**The Conserved P1019R Mutation of WASH-complex protein Swip (Washc4) Results in WASH Disruption, Endo-lysosomal Dysfunction, and Cognitive-Movement Deficits in Mice and Humans**

**The Conserved SWIP*P1019R* Mutation Results in WASH-Complex Disruption, Endo-lysosomal Dysfunction, and Cognitive-Movement Deficits in Mice and Humans**

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**Abstract:**

Loss of WASH complex function is implicated in human cognitive and movement disorders, yet its functional role in the nervous system remains poorly understood. Using *in vivo* BioID proteomics, we identify the neuronal WASH complex proteome, revealing a network of endosomal interactors. To uncover links between WASH complex dysfunction and disease, we generate a mouse model of the human P1019R mutation in WASH complex protein SWIP. Quantitative spatial proteomics analysis of SWIPP1019R brain uncovers perturbations in endo-lysosomal trafficking pathways. Cellular and histological analyses confirm these functional changes and reveal evidence of neurodegeneration. Behavioral analyses confirm that *SWIPP1019R* impacts cognition and demonstrate that SWIP*P1019R* results in significant deficits in motor function. Retrospective analysis of the patients first studied confirms this deficit in humans. Combined, these findings demonstrate WASH’s critical role in neuronal endo-lysosomal function and reveal endo-lysosomal dysfunction as a key driver of cognitive-movement disorders.

**Introduction:**

Neurons maintain precise control of their subcellular proteome using a sophisticated network of vesicular trafficking pathways that shuttle cargo throughout their elaborate processes. Endosomes function as a central hub in organizing this intricate vesicular relay system, coordinating protein sorting between multiple cellular compartments, including surface receptor endocytosis and recycling, as well as degradative shunting to the lysosome. How endosomal trafficking in neurons is modulated remains a vital area of research due to the unique degree of spatial organelle segregation within neuronal cells and its strong implication in developmental and degenerative diseases.

In non-neuronal cells an evolutionarily conserved complex, termed the Wiskott Aldrich Syndrome protein and SCAR Homology (WASH) complex, coordinates endosomal trafficking7,8. WASH is composed of five proteins as its core components: WASH1, Fam21, ccdc53, SWIP, and Strumpellin (encoded by genes *Washc1-Washc5*, respectively), which are broadly expressed in multiple organ systems1,9–12. The WASH complex plays a central role in non-neuronal endosomal trafficking by activating Arp2/3-dependent actin branching at the outer surface of endosomes to influence cargo sorting and vesicular scission9,13–16. It also interacts with at least three main cargo adaptor complexes — Retromer, Retriever, and the COMMD/CCDC22/CCDC93 (CCC) complexes — all of which associate with distinct sorting nexins to select specific cargo and enable their trafficking to other cellular locations10,16–20. Loss of the WASH complex in non-neuronal cells has detrimental effects on endosomal structure and function, such as endosomal tubule elongation and cargo mislocalization13,14,16,21–24. In addition, expression of a dominant-negative form of WASH1 in amoeba impairs recycling of lysosomal V-ATPases, supporting a role for the WASH complex in endo-lysosomal trafficking25,26. Studies in *Drosophila* and mammalian cell lineshave shown similar endo-lysosomal contributions, as loss of WASH components affects integrin receptor trafficking, as well as lysosomal acidification1,27,28. While these studies have uncovered the mechanistic actions of WASH in endo-lysosomal networks *in vitro*, whether these roles apply to neuronal cells or produce functional ramifications *in vivo* remains to be elucidated.

It has become clear that preservation of the endo-lysosomal system is critical to neuronal function, as mutations in mediators of this process are implicated in neurological diseases such as Parkinson’s disease, Huntington’s disease, Alzheimer’s disease, Frontotemporal Dementia, Neuronal Ceroid Lipofuscinoses, and Hereditary Spastic Paraplegia 4,23,31–39. These links to predominantly neurodegenerative conditions have supported the proposition that loss of endo-lysosomal integrity can have compounding effects over time and contribute to progressive disease pathology. Consistent with the association between the endo-lysosomal system and disease, as well as the potential role of WASH in modulating neuronal endo-lysosomal fidelity, dominant missense mutations of *Washc5* (protein: Strumpellin) are associated with hereditary spastic paraplegia (SPG8)4,40, and autosomal recessive point mutations in *Washc4* (protein: SWIP) and *Washc5* are associated with syndromic and non-syndromic intellectual disabilities3,6,41. In particular, an autosomal recessive mutation in *Washc4* (*KIAA1033c.3056c>g*, encoding SWIPP1019R) was identified in a cohort of children with non-syndromic intellectual disability3. Cell lines derived from these patients exhibited decreased abundance of WASH proteins. These authors suggested that the observed cognitive deficits in humans with the SWIPP101R mutation could result from disruption of neuronal WASH-endosomal signaling. However, whether this mutation leads to perturbations in endo-lysosomal integrity or neurodegeneration over time is not known.

Here we report the analysis of neuronal WASH and its molecular role in disease pathogenesis. We use *in vivo* proximity proteomics (iBioID) to uncover the neuronal WASH proteome, and demonstrate that it is highly enriched for components of endosomal components in mouse brain. We then generate a mouse model of the human *WASHC4* (aka *KIAA1033)*mutation (*WASHC4.3056c>g* encoding*SWIPP1019R*)3 to define whether this mutation alters neuronal trafficking pathways and test whether it leads to phenotypes congruent with human patients. Using an adapted quantitative spatial-proteomics approach42 and protein covariation analysis , we find strong evidence for substantial disruption of neuronal endo-lysosomal pathways *in vivo*. Cellular analyses confirm a significant impact on neuronal endo-lysosomal trafficking *in vitro* and *in vivo*, with evidence of lipofuscin accumulation and progressive activation of apoptotic pathways indicative of neurodegenerative pathology. Behavioral analyses of SWIPP1019R mice at adolescence and adulthood confirm a role of WASH in cognitive processes, as well as profound, progressive motor dysfunction. Retrospective examination of *SWIPP1019R* patient data confirms motor dysfunction coincident with cognitive impairments. Our results reveal a central role of the WASH complex in neuronal endo-lysosomal pathways, and demonstrate its dysfunction as a driver of endo-lysosomal dysfunction and cognitive/movement impairments.

**Results:**

**Identification of the WASH complex proteome *in vivo* reveals a conserved role in endosomal trafficking.** While multiple mutations within the WASH complex have been identified in humans3,4,6,41, how these mutations lead to neurological dysfunction remains unknown (Fig 1a). Given that previous work in non-neuronal cell culture systems and non-mammalian organisms has established that the WASH complex functions in endosomal trafficking, we aimed to determine whether this role was conserved in the mouse nervous system1–3,14,21. To determine the likely molecular functions of the neuronal WASH complex, we utilized an *in vivo* BioID (iBioID) paradigm developed in our laboratory to identify the WASH complex proteome from brain tissue *in vivo*43. BioID probes were generated by fusing a component of the WASH complex, WASH1 (gene *Washc1*), with a version of the promiscuous biotin ligase, BioID2 (WASH1-BioID2, Fig 1b), or by expressing BioID2 alone (negative control, soluble BioID2) under the neuron-specific, human Synapsin-1 promoter. We injected adenoviruses (AAV) expressing these constructs into the cortex of wild-type postnatal day zero (P0) mice (Fig 1c), and two-weeks post-injection, we administered daily subcutaneous biotin injections for seven days for *in vivo* biotinylation. The constructs displayed efficient expression and activity in brain tissue, as evidenced by colocalization of the WASH1-BioID2 viral epitope (HA) and biotinylated proteins (Streptavidin) (Fig 1d, Extended Data Fig 1). For label-free quantitative high-mass accuracy LC/MS/MS analyses, brain samples were collected at P22, snap-frozen, and processed as previously described43.A total of 2,242 proteins were identified in all three experimental replicates, which were further analyzed for those with significant enrichment in WASH1-BioID2 samples over solubleBioID2 negative controls (Supplementary Table 1).

