B16-F1 Pls3 knockout cell lines. As expected, CRISPR-Cas9-mediated homologous disruption of the Pls3 locus completely abolishes Pls3 expression (Supplementary Fig. 9a). Excitingly, already in this cell line, in a random migration assay, Pls3 knockout cells show increased migration speed and traveled distance (Supplementary Fig. 9b,c), the exact opposite phenotype displayed by Cul3 mutant cells. Then, employing the above validated Pls3 sgRNA, we independently knocked out or overexpressed Pls3 in wild-type neural progenitor cells (Supplementary Fig. 9d) and tracked their movement over the course of 15 h. In support of a direct role of Pls3 in neural cell migration, we found that Pls3 knockout cells move faster than wild-type cells, while its overexpression leads to slower cell migration (Fig. 6a–c). Furthermore, we determined that the Pls3 effect on neural migration is cell-autonomous since transfected and un-transfected cells could be tracked side by side due to the presence of a green fluorescent protein (GFP) (Supplementary Fig. 9e).
Together our results indicate that Pls3 is an important player in the fine regulation of cell migration, and its level is inversely proportional to migration speed. Although we clearly established that the amount of Pls3 protein is a determinant factor for cell migration dynamics, we went on and assessed whether the migration phenotype observed in Cul3 mutant NPCs can be completely attributed to the elevated levels of Pls3 (Supplementary Fig. 9f). Thus, we knocked out Pls3 in the context of Cul3 haploinsufficiency (Supplementary Fig. 9d), and re-assessed neural cell migration. As expected, we found that reducing Pls3 levels is sufficient to correct the migration pattern observed in Cul3+/− cells (Fig. 6d–f).

Given that Pls3 is an actin-binding protein, we analyzed actin conformation in neural stem cells in the context of Cul3 mutations 42, employing stimulated emission depletion (STED) super-resolution imaging. Specifically, we stained control and Cul3 mutant NPCs with SiR-actin, a fluorescent probe for F-actin, as well as a tubulin antibody and analyzed actin filament orientation at the leading edge in diffraction-unlimited images (Fig. 7a). We found that Cul3 deficiency leads to a disorganized
Fig. 5 Deregulation of cytoskeletal proteins in Cul3 mutant embryonic forebrain tissue. a Sample preparation for proteomic analysis (n(Cul3<sup>+/−</sup>) = 5 per genotype, n(Cul3<sup>+/−</sup> Emx1-Cre) = 3 per genotype, n(Cul3<sup>/−</sup> Emx1-Cre) = 3 per genotype; male mutant and control littermate pairs). b–d, Volcano plot of deregulated proteins at 10% FDR cut-off in the Cul3<sup>+/−</sup> developing cortex (b), Cul3<sup>+/−</sup> Emx1-Cre (c), and Cul3<sup>/−</sup> Emx1-Cre (d) developing forebrain; cytoskeleton related proteins (purple), proteins differently regulated in more than one data set are underlined, Cul3 (red) (Fig. 7e) (details: Supplementary Data 3–5). e DAVID functional annotation identified down- (left) and up- (right) regulated proteins at 20% FDR of the Cul3<sup>/−</sup> Emx1-Cre forebrain involved in regulating the activity of RNA polymerase II, the proteasome core complex, neurogenesis and actin and microtubule cytoskeletal organization (selected GO-terms, significant GO-terms in red; RNA polymerase II core complex, GO: 0005665, adj. P-value = 0.002; proteasome core complex GO: 0005839, adj. P-value< 0.007; actin cytoskeleton GO: 0006351, adj. P-value< 0.007; cell adhesion GO: 0007155, adj. P-value< 0.007; and neuronal cell development GO: 0002501, adj. P-value< 0.007). f, g Comparison of ratios of up- and downregulated proteins in the Cul3<sup>+/−</sup> Emx1-Cre and Cul3<sup>/−</sup> Emx1-Cre cortex at log 0.1 change (details: Supplementary Data 7). h, i Western blot (left) and analysis of Gapdh normalized intensities (right) confirm increased levels of the cytoskeletal proteins Pls3 and INA in Cul3<sup>/−</sup> lysates (n = 5 per genotype; *P = 0.04, **P < 0.001; unpaired two-tailed t-tests). j Western blot and analysis of Gapdh normalized intensities confirm increased levels of cytoskeletal proteins Nisch, Pls3, and INA in Cul3<sup>/−</sup> Emx1-Cre cortical lysates (samples on the same membrane cut for better visualization, n = 5 per genotype; *P = 0.04, **P < 0.001; unpaired two-tailed t-tests). k Quantitative real-time PCR analysis of mRNA levels of Pls3, Pls3, and INA normalized to wild-type levels (ΔΔCq expression values; n = 5 per genotype; *P = 0.02; unpaired two-tailed t-tests). Data presented as or as box and whiskers, min to max (b) and mean ± SEM in i–k. Detailed statistics are provided in Supplementary Data 1.

Fig. 6 Pls3 regulates cell migration dynamics in Cul3 haploinsufficient NPCs. a 2D random migration assay was performed over a period of 15 h in Pls3 wt, Pls3 ko, and Pls3 over-expressing NPCs. Representative cell paths/trajectories of Pls3 wild-type, Pls3 ko, and Pls3 over-expressing cells indicate a larger migration area being covered by cells upon Pls3 ko (a middle), while over-expression of Pls3 leads to a smaller migration area (a right). b, c Quantification revealed a significant increase in migration velocity and cumulative distance in Pls3 ko cells, and a significant decrease in Pls3 over-expressing NPCs relative to Pls3 wild-type cells (n(Pls3 wt) = 49 cells; n(Pls3 ko) = 31 cells and n(Pls3 over-exp.) = 37 cells; *P = 0.02, **P < 0.0001; 1-way ANOVA and Sidak’s multiple comparison test). d 2D random migration assay in Cul3<sup>+/−</sup>, Cul3<sup>+/−</sup> and Cul3<sup>/−</sup> Pls3 ko NPCs. Representative cell paths/trajectories show a smaller migration area covered by Cul3<sup>+/−</sup> NPCs (d middle) as compared to Cul3<sup>+/−</sup> cells (d left). Reducing Pls3 levels in Cul3<sup>/−</sup> NPCs by Pls3 ko is sufficient to rescue the Cul3 haploinsufficient phenotype (d right). e, f Quantification revealed a significant decrease in migration velocity and traveled distance in Cul3<sup>+/−</sup> NPCs, a phenotype rescued when downregulating Pls3 levels through Pls3 ko (n(Cul3<sup>+/−</sup>) = 34 cells; n(Cul3<sup>/−</sup>) = 40 cells and n(Cul3<sup>+/−</sup> Pls3 ko) = 35 cells; **P < 0.0001; one-sided Kruskal–Wallis test and Dunn’s multiple comparison test). Data are shown as mean ± SEM and scatter plots in (b, c, e, f). Detailed statistics are provided in Supplementary Data 1.

actin architecture at the cell front of adherent Cul3<sup>+/−</sup> NPCs (Fig. 7b–d), while the microtubule organization appears normal (Fig. 7e–g). Decreased directionality of actin cables and stress fibers may in principle be caused by an increased number of focal adhesion sites, as these puncta contain all possible angles and would thereby decrease the measured dominant direction. Thus, we further analyzed the number of adhesion points in control and mutant cells. Surprisingly, adhesion site counting revealed a slight, yet significant, decrease in focal adhesions in the mutant cells (Supplementary Fig. 10a–c). A reduction of adhesion points is also in line with our proteomic data, which identified a reduction of cell adhesion proteins (such as Podxl, Nid2, and Emb). To understand whether increased levels of Pls3 alone are sufficient to change the actin architecture in
NPCs, we overexpressed Pls3 in wild-type NPCs and analyzed their actin conformation by STED microscopy. Indeed, cells that overexpress Pls3 in a wild-type background, show the same actin cytoskeletal organization as Cul3+/− NPCs (Fig. 7h–j and Supplementary Fig. 9g). Interestingly, Pls3 over-expression additionally leads to an increase in focal adhesion counts (Supplementary Fig. 10d–f), suggesting that in Cul3+/− additional compensatory, factors may come into play. Taken together, high-resolution imaging indicates a disorganized actin cytoskeleton at the cell front, probably constituting the underlying cell biological correlate of the migration defects observed in Cul3 mutant cells.
Cul3 transcription activation rescues cell migration defects. While Pls3 may represent an optimal target to rescue Cul3 haploinsufficiency-associated phenotypes there are no compounds currently available to reduce its activity. In addition, downregulating or blocking the function of an actin-binding protein may be harmful in vivo. Therefore, an alternative solution may be required.