The resulting neuronal WASH proteome included 176 proteins that were significantly enriched (Fig 1e). Of these proteins, we identified all five WASH complex components (Fig 1f), as well as 13 previously reported WASH complex interactors (Fig 1g, Supplementary Table X)9,16, which provided strong validity for our proteomic approach. Additionally, bioinformatic analyses of the neuronal WASH proteome identified a network of proteins implicated in vesicular trafficking, including 23 proteins enriched for endosomal functions (Fig 1h) and 24 proteins enriched for endocytic functions (Fig 1i). Among these endosomal and endocytic proteins were components of the recently identified endosomal sorting complexes, CCC (ccdc93 and commd9) and Retriever (vps35l)16,20, as well as multiple sorting nexins important for recruitment of trafficking regulators to the endosome and cargo selection, such as snx1-3, and snx1644–46*.* These data demonstrated that the WASH complex interacts with many of the same proteins in neurons as it does in yeast, flies, amoeba, and human cell lines. Furthermore, there were 32 proteins enriched for cytoskeletal regulatory functions (Fig 1j), including actin-modulatory molecules such as the Arp2/3 complex subunit Arpc5, which is consistent with its role in activating this complex to stimulate actin polymerization at endosomes for vesicular scission2,21.The WASH1-BioID2 isolated complex also contained 28 proteins known to localize to the excitatory post-synapse (Fig 1k). This included many scaffolding proteins, such as Shank2-3 and Dlgap2-447–50, as well as modulators of synaptic receptors such as Syngap1and Shisa651–54, which was consistent with the idea that vesicular trafficking plays an important part in synaptic function and regulation. Taken together, the results of this analysis supported a role of the WASH complex in endosomal trafficking in mouse brain.

**SWIPP1019R does not incorporate into the WASH complex, reducing its stability and levels *in vivo*.** To determine how the WASH complex’s endosomal role is affected in disease, we generated a mouse model of a human WASHC4 missense mutation found in children with intellectual disability *WASHC4*. Due to sequence homology between human and murine gene sequences, we were able to introduce the same point mutation in exon 29 of murine *Washc4* using CRISPR (Fig 2a)3,8. This C>G point mutation results in a Proline>Arginine amino acid substitution at position 1019 of SWIP’s amino acid sequence (Fig 2a)3. Analysis of brain lysate from adult homozygous SWIPP1019R mutant mice (referred to from here on as MUT) displayed significantly decreased expression of two other WASH complex members, Strumpellin and WASH1 (Fig 2b). This phenocopied data from the human patients3 and suggested that the WASH complex is unstable in the presence of this SWIP point mutation *in vivo*. To test whether this mutation disrupted interactions between WASH complex subunits, we measured the binding affinities between SWIPP1019R and WASH components, Strumpellin and WASH1, by co-immunoprecipitation in HEK cells. Compared to wild-type, MUT SWIP had a significant reduction in binding to WASH1 and Strumpellin (54.8% and 41.4%, respectively), suggesting that the SWIPP1019R mutation hinders WASH complex formation (Extended Data Fig 2). Together these data support the notion that SWIPP1019R is a damaging mutation that impairs not only the function of SWIP, but also results in significant reductions of the WASH complex as a whole.

**Subcellular fractionation and 16-plex TMT proteomics reveal significant disruptions in the endo-lysosomal pathway of SWIPP1019R mutant mouse brain.** Next we aimed to understand the molecular consequences of the SWIPP1019R mutation.on the subcellular organization of the brain proteome.. To do so, we used a tandem-mass-tag (TMT) spatial-proteomics approach to determine which proteins have altered abundance in adult SWIPP1019R mutant (MUT) versus wild-type control (WT) mouse brain42,55. Brains from 10-month-old mice were gently homogenized to release intact organelles, followed by successive centrifugation steps to enrich subcellular compartments into different fractions (Fig 2c)42. Seven WT and seven MUT fractions (each prepared from one brain) were labeled with unique isobaric tandem-mass tags and concatenated. In each 16-plex LC MS/MS experiment we included two quality control which were generated by pooling equivalent fractions of all WT and MUT samples, These samples allowed us to assess experimental variability and perform normalization between experimentsBy performing this experiment in triplicate, deep coverage of the mouse brain proteome was obtained—across all 48 samples we quantified 86,551 peptides, corresponding to 7,488 proteins. After data preprocessing, normalization, and filtering we retained 5,897 highly reproducibly quantified proteins in the final dataset. (Supplementary Table XX).

We assessed protein differential abundance at two different levels: 1) at the level of intra-fraction comparisons between WT and MUT genotypes, and 2) comparisons between WT and MUT genotypes regardless of fraction differences. At level 1 there were85 proteins with significantly altered abundance in at least one of the 7 fractions.… (Extended Data Fig 3b; 5 Proteins were differentially abundant in all 7 fractions (Washc4, Washc2, Washc5, Washc1), these included the four WASH proteins and Rab21a—a known WASH interactor that functions in eary endosome trafficking[REF]. The abundance of the remaining WASH complex protein, Washc1, was found to be very low and was not retained in the final dataset due to its sparse quantification). At level 2 we are interested in the effect of genotype, that is adjusted for differences in fraction, on protein abundance. This is the average effect of genotype across all fractions. At this level, there were 62 proteins with an FDR of <0.1 and +/- 20% change. In all there were 398 proteins with an FDR < 0.1 (May consider adding Supplementary proteomics fig).

In order to identify groups of proteins that are changing together Clustering all identified proteins based on their distribution profiles and known interactions revealed 252 distinct modules of proteins that strongly covary together (Supplemental Table X). Many of these modules exhibited significant differences in WT versus MUT brain (Extended Data Fig 4). Of note, the module containing the WASH complex, M19, was predicted to have endosomal function by gene ontology annotation, and was enriched for proteins identified by WASH1-BioID2 (bold node edges, Fig 2d). Similar to the WASH BioID proteome (Fig 1), M19 contained components of the CCC (Ccdc22, Ccdc93, Commd1-3, Commd7, and Commd9) and Retriever sorting complexes (Vps26c and Vps35l), but not the Retromer sorting complex, suggesting that in the brain, the WASH complex may not interact as closely with Retromer as it does in other cells (Fig 2d). The abundance of proteins in M19 was significantly lower in MUT brain compared to WT, supporting that the SWIPP1019R mutation hinders this network’s function (Fig 2e). Interestingly, some modules displayed increased, rather than decreased abundance in MUT brain, such as M2. This network contained multiple lysosomal proteins (Ctsl, Ctss, Ids, Man2b1, Grn; Fig 2f,g), suggesting that disruption of the WASH complex may increase demand for lysosomal substrate degradation56–59. Together, these altered modules and changes in protein levels demonstrate that loss of the WASH complex not only influences endosomal trafficking interactions, but also the stability of endo-lysosomal machinery.

In addition to endo-lysosomal changes, network alterations were evident for an endoplasmic reticulum (ER) module (M84), as well as two synaptic modules (M35, M143) in MUT mouse brain, supporting a change in the proteostasis of mutant neurons (Extended Data Fig 4). Notably, within the ER module, M84, there was increased abundance of chaperones (e.g. Hspa5) and foldases, (e.g. Pdia4), which are commonly engaged in the presence of misfolded proteins, such as those seen in Alzheimer’s disease or Parkinson’s disease (Extended Data Fig 4d)60–64. Taken together, these data demonstrate that the WASH complex functions in close proximity to endosomal compartments and its loss from these locations likely alters not only endosomal, but also lysosomal trafficking, with increased stress on cellular homeostasis.

**Mutant neurons display structural abnormalities in endo-lysosomal compartments *in vitro.***  Combined, the proteomics data strongly suggested that the ndo-lysosomal pathway was altered in adult SWIPP1019R mutant mouse brain. Next, we next analyzed whether structural changes in this system were evident in primary neurons. Cortical neurons from littermate WT and MUT P0 pups were cultured for 15 days *in vitro* (DIV15, Fig 3a), then fixed and stained for verified markers of early endosomes (EEA1; Eea1, Fig 3b,c) and lysosomes (Cathepsin D; Ctsd, Fig 3d,e). Reconstructed three-dimensional volumes of EEA1 and Cathepsin D puncta revealed that MUT neurons display larger EEA1+ somatic puncta than WT neurons (Fig 3g,j), but no difference in the total number of EEA1+ puncta (Fig 3f). This finding is consistent with a loss of function mutation, as loss of WASH activity prevents cargo scission from endosomes and leads to cargo accumulation22,24. On the other hand, MUT neurons exhibited significantly less Cathepsin D+ puncta than WT neurons (Fig 3h), but the remaining puncta were significantly larger than those of WT neurons (Fig 3i,k). These data confirmed that the SWIPP1019R mutation results in both molecular and morphological abnormalities in the endo-lysosomal pathway.

**­­SWIPP1019Rmutant brains exhibit increased cell death and abnormal endo-lysosomal structures *in vivo*.** As there is strong evidence that dysfunctional endo-lysosomal trafficking can be a basis for neurodegenerative disorders, adolescent (P42) and adult (10 month-old, 10mo) WT and MUT brain tissues were analyzed for the presence of cleaved caspase-3, a marker of apoptotic pathway activation, in three brain regions65,66. Very little cleaved caspase-3 staining was present in WT and MUT mice at adolescence (Fig 4a,b, Extended Data Fig 5-6). However, at 10mo, the MUT motor cortices displayed significantly greater cleaved caspsase-3 staining compared to age-matched WT controls (Fig 4c-f), but did not exhibit any significant difference in the number of total cells (Fig 4g-k). Furthermore, this difference appeared to be selective for the motor cortex, as we did not observe significant differences in cleaved caspase-3 staining at either age for striatal or cerebellar regions (Extended Data Fig 5-6). These data suggested that neurons of the motor cortex were particularly susceptible to disruption of the endo-lysosomal pathway, perhaps because long-range corticospinal neurons require high fidelity of trafficking pathways[REF].

To further examine motor neurons of the primary motor cortex at a subcellular resolution, samples from age-matched 7-month-old WT and MUT mice (7mo, 3 animals each) were imaged by transmission electron microscopy (TEM). Strikingly, the somas of MUT cortical motor neurons had large electron-dense inclusions with associated electron-lucent lipid-like inclusions (arrows, Fig 4l,m), which were consistent with lipofuscin accumulation at lysosomal residual bodies67–69. Lipofuscin is the result of lysosomal breakdown of lipids, proteins, and carbohydrates, which naturally accumulates over time in non-dividing cells such as neurons70–72. The marked increase in lipofuscin area and number in MUT electron micrographs (Fig 4n,o respectively), is consistent with dysregulated lysosomal breakdown of cellular material and is associated with neurodegenerative disorders, including Alzheimer’s disease, Parkinson’s disease, and Neuronal Ceroid Lipofuscinoses72. Together these data indicate that SWIP is important for maintaining proper lysosomal-mediated cargo degradation and its dysfunction could lead to neurodegeneration.

**SWIPP1019Rmutant mice display persistent deficits in cued fear memory recall.** To observe the functional neuronal consequences of the SWIPP1019R mutation, we next studied WT and MUT mouse behavior. Given that children with homozygous SWIPP1019R point mutations display intellectual disability3 and SWIPP1019R mutant mice exhibit endo-lysosomal disruptions implicated in neurodegenerative processes, behavior was assessed at two ages: adolescence (P40-50), and mid-late adulthood (5.5-6.5 mo). Interestingly, MUT mice performed equivalently to WT mice in episodic and working memory paradigms, including novel object recognition and Y-maze alternations (Extended Data Fig 7-8). However, in a fear conditioning task, MUT mice displayed a significant deficit in cued fear memory (Fig 5). This task tests the ability of a mouse to associate an aversive event (a mild electric footshock) with a paired tone (Fig 5a). Freezing behavior of mice during tone presentation is attributed to hippocampal or amygdala-based fear memory processes73–75. Forty-eight hours after exposure to the paired tone and footshock, MUT mice showed a significant decrease in conditioned freezing to tone presentation compared to their WT littermates (Fig 5b,c). To ensure that this difference was not due to altered sensory capacities of MUT mice, we measured the startle response of mice to both electric foot shock and presented tones. In line with intact sensation, MUT mice responded comparably to WT mice in these tests (Extended Data Fig 9). These data demonstrate that although MUT mice perceive footshock sensations and auditory cues, it is their memory of these paired events that is significantly impaired. Additionally, this deficit in fear response was evident at both adolescence and adulthood (top panels, and bottom panels, respectively, Fig 5b,c), consistent with cognitive impairments presenting as an early phenotype downstream of SWIPP1019R.

**SWIPP1019Rmutant mice exhibit motor deficits that progressively worsen with age.** Because SWIPP1019R results in endo-lysosomal pathology consistent with neurodegenerative disorders in the motor cortex, we next analyzed motor function in the mice over time. First, we tested the ability of WT and MUT mice to remain on a rotating rod for five minutes (Rotarod, Fig 6a-c). Surprisingly, at both adolescence and adulthood the MUT mice performed markedly worse than WT littermate controls (Fib 6c). Interestingly, mouse performance was not significantly different across trials, which suggested that this difference in retention time was not due to progressive fatiguing, but more likely due to an overall difference in motor control76.

To study the animals’ movement at a finer scale, the gait of WT and MUT mice was also analyzed using a Treadscan system containing a high-speed camera coupled to a transparent treadmill (Fig 6d)77. Interestingly, while the gait parameters of mice were largely indistinguishable across genotypes at adolescence, a striking difference was seen when the same mice were aged to adulthood (Fig 6e-g). In particular, MUT mice took slower (Fig 6e), longer strides (Fig 6f), stepping closer to the midline of their body (track width, Extended Data Fig 10), and their gait symmetry was altered so that their strides were no longer perfectly out of phase (out of phase=0.5, Fig 6g). While these differences were most pronounced in the rear limbs (as depicted in Fig 6e-g), the same trends were present in front limbs (Extended Data Fig 10). These findings demonstrate that SWIPP1019R results in a progressive motor function decline that was detectable by the rotarod task at adolescence, but which became more prominent with age, as both gait and strength functions deteriorated.

These marked motor findings prompted us to re-evaluate the original human SWIPP1019R patients3. While developmental delay or learning difficulties were the primary impetus for medical evaluation, all patients also exhibited motor symptoms at initial presentation (mean age = 10.4 years old, Fig 6h). The patients’ movements were described as “clumsy” with notable fine motor difficulties, dysmetria, dysdiadochokinesia, and mild dysarthria on clinical exam (Fig 6h). Recent communication with the parents of these patients who are now on average 21 years old, revealed no notable exacerbation of these symptoms (personal communication with Dr. Anna Rajab). It is therefore possible that our mouse model could portend disease progression for these individuals, given that we observed significant worsening at 5-6 months old in mice (equivalent to ~30-35 years old in humans).