Autism-associated gene variants are often de novo, heterozygous, loss of function mutations. This is indeed the case for all the CUL3 ASD-associated mutations identified so far1,2,4,6–11. Thus, one potential therapeutic strategy could be to enhance gene expression from the non-mutated allele. In order to test this possibility, we made again use of the CRISPR system. This time, however, we expressed two independent sgRNAs targeting the promoter of the Cul3 gene and a catalytically inactive Cas9 enzyme fused to a transcriptional activator (CRISPRa)43 (Fig. 8a). First, we tested the ability of this system to increase Cul3 expression in Cul3+/− NPCs by transfecting wild-type and mutant Cul3 cells with the CRISPRa and either one of two guides targeting the Cul3 promoter or a control guide (Fig. 8a) and performing real-time PCR to assess Cul3 expression under the different conditions. Of note, both guides were able to enhance Cul3 mRNA expression with one of the two guides (i.e., sgRNA 2) completely rescuing Cul3 expression in Cul3+/− NPCs (Fig. 8b). In contrast, transfecting wild-type cells with either one of the Cul3-targeting sgRNA does not increase expression of the Cul3 mRNA (Supplementary Fig. 11a), suggesting that other mechanisms may be in place to avoid Cul3 accumulation. Next, we tested neural cell migration under these conditions. As hypothesized, we found that increasing Cul3 expression by leveraging on the intact allele is sufficient to rescue the neural cell migration defects presented by Cul3 mutant cells (Fig. 8c–e). Importantly, we found that cells transfected with the Cul3 specific RNA guides do not outrun wild-type cells but migrate exactly with the same speed and the same distance as in control conditions (Fig. 8c–e). In addition, enhancing Cul3 mRNA levels is sufficient to normalize Pls3 protein levels in Cul3+/− cells, further underlining the tight regulatory link between Cul3 and Pls3 (Supplementary Fig. 11b). Altogether, our analysis shows that enhancing gene transcription of the wild-type allele is capable of rescuing phenotypes caused by ASD-gene haploinsufficiency and therefore may represent an optimal therapeutic strategy for this group of conditions.

Discussion
De novo loF mutations in CUL3 are an important cause of ASD, motor deficits, and intellectual disability in humans. Therefore, understanding the role of CUL3 in the mammalian brain is of utmost importance. While the molecular function of CUL3 is well described, its role in the brain, particularly during development, remains largely unclear. To address these questions and model patients’ Cul3 haploinsufficiency, we analyzed a Cul3 construct valid mouse model.

We found that constitutive Cul3 heterozygous deletion leads to several behavioral abnormalities, including sociability issues, motor dysfunctions, and olfactory hyper-reactivity in adult animals. While this confirms the importance of maintaining correct Cul3 dosage in the mammalian brain, it also implicates Cul3 in the function of different brain regions, in line with the complex presentation of CUL3 mutant patients. Importantly, for our behavioral studies, we invested effort in generating and analyzing male and female cohorts separately but we did not observe major phenotypic differences between genotypes of different sexes, supporting the observation that in humans CUL3 mutations similarly affect males and females1,2,8,10,11,43. Thus, CUL3 mutations do not appear to contribute to the skewed sex ratio in ASD cases.

While a gross morphological analysis did not reveal major abnormalities in the adult mutant brain, we found that Cul3 haploinsufficiency leads to cortical lamination defects. Lamination abnormalities in Cul3+/− animals are linked to migration defects, which lead to retention of neuronal cells in lower cortical layers. Cul3 mutation-associated neuronal migration defects are cell-autonomous and do not depend on brain-specific cues, as we also observe migration phenotypes in vitro. Furthermore, our data indicate that Cul3 deficiency does not affect the number of astrocytes and microglial cells in the cortex, thus suggesting that Cul3 regulates neuronal-specific processes, possibly due to its much higher expression in neuronal cell types compared to other brain cells types. Lamination defects, even when subtle, can have a profound effect on the physiology of the brain and disrupt the stereotyped organization of the microcolumnar structures typical of the neocortex. Accordingly, lamination defects have been previously associated with ASD in human and mouse models due to alteration of neuronal connectivity44. Similarly, Cul3 haploinsufficient mice show decreased spontaneous activity of layer 2/3 cortical excitatory and inhibitory neurons, possibly due to a reduction of total neurons reaching these upper cortical layers and an overall incorrect laminar organization. Furthermore, when we calculated the E/I ratio, reflecting the interplay of the activity converging on an individual neuron, we found that Cul3 haploinsufficiency results in a reduced E/I balance. Interestingly, this is in contrast with the work of Dong and colleagues45, who observed an increased E/I ratio in pyramidal neurons of the CA1 in adult Cul3+/− GFAP-Cre mice. This difference may be explained by the different brain regions analyzed, or by the mosaicism inherent to the used GFAP-Cre line, in which only 20–38% of the inhibitory neurons in the neocortex show Cre-expression. In any case, such circuit dysfunctions are a commonly described feature in a large number of other ASD models and are believed to underlie the ASD-relevant behavioral defects of these mouse lines, in agreement with the so-called “E/I imbalance hypothesis”46,47.

Interestingly, complete deletion of Cul3 leads to additional phenotypes including increased apoptosis during the neural stem
Fig. 8 Transcriptional activation of Cul3 rescues the cell migration defects displayed by Cul3 haploinsufficient cells. a Scheme of CRISPR-mediated activation (CRISPRa). While Cul3+/− + Ctrl sgRNA NPCs express wild-type mRNA levels and migrate physiologically (a top, gray), Cul3+/− + Ctrl sgRNA NPCs show reduced Cul3 mRNA levels and are characterized by cell migration defects (a middle, blue). Upregulation of Cul3 transcription, through a nuclease-deficient dCas9 fused to a transcriptional activator targeting the genomic Cul3 promoter (a bottom, green), increases Cul3 mRNA levels and thereby restores migration dynamics. b Quantitative real-time PCR analysis of Cul3 mRNA levels normalized to the housekeepers Gapdh and Pgk1 as well as the Cul3 levels of wild-type NPCs transfected with their respective guides. Transfection of Cul3+/−/+ cells employing two different sgRNAs sequences (sgRNA 1 and 2) targeting the Cul3 promoter increased Cul3 expression levels (∆Cq expression values; n = 3 per condition; *P < 0.05, n.s. not significant; 1-way ANOVA and Sidak’s multiple comparison test). c–e 2D random migration assay was performed over a period of 15 h in Cul3+/+ and Cul3+/− NPCs transfected with Ctrl sgRNA, as well as Cul3+/− cells transfected with sgRNA1 and 2. Representative cell trajectories indicate reduced migration by Cul3+/−/+ + Ctrl sgRNA NPCs (c top right) as compared to Cul3+/−/+ + Ctrl sgRNA controls (c top left). This Cul3+/− haploinsufficient phenotype is rescued through transcriptional activation of the Cul3 promoter using two different sgRNAs (Cul3+/−/+ + sgRNA 1 and 2; c bottom left and right). Quantification revealed a significant increase in migration velocity (d) and traveled distance (e) when restoring Cul3 expression levels in transfected Cul3+/−/− NPCs (n(Cul3+/−/+ + Ctrl sgRNA)= 53; n(Cul3+/−/+ + Ctrl sgRNA)= 62 cells, n(Cul3+/−/+ + sgRNA1)= 48 cells, and n(Cul3+/−/+ + sgRNA2)= 28 cells; **P < 0.01, n.s. not significant; one-sided Kruskal–Wallis test and Dunn’s multiple correction test). Data are shown as mean ± SEM and scatter plots in b, d, e. Detailed statistics are provided in Supplementary Data 1.