**Conclusions:**

Taken together, the data presented here support a mechanistic model whereby SWIPP1019R causes a loss of WASH complex function, resulting in endo-lysosomal disruption and accumulation of neurodegenerative markers, such as upregulation of unfolded protein response modulators and lysosomal enzymes, as well as build-up of lipofuscin and cleaved caspase-3 over time. To our knowledge, this study provides the first mechanistic evidence of WASH complex impairment having direct and indirect effects that lead to cognitive deficits and progressive motor impairments (Fig 7).

Using *in vivo* proximity-based proteomics in wild-type mouse brain, we identify that the WASH complex interacts with the CCC (commd9 and ccdc93) and Retriever (Vps35l) cargo selective complexes20,22. Interestingly, we did not find significant enrichment of the Retromer sorting complex, a well-known WASH interactor, suggesting that it may not play as large a role in neuronal WASH-mediated cargo sorting (Fig 1). These data are supported by our tandem-mass-tag (TMT) and modular network analyses of SWIPP1019R mutant brain, which clustered the WASH, CCC, and Retriever complexes together in M19, but not the Retromer complex (Fig 2). TMT analyses also revealed that disruption of these WASH-CCC-Retriever interactions may have multiple downstream effects on the endosomal machinery, since endosomal modules displayed significant changes in SWIPP1019R brain (including M19, Fig 2 and M14, Extended Data Fig 4b), with corresponding decreases in abundance of endosomal proteins including Retromer subunits (vps29, vps35; M14)10, associated sorting nexins (snx17 (NC), snx27; M137!)19,78, known WASH interactors (Rab21 M137, Fkbp15, NC)79–81, and cargos (Lrp1, Itga3 M137; M85) (Extended Data Fig 3, 4b)10,82. While previous studies have indicated that Retromer and CCC influence endosomal localization of WASH16,20,80, our findings of altered endosomal networks containing decreased Retromer, Retriever, and CCC protein levels in SWIPP1019Rmutant brain point to a possible feedback mechanism wherein WASH impacts the protein abundance and/or stability of these interactors (Fig 2, Extended Data Figs 3, 4b). Perhaps redundancies exist within these endosomal sorting machineries to ensure trafficking accuracy. Future studies defining the hierarchical interplay between WASH, Retromer, Retriever, and CCC in neurons could provide clarity on how these mechanisms are organized.

In addition to highlighting an intricate endosomal sorting network, our TMT proteomics also identified proteins networks with increased abundance in SWIPP1019Rmutant brain (M2, Fig 2f-g; Extended Data Fig 4b). The proteins in these modules fell into two interesting categories: lysosomal enzymes and proteins involved in the endoplasmic reticulum (ER) stress response. Of note, some of the lysosomal enzymes with elevated levels in MUT brain (Grn (M2), Hexb (M6), and Gns (M208); Extended Data Fig 3, 4c) are implicated in lysosomal storage disorders, where they generally have decreased, rather than increased, function or expression59,83,84. We speculate that loss of WASH function in our mutant mouse model may lead to increased accumulation of cargo and associated machinery at early endosomes (as seen in Fig 3, enlarged EEA1+ puncta), eventually overburdening the vesicles and triggering transition to late endosomes for subsequent fusion with degradative lysosomes (Fig 7). This would effectively increase delivery of endosomal substrates to the lysosome compared to baseline, resulting in enlarged lysosomal structures and increased demand for these degradative enzymes. For example, since mutant neurons display larger lysosomal structures (Fig 3,4), they may require higher quantities of progranulin (Grn, Extended Data Fig 3) for sufficient lysosomal acidification85.

Similarly, endoplasmic reticulum (ER) stress is commonly observed in neurodegenerative states, where accumulation of misfolded proteins disrupts cellular proteostasis64,86,87. This cellular strain triggers the adaptive unfolded protein response (UPR), which attempts to restore cellular homeostasis by increasing the cell’s capacity to retain misfolded proteins within the ER, remedy misfolded substrates, and trigger degradation of persistently misfolded species. Involved in this process are ER chaperones and foldases that we identified as increased in SWIPP1019Rmutant brain including BiP (Hspa5), calreticulin (Calr), calnexin (Canx), and the protein disulfide isomerase family members (Pdia1, Pdia4, Pdia6) (Extended Data Fig 3)88. Many of these proteins were identified in the ER protein module found to be significantly altered in MUT mouse brain (M84), supporting a network-level change in the ER stress response (Extended Data Fig 4d). One notable exception to this trend was endoplasmin (Hsp90b1), which exhibited significantly decreased abundance in SWIPP1019Rmutant brain (Extended Data Fig 3). This is surprising given that endoplasmin has been shown to coordinate with BiP in protein folding89, however it may highlight a possible compensatory mechanism. Additionally, prolonged UPR can stimulate autophagic pathways in neurons, where misfolded substrates are delivered to the lysosome for degradation87. These data highlight a relationship between ER and endo-lysosomal disturbances as an exciting avenue for future research.

Importantly, the cellular disruptions we observed in our SWIPP1019R model are also seen in disorders such as Alzheimer’s disease, Parkinson’s disease, Frontotemporal Dementia, and Neuronal Ceroid Lipofuscinoses (NCLs)90. In particular, NCLs are lysosomal storage disorders primarily found in children, with heterogenous presentations and multigenic causations31. The majority of genes implicated in NCLs affect lysosomal enzymatic function or transport of proteins to the lysosome31,67,68,91. Most patients present with marked neurological impairments, such as learning disabilities, motor abnormalities, vision loss, and seizures, and have the unifying feature of lysosomal lipofuscin accumulation upon pathological examination31. While the human SWIPP1019Rmutation has not been classified as an NCL3, findings from our mutant mouse model suggest that loss of WASH complex function leads to phenotypes bearing strong resemblance to NCLs, including lipofuscin accumulation (Fig 3-6). As a result, our mouse model could provide the opportunity to study these pathologies at a mechanistic level, while also enabling preclinical development of treatments for their human counterparts.

Currently there is an urgent need for greater mechanistic investigations of neurodegenerative disorders, particularly in the domain of endo-lysosomal trafficking. Here we demonstrate the power of combining *in vivo* proteomics with *in vitro* and *in vivo* functional studies to uncover genetic and organelle trafficking relationships in disease pathology. Applying these approaches to other neurodegenerative disorders with organellar dysfunction could uncover convergent disease pathways. By investigating fundamental cellular processes in neurological diseases, neuron-specific modulators of the endo-lysosomal pathway may be identified, providing new future avenues for therapeutic endeavors.

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**Author Contributions:**

Conceptualization: JLC, TWAB, IHK, and SHS; Methodology: JLC, TWAB, IHK, and SHS; Investigation: JLC, TWAB, IHK, GW, TH, RV, ES, and AR; Resources: ES, RV, and SHS; Writing—Original Draft: JLC and SHS; Writing—Editing: JLC, TWAB, IHK, and SHS; Visualization: JLC; Funding Acquisition: JLC, TWAB, IHK, and SHS. All authors discussed the results and commented on the manuscript.

**Competing Interests:**

The authors declare no competing financial interests.

**Methods:**

**Animals**

We generated *Washc4* mutant (SWIPP1019R) mice in collaboration with the Duke Transgenic Core Facility to mimic the *de novo* human variant at amino acid 1019 of human *WASHc4.* A CRISPR-induced CCT>CGT point mutation was introduced into exon 29 of *Washc4*. 50ng/ µl l of the sgRNA (5’-ttgagaatactcacaagaggagg-3’), 100ng/ µl l Cas9 mRNA, and 100ng/µl of a repair oligo (5’-atttcgaaggccaaagaatatacatctccgaaatttctatatcattgttc**g**tcctcttgtgagtattctcaaaactagaagtgagttattgatgggtgttaatacagattcagtttccataaagca-3’) were injected into the cytoplasm of B6SJLF1/J mouse embryos (Jax #100012). Mice with germline transmission were then backcrossed into a C57BL/ 6J background (Jax #000664). At least 5 backcrosses were obtained before animals were used for behavior. We bred het x het SWIPP1019R mice to obtain age-matched mutant and wild-type genotypes for cell culture and behavioral experiments. Genetic sequencing was used to screen for germline transmission of the C>G point mutation (*FOR:* 5’-tgcttgtagatgtttttcct-3’, *REV*: 5’-gttaacatgatcctatggcg-3’). All mice were housed in the Duke University′s Division of Laboratory Animal Resources or Behavioral Core facilities at 2-5 animals/cage on a 14:10h light:dark cycle. All experiments were conducted with a protocol approved by the Duke University Institutional Animal Care and Use Committee in accordance with NIH guidelines.

**Constructs**

For immunoprecipitation experiments, a pmCAG-SWIP-WT-HA construct was generated by PCR amplification of the human *Washc4* sequence, which was then inserted between NheI and SalI restriction sites of a pmCAG-HA backbone generated in our lab. Site-directed mutagenesis (Agilent #200517) was used to introduce a C>G point mutation into this pmCAG-SWIP-WT-HA construct for generation of a pmCAG-SWIP-MUT-HA construct *(FOR:* 5'-ctacaaagttgagggtcagacggggaacaattatatagaaa-3', *REV:* 5'-tttctatataattgttccccgtctgaccctcaactttgtag-3’)*.* For iBioID experiments, an AAV construct expressing hSyn1-WASH1-BioID2-HA was generated by cloning a *Washc1* insert between SalI and HindIII sites of a pAAV-hSyn1-Actin Chromobody-Linker-BioID2-pA construct (replacing Actin Chromobody) generated in our lab. This backbone included a 25nm GS linker-BioID2-HA fragment from Addgene #80899, generated by Roux et al92. An hSyn1-solubleBioID2-HA construct was created similarly, by removing Actin Chromobody from the above construct.

**Antibodies**

*Immunocytochemistry:*

Primary antibodies: Rabbit anti-EEA1 (Cell Signaling Technology #C45B10, 1:500), Rat anti-CathepsinD (Novus #204712, 1:250), Guinea Pig anti-MAP2 (Synaptic Systems #188004, 1:500)

Secondary antibodies: Goat anti-Rabbit Alexa Fluor 568 (Invitrogen #A11036, 1:1000), Goat anti-Guinea Pig Alexa Fluor 488 (Invitrogen #A11073, 1:1000), Goat anti-Rat Alexa Fluor 488 (Invitrogen #A11006, 1:1000), Goat anti-Guinea Pig Alexa Fluor 555 (Invitrogen #A21435, 1:1000)

*Immunohistochemistry:*

Primary antibodies:

Rabbit anti-Cleaved Caspase-3 (Cell Signaling Technology #9661, 1:2000), Mouse anti-Calbindin (Sigma #C9848, 1:2000), Rat anti-HA 3F10 (Sigma #12158167001, 1:500)

Secondary antibodies:

Donkey anti-Rabbit Alexa Fluor 488 (Invitrogen #A21206, 1:2000), Goat anti-Mouse Alexa Fluor 594 (Invitrogen #A11032, 1:2000), Goat anti-Rat Alexa Fluor 488 (Invitrogen #A11006, 1:5000), Streptavidin Alexa Fluor 594 conjugate (Invitrogen #S32356, 1:5000), 4′,6-diamidino-2-phenylindole (DAPI, Sigma #D9542, 1:1000 for 10min at RT)

*Western blotting:*

Primary antibodies: Rabbit anti-Strumpellin (Santa Cruz #sc-87442, 1:500), Rabbit anti-WASH1 c-terminal (Sigma #SAB4200373, 1:500), Mouse anti-Beta Tubulin III (Sigma #T8660, 1:10,000), Mouse anti-HA (BioLegend #MMS-101P, 1:5000)

Secondary antibodies: Donkey anti-Rabbit-HRP (GE Life Sciences #NA934, 1:5,000), Goat anti-mouse-HRP (GE Life Sciences #NA931, 1:5000)

**AAV viral preparation**

Large-scale AAV preparations were performed as described previously43. The day before transfection, HEK293T cells were plated at a density of 1.5x107 cells per 15cm2 plate in DMEM media with 10% fetal bovine serum and 1% Pen/Strep (Thermo #11965-092, Sigma #F4135, Thermo #15140-122). Six HEK293T 15cm2 plates were used per viral preparation. The next day, 30µg of pAd-DeltaF6 helper plasmid, 15µg of AAV2/9 plasmid, and 15µg of an AAV plasmid carrying the transgene of interest were mixed in OptiMEM with PEI-MAX (final concentration 80µg/ml, Polysciences #24765). 2ml of this solution were then added dropwise to each of 6 HEK293T plates. Eight hours later, the media was replaced with 20ml DMEM+10%FBS. 48 hours after that, cells were scraped and collected in the media, pooled, and centrifuged at 1500rpm for 5min at RT. The final pellet from the 6 cell plates was resuspended in 5ml of cell lysis buffer (15 mM NaCl, 5 mM Tris-HCl, pH 8.5), and freeze-thawed three times using an ethanol/dry ice bath. The lysate was then treated with 50U/ml of Benzonase (Novagen #70664), for 30min in a 37ºC water bath, vortexed, and then centrifuged at 4500rpm for 30min at 4ºC. The resulting supernatant was added to the top of an iodixanol gradient (15%, 25%, 40%, 60% top to bottom) in an Optiseal tube (Beckman Coulter #361625). The gradient was then centrifuged using a Beckman Ti-70 rotor in a Beckman XL-90 ultracentrifuge at 67,000rpm for 70min, 18ºC. The viral solution was extracted from the 40%/60% iodixanol interface using a syringe, and placed into an Amicon 100kDa filter unit (#UFC910024). The viral solution was washed in this filter 3 times with 1X ice-cold PBS by adding 5ml of PBS and centrifuging at 4900rpm for 45min at 4ºC, which concentrated the virus to 200µl total volume. 5-10µl aliquots of virus were stored at -80ºC until use.

**Cell Lines**

HEK293T cells (ATCC #CRL-11268) were purchased from the Duke Cell Culture facility, and were tested for mycoplasma contamination. HEK239T cells were used for coimmunoprecipitation experiments and preparation of AAV viruses.

**Primary Neuronal Culture**

Primary neuronal cultures were prepared from mouse cortex. P0 pups were rapidly decapitated and cortices were dissected and kept individually in 5ml Hibernate A (Thermo #A1247501) supplemented with 2% B27(Thermo #17504044) at 4ºC for one overnight to allow for individual animal genotyping before plating. Neurons were then treated with Papain (Worthington #LS003120) and DNAse (VWR #V0335)-supplemented Hibernate A for 18min at 37ºC and washed twice with plating medium (plating medium: Neurobasal A (Thermo #10888022) supplemented with 10% horse serum, 2% B-27, and 1% GlutaMAX (Thermo #35050061)), and triturated before plating at 250,000 cells/well on poly-L-lysine-treated coverslips (Sigma #P2636) in 24-well plates. Plating medium was replaced with growth medium (Neurobasal A, 2% B-27, 1% GlutaMAX) 2 hours later. Cell media was supplemented and treated with AraC at DIV5 (5uM final concentration/well). Half-media changes were then performed every 4 days.