A role of Cul3 in cell migration was already hypothesized due to its connection with Kctd13, one of the genes involved in the 16p11.2 deletion syndrome, associated with neurological symptoms. Kctd13 encodes a substrate-linking protein for Cul3 and was suggested to bind RhoA, a regulator of the actin cytoskeleton, and thereby targeting it for ubiquitylation and degradation. However, Kctd13 deletion does not lead to elevated RhoA until after P7, and adult Kctd13 heterozygous knockout mice do not have major structural brain differences as assessed by MRI. Thus, in agreement with a lack of RhoA increase in

cell proliferation phase, possibly due to a defect in cell cycle progression. This is matched by a more severely affected proteostasis in the developing brain of Cul3+/−Emx1-Cre embryos. Defects of cell cycle progression were already associated with complete depletion of Cul3 and do underlie the lethality of Cul3 null mice. Thus, studying construct valid Cul3 models is critical to understand the bases of ASD in patients, as due to its stronger effect, homozygous deletion can obscure the underlying cellular and molecular drivers of Cul3 haploinsufficiency-linked phenotypes.

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Cul3+/− embryonic forebrain tissue, as seen in our proteomics data, the observed migration and lamination defects are not driven by RhoA.

Importantly, while two recent studies35,36 analyzed the effect of Cul3 deficiency and haploinsufficiency in the adult mouse brain, both studies fell short of studying the developmental consequences of Cul3 mutations in detail, employed Cre-lines that may miss critical developmental issues. In contrast, here we focused on the constitutive effects of Cul3 haploinsufficiency and discovered its crucial role in cell migration and proper organization of the cortex. Interestingly, however, when comparing our proteomic data set with these recently published studies we made a few remarkable observations. First, in mutant embryos, we found down-regulation of Map2, a microtubule-associated protein and known component of the neuronal cytoskeleton. Reduced levels of Map2 may be due to the reduced cortical layer thickness and numbers of neurons. Second, we found that the only protein consistently up-regulated in mutant animals is Pk3, under-investigated actin-bundling protein31,32. Pk3 is elevated in the developing and adult cortex, the adult hippocampus, but not in the adult striatum35,36. Thus, although there are some discrepancies, probably due to different experimental choices (e.g., mouse model employed, time point analyzed, etc.), the combined evidence suggests a role of Cul3 in regulating cytoskeletal organization by controlling Pk3 levels in the brain. In addition, our data shows a direct correlation between Cul3 activity and Pk3 levels, suggesting that Pk3 levels may be a good readout to test the pathogenicity of Cul3 mutations in humans. Interestingly, a pathogenic variant of Pk3 in humans. Interestingly, a pathogenic variant of Cul3 may be a good readout to test the pathogenicity of Pk3 levels in the brain. In addition, our data shows a direct correlation between Cul3 activity and Pls3 levels, suggesting that Pls3 expression may be therapeutically relevant for ASD-gene haploinsufficiency and haploinsufficiency in the adult mouse brain, both studies fell short of studying the developmental consequences of Cul3 mutations in detail, employed Cre-lines that may miss critical developmental issues. In contrast, here we focused on the constitutive effects of Cul3 haploinsufficiency and discovered its crucial role in cell migration and proper organization of the cortex. Interestingly, however, when comparing our proteomic data set with these recently published studies we made a few remarkable observations. First, in mutant embryos, we found down-regulation of Map2, a microtubule-associated protein and known component of the neuronal cytoskeleton. Reduced levels of Map2 may be due to the reduced cortical layer thickness and numbers of neurons. Second, we found that the only protein consistently up-regulated in mutant animals is Pk3, under-investigated actin-bundling protein31,32. Pk3 is elevated in the developing and adult cortex, the adult hippocampus, but not in the adult striatum35,36. Thus, although there are some discrepancies, probably due to different experimental choices (e.g., mouse model employed, time point analyzed, etc.), the combined evidence suggests a role of Cul3 in regulating cytoskeletal organization by controlling Pk3 levels in the brain. In addition, our data shows a direct correlation between Cul3 activity and Pk3 levels, suggesting that Pk3 levels may be a good readout to test the pathogenicity of Cul3 mutations in humans. Interestingly, a pathogenic variant of Pk3 in humans. Interestingly, a pathogenic variant of Cul3 mutations in humans. Interestingly, a pathogenic variant of Cul3 may be a good readout to test the pathogenicity of Cul3 levels in the brain. In addition, our data shows a direct correlation between Cul3 activity and Pls3 levels, suggesting that Pls3 expression may be therapeutically relevant for ASD-gene haploinsufficiency.

In summary, our work provides novel insights into the pathophysiological and temporal basis of Cul3-linked behavioral abnormalities and offers important information on drug development and clinical trial design for this and other forms of ASD.

Methods

Mice. We thank Dr. Jeffrey Singer (Portland State University, US) for providing us with the Cul3fl/fl (Cul3P) conditional mouse line in which the exons 4 to 7 are flanked by loxP sites18. The Cul3 constitutive knockout mouse line (Cul3−/−) was obtained by mating Cul3fl/fl mice with a CMV-Cre line (B6.C-Tg(CMV-Cre)1CreFlj) and back-crossings to C57BL/6J wild-type animals (Supplementary Fig. 1a).

Genotyping for the Cul3 knockout allele was performed using the following primers forward GGAACATCTAAGTTTGTATGTCG and reverse TTTGCTTGGACCAAATATGGCAGCCCAAACC. Details of all primers used can be found in Supplementary Table 1. The Cul3 Em1t-Cre conditional line was generated by crossing male Cul3+/− mice with a Em1t-Cre expressing line (B6.129S2 (Em1t1tm1cre)Koj))39. Cul3P Em1t-Cre mutant pups were sacrificed as soon as the phenotype was clearly detectable (strongly reduced size and very weak animals) within 24 h of life, to comply with ethical requirements (3R principle).

Embrionic time points were determined by plug checks, defining embryonic day (E) 0.5 as the morning after copulation. Animals were housed in groups of 3–4 animals per cage and kept on a 12 h light/dark cycle (lights on at 7:00 am), with water and food available ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee at IST Austria and the Bundesministerium für Bildung, Wissenschaft und Forschung, Austria, approval number: BMWFUW-66.018/0021-WF/V3b/.

Behavior. All behavioral tests were performed on adult, 2- to 5-month-old, sex-matched littermate animals during the light period. Animal cohort sizes ranged between 8 and 20 animals per genotype and sex. Male and female cohorts were sex-matched separately, and the data was only pooled in case wild-type male and wild-type female data was comparable. Before testing, animals were habituated at least for 1 h to the testing room, equipment was cleaned with 70% EtOH after each animal. Different behavioral tests in the same mouse cohort were separated by at least one day of break. Tests were performed starting with the least aversive task and ending with the most aversive, and either scored automatically or by an experimenter blind to the genotype.