**Immunocytochemistry**

At DIV15, neurons were fixed for 15 minutes using ice-cold 4%PFA/4% sucrose in 1X PBS, pH 7.4 (for EEA1 staining), or 30 minutes with 50% Bouin’s solution/4% sucrose (for CathepsinD staining, Sigma #HT10132), pH 7.493. Fixed neurons were washed with 1X PBS, then permeabilized with 0.25% TritonX-100 in PBS for 8 minutes at RT, and blocked with 5%normal goat serum/0.2%Triton-X100 in PBS (blocking buffer) for 1 hour at RT with gentle rocking. For EEA1/MAP2 staining, samples were incubated with primary antibodies diluted in blocking buffer at RT for 1 hour. For CathepsinD/MAP2 staining, samples were incubated with primary antibodies diluted in blocking buffer overnight at 4ºC. For both conditions, samples were washed three times with 1X PBS, and incubated for 30min at RT with secondary antibodies, protected from light. After secondary antibody staining, coverslips were washed three times with 1X PBS, and mounted with FluoroSave mounting solution (Sigma #345789). See antibody section for staining dilutions.

**Imaris 3D reconstruction**

For EEA1+ and CathepsinD+ puncta analyses, coverslips were imaged on a Zeiss LSM 710 confocal microscope. Images were sampled at a resolution of 1024 x 1024 pixels with a dwell time of 0.45µsec using a 63x/1.4 oil immersion objective, a 2.0 times digital zoom, and a z-step size of 0.37 µm. Images were saved in the “.lsm” format, and quantification was performed on a POGO Velocity workstation in the Duke Light Microscopy Core Facility using Imaris 9.2.0 software (Bitplane, South Windsor, CT). For analyses, we first used the “surface” tool to make a solid fill surface of the MAP2-stained neuronal soma and dendrites, with the background subtraction option enabled. We selected a threshold that demarcated the neuron structure accurately while excluding background. For EEA1 puncta analyses, a 600 x 800 µm selection box was placed around the soma in each image and surfaces were created for EEA1 puncta within the selection box. Thresholding was similar to that of MAP2. Similarly, for CathepsinD puncta analyses, a 600 x 600 µm selection box was placed around the soma(s) in each image for surface creation. The same threshold settings were used across images, and individual surface data from each soma were exported for aggregate analyses. The experimenter was blinded to sample conditions for both image acquisition and analysis.

**Immunohistochemistry**

Mice were deeply anesthetized with isoflurane and then transcardially perfused with ice-cold heparinized PBS (25U/ml) by gravity flow. After clearing of liver and lungs (~2min), perfusate was switched to ice-cold 4% PFA in 1X PBS (pH 7.4) for 15 minutes. Brains were dissected, post-fixed in 4%PFA overnight at 4ºC, then cryoprotected in 30% sucrose/1X PBS for 48hr at 4ºC. Brains were then mounted in OTC (Sakura TissueTek #4583) and stored at -20ºC until cryosectioning. Every third sagittal section (30 µm thickness) was collected from the motor cortex and striatal regions. Free-floating sections were then permeabilized with 1%TritonX-100 in 1X PBS at RT for 2 hr, and blocked in 1X blocking solution (Abcam #126587) diluted in 0.2%TritonX-100 in 1X PBS for 1hr at RT. Sections were then incubated in primary antibodies diluted in the 1X blocking solution for two overnights at 4ºC. After three washes with 0.2%TritonX-100 in 1X PBS, the sections were then incubated in secondary antibodies diluted in 1X blocking buffer for one overnight at 4ºC. Sections were then washed four times with 0.2%TritonX-100 in 1X PBS at RT, and mounted onto coverslips with FluoroSave mounting solution (Sigma #345789).

**Cleaved Caspase-3 Image Analysis**

Z-stack images were acquired on a Zeiss 710 LSM confocal microscope. Images were sampled at a resolution of 1024 x 1024 pixels with a dwell time of 1.58µsec, using a 63x/1.4 oil immersion objective (for cortex and striatum) or 20x/0.8 dry objective (cerebellum), a 1.0 times digital zoom, and a z-step size of 0.67 µm. Images were saved in the “.lsm” format, and then converted into maximum intensity projections (MIP) using Zen 2.3 SP1 software. Quantification of CC3 colocalization with DAPI was performed on the MIPs using the Particle Analyzer function in FIJI ImageJ software. The experimenter was blind to sample conditions for both image acquisition and analysis.

**Western blotting**

Ten micrograms of each sample were electrophoresed through a 12-well, 4-20% SDS-PAGE gel (Bio-Rad #4561096) at 100V for 1hr at RT, transferred onto a nitrocellulose membrane (GE Life Sciences #GE10600002) at 100V for 70min at RT on ice, and blocked with 5% nonfat dry milk in TRIS-buffered saline containing 0.05% Tween-20 (TBST, pH 7.4). Gels were saved for Coomassie staining at RT for 30 min. Membranes were probed with one primary antibody at a time for 24hr at 4ºC, then washed the membranes four times with TBST at RT before incubating with the corresponding species-specific secondary antibody at RT for 1hr. Membranes were washed with TBST, and then enhanced chemiluminescence (ECL) substrate was added (Thermo Fischer #32109). Membranes were exposed to autoradiography films and scanned with an Epson 1670 at 600dpi. We probed with one antibody at a time, stripped the membrane with stripping buffer (Thermo Fischer #21059) for 10min at RT, and then blocked for 1hr at RT before probing with the next antibody. Order of probes: Strumpellin, then B-Tubulin, then WASH1. We determined the optical density of the bands using Image J software (NIH). Data obtained from three independent experiments were plotted and statistically analyzed using Prism8 software.

**Immunoprecipitation**

HEK293T cells were transfected with pmCAG-SWIP-WT-HA or pmCAG-SWIP-MUT-HA constructs for three days, as previously described94. Cells were lysed with lysis buffer [25mM HEPES (pH7.4), 150mM NaCl, 1mM EDTA, 1% NonidetP-40, 5mM NaF, 1mM orthovanadate, 1mM AEBSF, 2 μg/mL leupeptin/pepstatin] and centrifuged at 1700g for 5 min. Collected supernatant was incubated with 30µl of pre-washed anti-HA agarose beads (Sigma #A2095) on a sample rotator (15 rpm) for 2 hrs at 4ºC. Beads were then washed 3 times with lysis buffer, and sample buffer was added before subjecting to immunoblotting as described above. The protein-transferred membrane was probed individually for WASH1, Strumpellin, and HA. Data were collected from four separate preparations of WT and MUT conditions.

**iBioID**

AAV2/9 viral probes, hSyn1-WASH1-BioID2-HA or hSyn1-solubleBioID2-HA, were injected into wild-type CD1 mouse brains using a Hamilton syringe (#7635-01) at age P0-P1 to ensure spread throughout the forebrain95. 15 days post-viral injection, biotin was subcutaneously administered at 24mg/kg for seven consecutive days for biotinylation of proteins in proximity to BioID2 probes. Whole brains were extracted on the final day of biotin injections, snap frozen, and stored in liquid nitrogen until protein purification. Seven brains were used for protein purification of each probe, and each purification was performed three times independently (21 brains total for WASH1-BioID2, 21 for solubleBioID2).

We performed all homogenization and protein purification on ice. A 2ml Dounce homogenizer was used to individually homogenize each brain in a 1:1 solution of Lysis-R:2X-RIPA buffer solution with protease inhibitors (Roche cOmplete tablets #11836153001). Each sample was sonicated three times for 7 seconds and then centrifuged at 5000g for 5min at 4ºC. Samples were transferred to Beckman Coulter 1.5ml tubes (#344059), and then spun at 45,000rpm in a Beckman Coulter tabletop ultracentrifuge (TLA-55 rotor) for 1hr at 4ºC. SDS was added to supernatants (final 1%) and samples were then boiled for 5min at 95ºC. We next combined supernatants from the same condition together (WASH1-BioID2 vs. soluble\_BioID2) in 15ml conical tubes to rotate with 30µl high-capacity NeutrAvidin beads overnight at 4ºC (Thermo #29204).

The following day, all steps were performed under a hood with keratin-free reagents. Samples were spun down at 6000rpm, 4ºC for 5min to pellet the beads and remove supernatant. The pelleted beads then went through a series of washes, each for 10 min at RT with 500ul of solvent, and then spun down on a tabletop centrifuge to pellet the beads for the next wash. The washes were as follows: 2% SDS twice, 1% TritonX100-1%deoxycholate-25mM LiCl2 once, 1M NaCL twice, 50mM Ammonium Bicarbonate (Ambic) five times. Beads were then mixed 1:1 with a 2X laemmli sample buffer that contained 3mM biotin/50mM Ambic, boiled for 5 mins at 95ºC, vortexed three times, and then biotinylated protein supernatants were stored at -80ºC until LC-MS/MS.

**LC-MS/MS for iBioID**

*Sample Preparation.* We gave the Duke Proteomics Core Facility (DPCF) six eluents from streptavidin resins (3x WASH1-BioID2, 3x soluble\_BioID2), stored on dry ice. Samples were reduced with 10 mM dithiolthreitol for 30 min at 80ºC and alkylated with 20 mM iodoacetamide for 30 min at room temperature. Next, samples were supplemented with a final concentration of 1.2% phosphoric acid and 256 μL of S-Trap (Protifi) binding buffer (90% MeOH/100mM TEAB). Proteins were trapped on the S-Trap, digested using 20 ng/μl sequencing grade trypsin (Promega) for 1 hr at 47ºC, and eluted using 50 mM TEAB, followed by 0.2% FA, and lastly using 50% ACN/0.2% FA. All samples were then lyophilized to dryness and resuspended in 20 μL 1%TFA/2% acetonitrile containing 25 fmol/μL yeast alcohol dehydrogenase (UniProtKB P00330; ADH\_YEAST). From each sample, 3 μL was removed to create a pooled QC sample (SPQC) which was run analyzed in technical triplicate throughout the acquisition period.

*Quantitative Analysis.* Quantitative LC/MS/MS was performed on 2 μL of each sample, using a nanoAcquity UPLC system (Waters Corp) coupled to a Thermo QExactive HF-X high resolution accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization source. Briefly, the sample was first trapped on a Symmetry C18 20 mm × 180 μm trapping column (5 μl/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.8 μm Acquity HSS T3 C18 75 μm × 250 mm column (Waters Corp.) with a 90-min linear gradient of 5 to 30% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55ºC. Data collection on the QExactive HF-X mass spectrometer was performed in a data-dependent acquisition (DDA) mode of acquisition with a r=120,000 (@ m/z 200) full MS scan from m/z 375 – 1600 with a target AGC value of 3e6 ions followed by 30 MS/MS scans at r=15,000 (@ m/z 200) at a target AGC value of 5e4 ions and 45 ms. A 20s dynamic exclusion was employed to increase depth of coverage. The total analysis cycle time for each sample injection was approximately 2 hours.

Following UPLC-MS/MS analyses, data was imported into Proteome Discoverer 2.2 (Thermo Scientific Inc.), and aligned based on the accurate mass and retention time of detected ions (“features”) using Minora Feature Detector algorithm in Proteome Discoverer. Relative peptide abundance was calculated based on area-under-the-curve (AUC) of the selected ion chromatograms of the aligned features across all runs. The MS/MS data was searched against the SwissProt *Mus musculus* database (downloaded in April 2018) with additional proteins, including yeast ADH1, bovine serum albumin, as well as an equal number of reversed-sequence “decoys” for false discovery rate determination. Mascot Distiller and Mascot Server (v 2.5, Matrix Sciences) were utilized to produce fragment ion spectra and to perform the database searches. Database search parameters included fixed modification on Cys (carbamidomethyl) and variable modifications on Meth (oxidation) and Asn and Gln (deamidation). Peptide Validator and Protein FDR Validator nodes in Proteome Discoverer were used to annotate the data at a maximum 1% protein false discovery rate.

Protein intensities were exported from Proteome Discoverer and processed using custom R scripts. Carboxylases and keratins, as well as 315 mitochondrial proteins96, were removed from the identified proteins as known contaminants. To assess for technical variability, the % coefficient of variation (%CV) was calculated for all proteins (n=2242) across the three injections of the QC pool (mean %CV=16.8%). To assess for biological and technical variability, %CVs were measured for each protein across the individual groups (WASH1-BioID2=24.8%, soluble\_BioID2 control=24.8%). Next, we performed sample loading normalization to account for technical variation between the 9 MS runs. This is done by multiplying intensities from each MS run by a scaling factor, such that the sum of all intensities in an experiment are equal. Then we performed sample pool normalization to SPQC samples to standardize protein measurements across all samples and correct for batch effects between analyses. As QC samples were created by pooling equivalent aliquots of peptides from each biological replicate, the average of all biological replicates should be equal to the average of all technical SPQC replicates. Sample pool normalization adjusts the protein-wise mean of all biological replicates to be equal to the mean of all SPQC replicates. Finally, proteins that were identified by a single peptide, and/or identified in less than 50% of samples were removed. Any remaining missing values were inferred to be missing not at random due to the left shifted distribution of proteins with missing values and imputed using the k-nearest neighbors algorithm (impute::impute.knn). Normalized protein data was analyzed using the R package edgeR. Differential enrichment of proteins in the WASH1-BioID2 pull-down relative to the soluble BioID2 control pull-down were evaluated with an exact test as implemented by the edgeR::exactTest. To consider a protein enriched in the WASH interactome, we required a protein’s Benjamini Hochberg adjusted p-value be less than 0.05 and exhibit a fold change greater than 3 over the negative control. With these criteria,176 proteins were identified as WASH1 interactors. Raw peptide and final normalized protein data can be found in Supplementary Tables 1-2., respectively.

Experimentally-determined, protein-protein interactions (PPIs) among the WASH1 interactome were compiled from the HitPredict database97 using a custom R package, getPPIs, (available online at twesleyb/getPPIs). We report PPIs among WASH1 interactome in Supplemental Table 2.

Bioinformatic GO analysis was conducted by manual annotation of identified proteins and confirmed with Metascape analysis98 of WASH1-BioID2 enriched proteins using the 2242 proteins identified in the mass spec analysis as background.

**LOPIT-DC Subcellular Fractionation**

We performed three independent fractionation experiments with one adult SWIP mutant brain and one WT mouse brain fractionated in each experiment. Each mouse was sacrificed by isoflurane inhalation and its brain was immediately extracted and placed into a 2ml Dounce homogenizer on ice with 1ml isotonic TEVP homogenization buffer (320mM sucrose, 10mM Tris base, 1mM EDTA, 1mM EGTA, 5mM NaF, pH7.455). A cOmplete mini protease inhibitor cocktail tablet (Sigma #11836170001) was added to a 50ml TEVP buffer aliquot immediately before use. Brains were homogenized for 15 passes with a Dounce homogenizer to break the tissue, and then this lysate was brought up to a 5ml volume with additional TEVP buffer. Lysates were then passed through a 0.5ml ball-bearing homogenizer for two passes (14 µm ball, Isobiotec) to release organelles. Final brain lysate volumes were approximately 7.5ml each. Lysates were then divided into replicate microfuge tubes (Beckman Coulter #357448) to perform differential centrifugation, following Geladaki et. al’s LOPIT-DC protocol42. Centrifugation was carried out at 4ºC in a tabletop Eppendorf 5424 centrifuge for spins at 200g, 1,000g, 3,000g, 5,000g, 9,000g, 12,000g, and 15,000g. To isolate the final three fractions, a tabletop Beckman TLA-100 ultracentrifuge with a TLA-55 rotor was used at 4ºC with speeds of: 30,000g, 79,000g, and 120,000g, respectively. Samples were kept on ice at all times and pellets were stored at -80ºC. Pellets from 5,000g-120,000g were used for proteomic analyses.