Hind limb clasping. During a 10 s tail suspension period hind limb clasping severity (scores 0–1: no hind limb clasping to 3: most severe phenotype) was assessed by the experimenter. The test was repeated three times per animal and the average scores was calculated, scores between 0 and 1 are not considered as hind limb clasping.

Gait analysis. Gait properties were assessed by footprint analysis as described previously20. Briefly, fore- and hind paws were colored with non-toxic dye and the animals allowed crossing a white sheet of paper, 70 cm in length, in a straight line. Stride, sway, and stance length, as well as paw distance, were measured as indicated in Fig. 1c by the experimenter.

RotaRod. Performance on the accelerating mouse RotaRod (Ugo Basile S.R.L., Cat. No. 47650) was analyzed on 3 trials per day over three consecutive days, with an acceleration from 5 to 40 rpm over 5 min (300 s). For each animal the latency to fall (s) and maximum speed at the end of each trial (rpm) were automatically determined, sex-matched littermates were tested simultaneously to avoid confounding factors. Initial coordination was assessed by analyzing the first trial on the first day of training.

Open field. As previously described34, animals were allowed to freely explore a brightly lit arena (45 × 45 × 30 cm3), made out of gray Plexiglas, over a 30 min time period. Locomotor activity (distance traveled and velocity) and center crossings were recorded by a video camera and analyzed using the EthoVision XT software (v.11.5; Noldus).

Elevated plus maze (EPM). Mice were placed in the center of the EPM apparatus facing the open arm and left to explore the maze for 6 min. The time spent in the open and closed arms along with the number of closed and open arm entries and distance traveled in each arm were determined using EthoVision XT (v.11.5) video tracking system and software. The ratio of the time (in s) spent on the open arm vs. the total time spent on the maze was calculated.

Three-chamber sociability test. Mice were tested for sociability and social novelty preference as described previously20. The testing apparatus was a rectangular clear Plexiglas three chambers box (60 cm (L) × 40 cm (W) × 20 cm (H)). The dividing walls had doorways allowing access to each chamber. Age and sex-matched C57BL/6 mice were used as stranger mice and were habituated to placement inside the wire cage. Each test animal was placed first into the center chamber with open access to both left and right chamber, each chamber containing an empty round wire cage. The wire cage (12 cm height, 11 cm diameter) allows nose contact between mice but prevents lighting. After 10 min of habituation, during the social phase, an age-matched stranger was placed in the left chamber while a novel object was placed into the right chamber. The test animal was allowed to freely explore the social apparatus for 10 min. Subsequently, each mouse was tested in a second 10 min session to evaluate the preference for a novel stranger, which was placed

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inside the right wire cage. The number of nose contacts (<5 cm proximity) with the caged mouse, as well as the time spent in each chamber, was calculated. Analysis was done using the Noldus EthoVision XT (v.11.5). In addition, the social preference/novelty index for each mouse was calculated, as described previously55.

Olfactory habituation and disambiguation test (ODHD). The test was performed as previously described56. In brief, mice were presented with a sequence of non-social, i.e., water, almond (McCormick) and banana (McCormick) (diluted 1:100 in water), and social cotton swabs (Social A and B). Social odors were obtained by wiping the cotton swabs in a zig-zag manner through the bedding of two distinct cages, housing each three, to the test mice, sex- and age-matched wild-type mice. Each cotton odor swab was presented for 3 × 2 min with a 1 min break. The time interval for the electronic odour was determined by the experimenter using a stop watch. The adapted ODHD, Fig. 2h, was performed as described above, however excluding the non-social odors almond and banana.

Contextual fear conditioning (CFC) task. As described previously54, mice were subjected to the CFC task in three sessions, each distance by 24 h: a training session (day 1) and two re-exposure sessions (days 2 and 3). On day one, mice were subjected to a single fear conditioning training session of 5 min, in which they learned to associate the conditioned stimulus (CS: context) to the unconditioned stimulus (US: a foot-shock). To this end, each mouse was placed in a fear-conditioning chamber (18 × 18 cm², Noldus) with an electrified grid floor. After 120 s of acclimatization the mouse was subjected to three times a foot-shock (0.5 mA; 2 s) delivered through the grid-floor, every 1 min. Mice remained in the conditioning chamber for 1 min after the last shock delivery. On days two and three, each mouse was placed back in the conditioned chamber for 10 and 5 min, respectively, while a US was applied to the electronic grid to test their retention and memory extinction. Behavior during all experimental sessions was recorded by a video camera mounted above the ceiling of the cage and connected to an electronic device equipped with the Ethovision XT software (Noldus). Percentage of time spent freezing (absence of all but respiratory movements for at least 3 s) was scored for each mouse during the emotional reactivity during training (day 1) and fear memory during retention test (the first 3 min of day 2 session) and extinction test (first 3 min of day 3 session). All behavioral parameters were scored by an experimenter blind to the animal experimental condition. Pairs of control-mutant littermates were randomly tested in the morning and in the afternoon to control for circadian rhythm.

Immunofluorescence; BrdU labeling and imaging

Immunofluorescent staining in adults. Adult male littermate mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde (PFA). Brains were dissected, postfixed in 4% PFA, dehydrated in 30% sucrose, and sliced at 40 µm on a sliding VT1200S vibratome (Leica Microsystems). Stainings on adult brains were performed on floating sections without antigen retrieval. In brief, sections were washed in 1× PBS and incubated overnight on a horizontal shaker at 4 °C in a primary antibody solution (14–16 h). Primary antibodies were diluted in 0.3% Triton X-100 and 2% normal goat serum in 1× PBS. In brief, fluorescent stainings, antigen retrieval was performed using 1× DAKO antigen retrieval solution (s1699) and immunofluorescent staining in embryos and newborn pups (P0/P1). Mice were killed for analysis at E14.5 and E16.5 and P0 by decapitation. Heads were drop fixed in 4% PFA overnight, dehydrated in 30% sucrose, embedded in O.C.T. (Tissue Tek) and 18 µm sections were prepared on a Microm HM560 cryostat (Thermo Scientific). For assessment of the cellular composition of the embryonic cortex by immunofluorescent stainings, antigen retrieval was performed using 1× DAKO antigen retrieval solution (s1699) and immunofluorescent staining was performed as outlined above. The following primary antibodies were used: anti-Cux1 (Santa Cruz, sc-13024, 1:500), anti-Ctip2 (Abcam, ab18465, 1:500), anti-Nkx2.1, mouse monoclonal (3G3, Cell Signaling, Cat. No. 3000), anti-PH3 (Millipore, cat. No. 06-570, 1:500), anti-Sox2 (Millipore, AB5603, 1:200), anti-Thrb2 (Abcam ab183991, 1:250). Analysis of Cux1/Ctip2 cell distributions at P0 was performed by counting the relative number of Cux- and Ctip2- cells in each of 10 bins (bin height adjusted to cortical thickness, bin width 280 µm) in the cortex, normalized to the total number of positive cells in the respective region (n = at least 3 littermate mice per genotype, at least 4 images/animal).

BrdU-based birthdate labeling for migration and proliferation analysis. For cell cycle analysis, pregnant mice were injected with 0.1 mg/kg bromodeoxyuridine (BrdU) at E13.5 and 12 h before sacrifice. Brains were dissected and processed for immunostaining as described above. Cells in S-phase that incorporated BrdU were detected using anti-BrdU (BioRad, MA2060T, 1:500). The number of BrdU+ cells was manually counted in cortical regions of 200 µm width in blinded images (n = 3 littermate animals per genotype, at least 5 images/animal). For cell cycle analysis, pregnant females were injected with BrdU at E13.5, as described elsewhere56. Upon delivery (P0), pups were decapitated and tissue was processed for immunostaining as outlined above. For migration analysis, sections were stained with anti-BrdU (BioRad, MA2060T, 1:150), and anti-Ctip2 (Abcam, ab18465, 1:500) antibodies. The P0 cortex was divided into ten bins of the same size and the relative number of BrdU+ nuclei per bin counted manually in blinded images (n = at least 3 littermate mice per genotype, at least 5 images/animal).

Imaging. Images from immunofluorescent stainings were acquired on a Zeiss LSM800 inverted confocal microscope using ZEN Blue imaging software (v.2.3), background corrected and adjusted for contrast and brightness, as well as analyzed in Fiji (v.1.52n) using the cell counter plugin.

Nissl staining and Golgi staining

Nissl staining. For Nissl staining, brains from perfused adult animals were post-fixed in 4% PFA overnight, dehydrated and paraffin-embedded. Sagittal and coronal sections were cut on a Microtome HM 355 at 10 µm thickness. For Nissl staining of newborn mice, perfused brains were incubated with PFA for 2 h, and equilibrated in 30% sucrose-containing buffer (20% sucrose, 100 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM MgCl2) before overnight in 1× PBS to remove the OCT. Nissl staining of adult and P0/P1 brains were captured using an Olympus Slide scanner VS120 and analyzed using Fiji (v.1.52n).

Golgi staining and analysis. Golgi-Cox staining was performed according to protocols using the FD Rapid GolgiStain KitTM (FD Neurotechnologies). After three weeks of Golgi impregnation, brains were cut coronally (120 µm) using a Leica Vibratome (Leica VT 1200 s) and mounted onto 1% gelatin-coated slides. Slides were then dehydrated through graded ethanol steps, cleared with RotiHistol (Carl Roth), and mounted with DPX mounting medium on coverslips (#1.5). To quantitatively analyze pyramidal neurons in Golgi-stained slides, impregnated pyramidal cells (~8–10 neurons per brain, n = 3 littermate brains per genotype) of layer 2/3 in the somatosensory cortex were selected and imaged with a Nikon Eclipse Ti2 using a ×40 magnification. For analysis, single pyramidal neurons were manually reconstructed using Imaris analysis software (version 9.3.1). The average filament area, filament length, and Sholl intersections were analyzed using the same software. Spine counting was performed using Fiji (v.1.52n), spines that started from 100 µm distance of the apical dendrite were counted within a 100 µm segment.

Ex vivo live-imaging of acute cortical slices

Lentiviral production. Lentivirus, Lenti-X 293T cells (Takara, cat. nr. 632180) used for virus production were grown in DMEM (Sigma cat. nr. D5796) at 37 °C and 5% CO2. Briefly, 660-kb LentiVirus was produced by co-transfecting Lentis-X 293T cells using polyethyleneimine (PEI) with 30 µg of hsyn-GFP plasmid (plasmid was generously provided by Yoav Ben Simon, IST Austria), 18 µg of packaging plasmid pPax2 (Addgene plasmid ID: 12260) and 7.5 µg of envelope plasmid pMD2.G (Addgene plasmid ID: 12259). The next day, the culture medium was exchanged and cell supernatant was collected 24 h after transfection and filtered through 45 µm filters (Millipore). The virus-containing supernatant was overlaid on a 20% sucrose-containing buffer (20% sucrose, 100 mM NaCl, 20 mM HEPES (pH 7.4), 1 mM EDTA (pH 7.9) in sterile water) at a 5:1 v/v ratio, transferred to an ultra-centrifuge (Thermo Scientific, Sorvall WX) and spun at 112,000 × g for 1.5 h at 4 °C. For as-solution-suspended in 200 µl PBS and aliquots were stored at −80 °C until used. To determine the viral titer, Lenti-C 293T cells were transduced with serially diluted (0.1: 0.01, 0.001) viral suspensions and the percentage of GFP+ cells was determined by FACS analysis using a BD FACS Canto II Analyzer. The viral titer was in the range of 2 × 1012 TU/ml.

Animal surgery. Pregnant mice were anesthetized at E13.5 with isoflurane in combination with a subcutaneous injection of Metacam (0.005 mg/kg) as an analgesic, mice were kept under constant isoflurane administration through a nose cone. After exposing both uterine horns, 2 µl of lentiviral suspension mixed with 0.1% Fast Green was injected through the uterine walls into the embryonic lateral ventricle as described in55. For injections, microdroplets were gently loaded into a 20G needle attached to a 30G needle. Connectors were inserted into the peritoneal cavity and the muscular walls and overlying skin were sutured. Following 4 days of lentiviral expression, brains were collected at E17.5 for slice culture preparation.
Acute slice culture preparation and live imaging of embryonic brains.Brains slices were obtained from E17.5 Csd+/− and Csd+/+ wild-type littermates. Embryos were decapitated and the cortex removed to yield a thin slice (500 µm) which was rapidly removed and placed in ice-cold cutting solution, containing (mM): 93 NMDG, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPS, 25 glucose, 5 sodium ascorbate, 2 thioourea, 3 sodium pyruvate, 10 MgCl2, 0.5 CaCl2 (320 mM Os, 7.2–7.4 pH). Acute coronal slices (250 µm) were sectioned using a McIlwain Tissue Chopper (Mc Ilwain, Redditch, UK) and stored in placid for 1 h at 4 °C for 24 h to allow for complete dissociation 

TMT labeling and High pH reversed-phase chromatography

NATURE COMMUNICATIONS| (2021) 12:3058 | https://doi.org/10.1038/s41467-021-23123-x | www.nature.com/naturecommunications

meninges were removed and samples were snap-frozen in liquid nitrogen and stored at −80°C until protein extraction. Embryonic E16.5 forebrain tissue (pooled developing cortex and hippocampus; male embryos n(constitutive KO) = 5 mice per genotype, n(Emx1Cre conditional KO) = 5 Csd+/−/−, 3 Csd+/−/Emx-Cre, and 3 Csd−/+/Emx-Cre respectively; for batch correction purposes each litter selected contained at least 30 mice) were homogenized 1:5 (w:v) in 1% (V/V) formic acid prior to analysis by nano-LC MSMS using an Orbitrap Elite (Thermo Scientific) and 1 mM 1-glutamine (Thermo Scientific). For recovery prior to imaging, slice cultures were placed at 37°C with 5% CO2 for 30 min. GTP time-lapse images were acquired with a ×10 objective on an inverted Nikon TiZ microscope at 37°C with 5% CO2 for 16 h every 15 min with Z stacks (Z-step 15 µm). Velocity and migration patterns of individual GFP-labeled neurons were tracked manually employing the built-in Fiji plugin Manual Tracking. Data of traced cells were then imported into the Chemotaxis and Migration Tool (Ibidi, https://www.ibidi.com/chemotaxis-analysis/171-chemotaxis-and-migration-tool.html), analyzing tracks of individual cells, quantifying the track velocity and cumulative distance.

LC-MS/MS/MS whole-proteome analysis

Samples. Adult male Csd+/− and Csd+/+ wild-type littermate animals (cortex and hippocampus: 4 littermates per genotype; cerebellum: 5 littermates per genotype) were deeply anesthetized and transcardially perfused with 15 ml of ice-cold 0.9% NaCl to clear the brain from blood. The cortex, hippocampus, and cerebellum were rapidly dissected on ice, snap-frozen in liquid nitrogen, and stored at −80°C until protein extraction. Embryonic E16.5 forebrain tissue (pooled developing cortex and hippocampus; male embryos n(constitutive KO) = 5 mice per genotype, n(Emx1Cre conditional KO) = 5 Csd+/−/−, 3 Csd+/−/Emx-Cre, and 3 Csd−/+/Emx-Cre respectively; for batch correction purposes each litter selected contained at least one control animal, see Statistical Data Analysis) were dissected on ice, meninges were removed and samples were snap-frozen in liquid nitrogen and stored at −80°C until protein extraction. Tissues were homogenized 1:25 (w:v) in modified RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF) supplemented freshly with protease and phosphatase inhibitors (Roche 04 693 159 001 and 04 906 837 001) and lysed for 30-45 min on ice, while occasionally being vortexed gently. Each sample was sonicated twice at 180 W in an ice-cold water bath and centrifuged at 12,000 × g for 20 min at 4°C. Lysates were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Cat. no. 23225).

TMT labeling and High pH reversed-phase chromatography. Aliquots of 100 µg of each sample were digested with trypsin (2.5 µg trypsin per 100 µg protein, 37°C, overnight), labeled with Tandem Mass Tag (TMT) 11plex reagents according to the manufacturer’s protocol (ThermoFisher Scientific, Loughborough, LE11 5RG, UK) and pooled. For the adult data set, where the number of samples exceeded the number of available TMT channel, one combined TMT sample was generated for each tissue. For the embryonic data set, one TMT sample contained Csd+/−/− and Csd+/−/+ samples, while the other sample contained the Csd−/+/−, Csd+/−/ Emx1-Cre, and Csd−/+/Emx1-Cre samples. Details of TMT labeling can be found in Supplementary Data 9 (adult) and 10 (embryonic). Pooled samples were evaporated to dryness, re-dissolved in 5% formic acid, and then desalted using a SepPak cartridge according to the manufacturer’s instructions (Waters, Milford, Massachusetts, USA). The eluate from the SepPak cartridge was again evaporated to dryness and re-dissolved in 20 mM Ammonium Hydroxide in 90% acetonitrile, pH 10) to 0 from 95% to 60 min. 15 fractions were collected by time per sample, evaporated to dryness, and re-dissolved in 1% formic acid prior to analysis by nano-LC MSMS using an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific).

Nano-LC mass spectrometry. High pH RP fractions were further fractionated using an Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (V/V) formic acid were dissolved using an Acclpin PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.1% (V/V) acetonitrile (25 µl), fractions were resolved on a 250 mm × 75 µm Acclpin PepMap C18 reverse-phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1–6 solvent B over 1 min, 6–15% B over 58 min, 15–32% B over 5 min, 32–40% B over 5 min, 40–90% B over 1 min, held at 90% for 6 min and then reducing to 32% B in 30 s). The eluate was directly infused at 300 nA in 0.5% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.0 kV using a stainless steel emitter with an internal diameter of 30 µm (Thermo Scientific) and a capillary temperature of 275°C.

All spectra were acquired using an Orbitrap Fusion Lumos mass spectrometer controlled by Xcalibur 4.1 software (Thermo Scientific) and operated in data-dependent acquisition mode using an SPS-MS/MS workflow. FTMS1 spectra were collected at a resolution of 120,000, with automatic gain control (AGC) target of 20,000 and maximum injection time of 70 ms. Precursor ions were then fragmented using constantcollision energy values calculated from those of individual peptidoforms using an in-house weighted average function (using the inverse of each peptidoform’s precursor mass tolerance was set at 10 ppm, and MS/MS tolerance was set at 0.6 Da. Search criteria included oxidation of methionine (+15.9955), phosphorylation of serine, threonine or tyrosine (+79.966) as variable peptide modifications and carbamidomethylation of cysteine (+57.021) and the addition of the TMT mass tag (+229.163) to peptide N termini and lysine as fixed modifications. Acetylation (+42.011) and Met-loss + Acetylation (−89.030) were included as possible modifications to the protein N-terminals. Searches were performed with full tryptic digestion and a maximum of 2 missed cleavages were allowed. The reverse database search option was enabled and all data was filtered to satisfy the false discovery rate (FDR) of 5%.

Statistical data analysis. PSM tables exported from the Proteome discoverer were reprojected in R in-house scripts. PSM reporter intensities were scaled by integrated precursor MS1 peak intensities and normalized (Levenberg–Marquardt procedure) across TMT channels, then summed across fractions for each peptidoform (peptide in a specific modification state). The resulting peptidoform expression matrix was then renormalized across samples using VSN normalization (variance stabilization) followed by the Levenberg–Marquardt procedure (alignment of the expression vectors). In addition, the combined embryonic data set was batch-corrected against the litter effect using the ComBat function from the SVAT package (batch correction was skipped for the adult data set because this revealed only minor litter effects within tissues, and correcting for TMT batch would have removed tissue-specific variation, since each TMT sample was tissue-specific). Peptidoforms were assembled into protein groups, and protein groups-level expression values calculated from those of individual peptidoforms using an in-house weighted average function (using the inverse of each peptidoform’s posterior error probability as weights), excluding phosphorylated peptides and their unmodified counterpart form. Average expression values and ratios were calculated for each condition, and an F-test performed using the limma package. For both, embryonic and adult data sets, P-value significance thresholds were calculated for a pre-agreed false discovery rate level of 10% (Benjamini–Hochberg procedure). As a second filtering step, regardless of P-value, ratios were also not considered if the absolute value of their base-2 logarithm did not exceed a threshold calculated as excluding all but the 5% most extreme ratios between individual conditions. For functional annotation clustering, proteins found significant for a particular condition were submitted to GO terms using the DAVID Bioinformatics Resources 6.8 online tool23 (done for proteins at 20% FDR, GO-terms filtered for P < 0.01. Benjamini-adjusted P-values are reported for significantly enriched terms).

Western blots. Littermate embryonic and adult animals (at least n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen in liquid nitrogen, and stored at −80°C until protein extraction. Tissues were homogenized in ice-cold RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 10 mM NaF) and freshly added protease inhibitors (Roche), lysed for 45 min on ice and centrifuged at 12,000 × g for 20 min at 4°C. Lysates were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher, Cat. no. 23223). All samples were run on the same gel, 50 µg of proteins were mixed with 6× Laemmli buffer (375 mM Tris pH = 6.8, 12% SDS, 60% glycerol, 600 mM DTT, 0.06% bromophenol blue), heat-denatured at 95°C and separated on 8–10% SDS-PAGE
gels in running buffer. Proteins were transferred to a PVDF membrane (Merck) using transfer buffer in a wet blotting apparatus (BioRad) for 2 h at 4 °C with 300 mA constant current. Membranes were incubated for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C. Secondary anti-IgG antibody coupled to horseradish peroxidase (HRP) was detected using a Pierce™ enhanced chemiluminescent substrate (ThermoFisher) on a GE Healthcare Amersham machine. The following primary antibodies for actin were used: anti-β-actin (1:400, Cell Signaling #2795), anti-Gapdh (1:1000, Merck AB16), anti-Pls3 (T-Plastin 1:500, ThermoFisher PA5-27883), anti-Pl3 (T-Plastin 1:1000, Proteintech 12917-1-AP), anti-Pli (1:500, Novus Biologicals H0000357-M04), anti-alpha internexin (1:5000, Abcam ab40758), anti-Nischarin B3 (1:1000, Santa Cruz sc-35364), anti-beta (β-Tubulin) 1:1000. Secondary antibodies used: donkey-anti-rabbit IgG (1:5000, Amersham NA934), goat-anti-mouse IgG (1:10,000, Pierce 31432), goat-anti-rabbit IgG (1:5000, Goat DiaNoia 111-035-045) and goat-anti-mouse IgG (1:5000, Goat DiaNoia 111-035-146).

G-actin/F-actin assay. The ratio of filamentous (F-)actin and monomeric actin (G-actin) was quantified using a G-actin/F-actin in vivo assay kit (Cytoskeleton Inc. cat. nr. BK037) according to manufacturer instructions. Male littermate P21 animals (n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen, and the tissue was homogenized using a steel homogenizer in lysis and F-actin stabilization buffer, lysed for 10 min at 37 °C, transferred to a pre-warmed (37 °C) ultracentrifuge (Beckman Coulter, cat. nr. BK037) according to manufacturer instructions. Male littermate P21 animals (n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen, and the tissue was homogenized in lysis and F-actin stabilization buffer, lysed for 10 min at 37 °C, transferred to a pre-warmed (37 °C) ultracentrifuge (Beckman Coulter, cat. nr. BK037) according to manufacturer instructions. Male littermate P21 animals (n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen, and the tissue was homogenized in lysis and F-actin stabilization buffer, lysed for 10 min at 37 °C, transferred to a pre-warmed (37 °C) ultracentrifuge (Beckman Coulter, cat. nr. BK037) according to manufacturer instructions. Male littermate P21 animals (n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen, and the tissue was homogenized in lysis and F-actin stabilization buffer, lysed for 10 min at 37 °C, transferred to a pre-warmed (37 °C) ultracentrifuge (Beckman Coulter, cat. nr. BK037) according to manufacturer instructions. Male littermate P21 animals (n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen, and the tissue was homogenized in lysis and F-actin stabilization buffer, lysed for 10 min at 37 °C, transferred to a pre-warmed (37 °C) ultracentrifuge (Beckman Coulter, cat. nr. BK037) according to manufacturer instructions. Male littermate P21 animals (n = 3 per genotype) were decapitated, the brain was dissected on ice, snap-frozen, and the tissue was homogenized in lysis and F-actin stabilization buffer, lysed for 10 min at 37 °C, transferred to a pre-warmed (37 °C) ultracentrifuge (Beckman Coulter, cat. nr. BK037) according to manufacturer instructions.
imported into R (3.6.2) environment and analyzed. The track velocity (track displacement/track duration) of detaching cells, their average instantaneous speed (average of all displacements per frame duration), track path length (sum of all displacements between frames), and persistence (track displacement/track path length) were quantified by TrackMate and with R. Finally, results were plotted with the help of ggplot2 (3.2.1), ggpubr (0.2.4) packages.

Alternatively, an adapted neurerophere migration assay was used49. Spheres were embedded centrally in 40 µl of 5 mg/ml matrigel (Corning cat. nr. 356234) diluted in ice-cold basal media on PLO/Laminin coated clear bottom 96-well imaging plates (Corning cat. nr. 3603), one sphere per well. After 30 min polymerization of the matrigel at 37 °C, complete media was added to the wells, and images were captured on a x4 objective on a brightfield inverted microscope, to determine initial sphere sizes. Neureospheres too close to the well-border were excluded from the analysis. 22 and 46 h after embedding, radial outgrowth of NPCs from the sphere was imaged using a x4 objective on a brightfield inverted microscope and the distance (radius) from the center of the neureospheres was measured in Fiji (v1.52x) and normalized to the initial sphere radius.

B16-F1 cell culture conditions. B16-F1 (ATCC® CRL-6323™) mouse melanoma cells were maintained in DMEM supplemented with 10% FCS, 100 U/ml Penicillin/Streptomycin (Gibco cat. nr. 15140-122) and 2mM L-glutamine (Gibco cat. nr. 25030-825).

Generation of Pls3 knockout cell lines using CRISPR/Cas9 and Pls3 over-expression. Generation of knockout cell lines was performed as previously described50. For the generation of Pls3 knockout B16-F1 cell lines (Pls3 KO, Supplementary Figure S1), a custom CRISPR/Cas9 guide design was performed using http://crispr.mit.edu by choosing a guide sequence targeting exon 3 of Pls3: 5′-TATGGCTAAAACCTTCCGAA-3′. The following forward and reverse oligo sequences were cloned into a BspCas9(BB)-2A-Puro (PX459) (Addgene), for stable B16-F1 cell line generation, and a pSpCas9(BB)-2A-GFP (PX458) (Addgene), for transfection of non- NPCs, using Bstl-mediated restriction: 5′-CACCGTATCGCT-3′ and 5′-AACACCTTCGGAAGGGTGGAGGGGGGAC-3′ for the Pls3 KO cell line was amplified using Phusion High-Fidelity Polymerase (NEB) from stable Pls3 knockout B16-F1 cell lines (3 different clones #8, #10, #12). Genotyping for the Pls3 knockout allele was performed using the following primers: forward: ATTCGACCTGAATGTTGTTG and reverse ACCAATATGCGCAGGAC ACCC. The amplified DNA sequence was cloned into a Zero blunt TOPo vector (Invitrogen) according to manufacturer instructions and sequenced using the following primer: forward: CAGGAAACACGCTATGAC. Clones with frameshift mutations causing stop codons downstream of the target site were selected for further characterization (Pls3 KO) and the absence of Pls3 was confirmed via western blot in the Pls3 knockout B16-F1 cell lines (3 different clones #8, #10, #12). The pSpCas9(BB)-2A-GFP plasmid, containing the validated Pls3: 5′-TATGGCTAAAACCTTCCGAA-3′ guide sequence was further used for transfections of mNPCs using Lipofectamine® LTX & PLUS® Reagent (Invitrogen), cells GFP positive were then analyzed in the random migration assay 48–48 h after transfection.

Transient Pls3 over-expression of Pls3-GFP in NPCs was achieved by the generation of a Pls3-GFP plasmid. To that end, the synthetic full-length Pls3 DNA sequence (mouse) was synthesized by Integrated DNA Technologies (IDT) (gblocks Gene Fragments) and cloned into Blunt II-TOPO Vector. The DNA sequence from the TOPo vector was then amplified via PCR, introducing XhoI and SalI restriction sites N- and C-terminally, respectively. The PCR fragment and the EGFP-C1 vector (Clonetech) were digested with the above-mentioned restriction enzymes and ligated. The following primers were used for cloning into EGFP-C1: Pls3-FW-XhoI-EGFP:C1 ATATCTCGAGATATGGATGAGATGGCGACC and Pls3-Rev-Sall-EGFPC1 TTGGTGACATCACATCTCTCATCC and successful cloning confirmed by sequencing. Pls3 depletion or over-expression was confirmed by qRT-PCR on FACS-sorted cell populations.

CRISPR-mediated activation of Cul3. Upregulation of Cul3 levels was performed as previously described using CRISPR-mediated activation49. For transcription-mediated activation of Cul3, CRISPR/Cas9 gene design was performed using http://crispor.tefor.net by choosing two guide sequences targeting the promoter of Cul3. The following forward and reverse oligo sequences were cloned into a pAAV-U6-sgRNA-CMV-GFP (Addgene plasmid ID: 85451) using Sapl-mediated restriction: 5′-ACCCGGAGGAGATCGCTGCGCA-3′ and 5′-AATCTGGCGCGGAGATCGCTGCGCA-3′ as well as 5′-ACCCGGAGGAGATCGCTGCGCA-3′ and 5′-AACCCCGAGGAGGAGGAGGAGGAGGAGGAC-3′. Successful ligation of sgRNAs into the vector was confirmed by sequencing using the following primer: forward: ATGACGCTGAATGTTGTTG and reverse ACCAATATGCGCAGGAC ACCC. The amplified DNA sequence was cloned into a Zero blunt TOPo vector (Invitrogen) according to manufacturer instructions and sequenced using the following primer: forward: CAGGAAACACGCTATGAC. Clones with frameshift mutations causing stop codons downstream of the target site were selected for further characterization (Pls3 KO) and the absence of Pls3 was confirmed via western blot in the Pls3 knockout B16-F1 cell lines (3 different clones #8, #10, #12). The pSpCas9(BB)-2A-GFP plasmid, containing the validated Pls3: 5′-TATGGCTAAAACCTTCCGAA-3′ guide sequence was further used for transfections of mNPCs using Lipofectamine® LTX & PLUS® Reagent (Invitrogen), cells GFP positive were then analyzed in the random migration assay 48–48 h after transfection.

Random migration assay. B16-F1 cells were seeded on #1.5H glass coverslips (ibidi GmbH cat. nr. 80841), which were coated with 10 µg/ml Poly-L-ornithine (PLO) (Sigma cat. nr. P3655) for 3 h at 1 room temperature. Brightfield time-lapse images were acquired with a 20x objective on an inverted Nikon Ti2 microscope at 37 °C with 5% CO2 every 1 min. 15–20 mg/l and Cul3KO+ cells were transfected using Accutase for 5 min at 37 °C, pelleted in basal media, resuspended in complete media, and kept on ice until sorted. Right before sorting, the cell suspension was passed through a 35 µm cell strainer cap into the test tube (Falcon). FACS was performed on a BD FACS Aria III using a 100, sample and 15 ml collection tubes were kept at RT, and NPCs were sorted directly into complete media. Duplex exclusion was performed to ensure the sorting of single cells. Following the sorting of 50,000–200,000 cells, cell suspensions were pelleted, snap-frozen in liquid nitrogen, and stored at −80 °C until RNA or protein extraction.
pinhole size 1 airy unit; excitation laser powers: 560 nm: 3.7 µW; 640: 6.2 µW; 20º lateral/axial power ratio 75/25. Pixel dwell time: 15 µs with 2 scans per line for 560 nm excitation and 4 scans per line for 640 nm excitation.

We exceeded a threshold of 0.25 in the LoG filtered manually prior to analysis. For each image of size 30 µm × 30 µm with pixel size 1 airy unit, excitation laser powers: 560 nm: 3.7 µW; 640: 6.2 µW; STED: 146 µW with lateral/axial power ratio 75/25. Pixel dwell time: 15 µs with 2 scans per line for 560 nm excitation and 4 scans per line for 640 nm excitation.

Adhesion site analysis. Adhesion sites in the actin channel were analyzed in custom-written Python routines using the scikit-image library51 (ver. 0.16.2). We extracted the orientation distributions based on the structure tensor. The structure tensor summarizes local orientations and their coherence (degree of anisotropy) at each image location56. For each image of size 30 µm × 30 µm with pixel size 30 nm × 30 nm (1000 pixel²), we first normalized the raw photon counts from 16-bit integers to values in the range [0, 1] by subtracting the minimum gray value, followed by dividing with the maximum. From the structure tensor computed at each pixel location, we obtained manually prior to analysis. For each image of size 30 µm × 30 µm with pixel size 30 nm × 30 nm (1000 pixel²), we first normalized the raw photon counts from 16-bit integers to values in the range [0, 1] by subtracting the minimum gray value, followed by dividing with the maximum. From the structure tensor computed at each pixel location, we obtained an oriented map of the local orientation distribution. The resulting scalar map was visualized in the hue, saturation, and brightness (HSB) color space, where we set hue as the orientation angle, saturation as coherence, and brightness the normalized gray-value of the source image. The distribution of orientations was built as the histogram of orientations weighted by the corresponding coherency. Image locations with coherence or energy <10% of their maximum value were excluded from the histogram. Each orientation histogram was aligned to the corresponding dominant orientation angle computed from the tubulin channel. The dominant orientation is computed as the average orientation angle inside the cell. Each cell was segmented manually prior to analysis. After alignment, we averaged all orientation histograms per group (Cul3+/−; n = 43; Cul3−/−; n = 43, from three independent NPC preparations respectively) and computed 95% confidence intervals per orientation angle. To test statistical significance, we measured the spread of each orientation distribution as the standard deviation of angles weighted by their occurrences. Reported P-values were computed using a two-tailed Welch’s t-test using the scopy library (ver.1.3.0).

Tamoxifen-induced Cul3 deletion. To induce Cul3 deletion in adult double transgenic mice, Cul3+/− Cage-CreER (Cage-CreER line: Jackson 04453), were injected intraperitoneally with Tamoxifen (Sigma T5648) (100 mg/kg body weight, 10 µg/ml stock solution in corn oil) or vehicle (corn oil). Animals, aged P30–P40, were injected for five consecutive days and behavior tests were performed >21 days post (last injection (animals age P55-P65) to ensure successful recombination, protein degradation, and Tamoxifen (and its metabolites) clearance, as previously described53.

The behavioral tests were performed as described above. After behavioral tests, animals were sacrificed, the brain dissected and the right hemisphere was used for tissue lysis and western blot analysis to determine Cul3 protein levels and success of Tamoxifen-induced deletion. As described previously54, in 5–20% of mice treated with Tamoxifen, induction appeared to fail altogether, and no changes on Cul3 protein levels could be observed. These mice were excluded from the analysis.

Statistics. Statistical analyses were performed using Microsoft® Excel® 2013, Origin Software (Origin Inc.) and GraphPad Prism 6/8. Shapiro–Wilks test was used to evaluate normal distribution, means and standard deviations of the data. Parametric tests were analyzed for significance using unpaired two-tailed t-tests, 1-way or 2-way ANOVAs with Sidak’s post-hoc test, using P < 0.05, **P < 0.01, and ***P < 0.001 for significance, and presented as a bar, box and whiskers, scatter dot plots and mean ± standard error of the mean (SEM), unless otherwise specified. Data sets with non-normal distributions were analyzed using the two-tailed Mann–Whitney U test. Adjustment for multiple comparisons was made using post-hoc tests. Cumulative probability plots of the amplitude and inter-event interval of synaptic currents were compared with the Kolmogorov–Smirnov two-sample test. Illustrations for figures were prepared in Adobe Illustrator or using BioRender (BioRenderer.com). Detailed statistics are presented in Supplementary Data 1. Experiments were replicated at least three times.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE51 partner repository with the data set identifier PXD017040. Publicly available data sets used are available from the Allen Cell Atlas (https://atlas.brain-map.org/atlas-data/mouse) and the BrainSpan Atlas [http://www.brainspan.org/static/download.html]. Source data are provided with this paper.

Received: 5 August 2020; Accepted: 14 April 2021; Published online: 24 May 2021

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Acknowledgements
We thank A. Coll Manzano, F. Freeman, M. Ladron de Guevara, and A. C. Yahya for technical assistance, S. Deixler, A. Lepold, and A. Schlerka for the management of our animal colony, as well as M. Schunn and the Preclinical Facility team for technical assistance, S. Deixler, A. Lepold, and A. Schlerka for the management of our animal colony, as well as M. Schunn and the Preclinical Facility team for technical assistance.
Author contributions
J.M. and L.A.S. designed and performed experiments, analyzed data, and prepared figures. B.B., G.D., and S.T. performed experiments and data analysis. A.N., C.S., and C.P.D. analyzed data. C.K., Z.D., L.S.K., and E.C. performed experiments. J.G.D. supervised STED-imaging. F.K.S. supervised generation and analysis of Pls3 mutant cells. G.N. conceived and supervised the study. G.N. wrote the paper together with J.M. and L.A.S. All authors read and approved the final version of the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-23123-x.

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Peer review information
Nature Communications thanks Camilla Bellone, Laurent Nguyen and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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