**16-plex TMT LC-MS/MS**

*Sample Preparation.* The Duke Proteomics Core Facility (DPCF) processed and prepared fraction pellets from 3 WT and 3 MUT brains simultaneously for tandem-mass-tag (TMT) proteomics. The DPCF received 42 frozen fractions (7 fractions per brain from 6 animals), each split into 3 tubes (due to volume constraints) for a total of 126 samples. Each fraction was combined in the following manner; 100µL of 8M Urea was added to the first aliquot then probe sonicated for 5 seconds with an energy setting of 30%. This volume was then transferred to the second then third aliquot after sonication. All tubes were centrifuged at 10,000g and any volume from tubes 1 and 2 were added to tube 3. Protein concentrations were determined by BCA on the supernatant in duplicate (5 μL each assay). Total protein concentrations for each replicate ranged from 1.1 mg/mL to 7.8 mg/mL with total protein quantities ranging from 108.3 to 740.81 µg. 60 µg of each sample was removed and normalized to 52.6µL with 8M Urea and 14.6µL 20% SDS. Samples were reduced with 10 mM dithiolthreitol for 30 min at 80ºC and alkylated with 20 mM iodoacetamide for 30 min at room temperature. Next, they were supplemented with 7.4 μL of 12% phosphoric acid, and 574 μL of S-Trap (Protifi) binding buffer (90% MeOH/100mM TEAB). Proteins were trapped on the S-Trap, digested using 20 ng/μl sequencing grade trypsin (Promega) for 1 hr at 47ºC, and eluted using 50 mM TEAB, followed by 0.2% FA, and lastly using 50% ACN/0.2% FA. All samples were then lyophilized to dryness.

*TMT Labeling Procedure.* Each sample was resuspended in 120 μL 200 mM triethylammonium bicarbonate, pH 8.0 (TEAB). 20µL of each sample was combined to form a SPQC pooled sample. Each 16-plex TMT experiment consisted of the control and mutant fractions from the respective experiment and 2 SPQC samples. Fresh TMTPro reagent (0.5 mg for each 16-plex reagent) was resuspended in 20 μL 100% acetonitrile (ACN) and was added to each sample. Samples were incubated for 1 hour at RT. After 1-hour reaction, 5 μL of 5% hydroxylamine was added and incubated for 15 minutes at room temperature to quench the reaction. Sample were combined, then lyophilized to dryness.

*Offline Fractionation Procedure.*Samples were resuspended in 800µL 0.1% formic acid. 400µg was fractionated into 48 unique high pH reversed-phase fractions using pH 9.0 20 mM Ammonium formate as mobile phase A and neat acetonitrile as mobile phase B. The column used was a 2.1 mm x 50 mm BEH C18 (Waters) and fractionation was performed on an Agilent 1100 HPLC with G1364C fraction collector. Throughout the method, the flow rate was 0.4 mL/min and the column temperature was 55ºC. The gradient method was set as follows: 0 min, 3%B; 1 min, 7% B; 50 min, 50%B; 51 min, 90% B; 55 min, 90% B; 56 min, 3% B; 70 min, 3% B. 48 fractions were collected in equal time segments from 0 to 52 minutes, then concatenated into 12 unique samples using every 12th fraction. For instance, fraction 1, 13, 25, and 37 were combined, fraction 2, 14, 26, and 38 were combined, etc. Fractions were frozen and lyophilized overnight. Samples were resuspended in 66 μL 1%TFA/2% acetonitrile prior to LC-MS analysis.

*Quantitative Analysis.*Quantitative LC/MS/MS was performed on 2 μL (1 μg) of each sample, using a nanoAcquity UPLC system (Waters Corp) coupled to a Thermo Orbitrap Fusion Lumos high resolution accurate mass tandem mass spectrometer (Thermo) equipped with a FAIMS device via a nanoelectrospray ionization source. Briefly, the sample was first trapped on a Symmetry C18 20 mm × 180 μm trapping column (5 μl/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical separation was performed using a 1.8 μm Acquity HSS T3 C18 75 μm × 250 mm column (Waters Corp.) with a 90-min linear gradient of 5 to 30% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55ºC. Data collection on the Fusion Lumos mass spectrometer was performed for three different compensation voltages (CV: -40v, -60v, -80v). Within each CV, a data-dependent acquisition (DDA) mode of acquisition with a r=120,000 (@ m/z 200) full MS scan from m/z 375 – 1600 with a target AGC value of 4e5 ions was performed. MS/MS scans were acquired in the Orbitrap at r=50,000 (@ m/z 200) from m/z 100 with a target AGC value of 1e5 and max fill time of 105 ms. The total cycle time for each CV was 1s, with total cycle times of 3 sec between like full MS scans. A 45s dynamic exclusion was employed to increase depth of coverage. The total analysis cycle time for each sample injection was approximately 2 hours.

Following UPLC-MS/MS analyses, data were imported into Proteome Discoverer 2.4 (Thermo Scientific Inc.) The MS/MS data were searched against a SwissProt Mouse database (downloaded November 2019) plus additional common contaminant proteins, including yeast alcohol dehydrogenase (ADH), bovine casein, bovine serum albumin, as well as an equal number of reversed-sequence “decoys” for false discovery rate determination. Mascot Distiller and Mascot Server (v 2.5, Matrix Sciences) were utilized to produce fragment ion spectra and to perform the database searches. Database search parameters included fixed modification on Cys (carbamidomethyl) and variable modification on Met (oxidation), Asn/Gln (deamindation), Lys (TMTPro) and peptide N-termini (TMTPro). Reporter ion intensities were calculated using the Reporter Ions Quantifier algorithm in Proteome Discoverer. Percolator node in Proteome Discoverer was used to annotate the data at a maximum 1% protein false discovery rate.

*Quantitative LC-MS/MS results.*Raw peptide intensities were exported from Proteome Discover for downstream analysis and processing in R. The total dataset had 94020 unique peptides following FDR adjustment to 1%. Following database searching and protein scoring using the Protein FDR Validator algorithm, the data was annotated at a 1% protein false discovery rate, resulting in the identification of **7521 proteins.** These results can be found in Supplemental Tables 3-4.

*TMT Peptide-level processing.* Peptides from contaminant and non-mouse proteins were removed. First, we performed sample loading normalization, normalizing the total ion intensity for each TMT channel within an experiment to be equal. Sample loading normalization corrects for small differences in the amount of sample analyzed and labeling reaction efficiency differences between individual TMT channels within an experiment.

We found that in each experiment there were a small number of missing values (mean = 1.16% +/- 0.173 % for all three experiments). Missing values were inferred to be missing at random based on the overlapping distributions of peptides with missing values and peptides without missing values. We imputed these missing values using the k-nearest neighbor algorithm from the R package impute, as it is suitable for imputing data missing at random. Missing values for SPQC samples were not imputed. Peptides with any missing SPQC data were removed.

Following sample loading normalization, SPQC replicates within each experiment should yield identical measurements. As peptides with irreproducible QC measurements are unlikely to be quantitatively robust, and their inclusion may bias downstream processing (see IRS normalization below), we sought to remove them. To assess intra-batch variability, we utilized the method described by Ping et al., 201999. Briefly, peptides were binned into 5 groups based on the average intensity of the two SPQC replicates. For each pair of SPQC measurements, the log ratio of SPQC intensities was calculated. To identify outlier QC peptides, we plotted the distribution of these log ratios for each bin. Peptides with ratios that were more than four standard deviations away from the mean of its intensity bin were considered outliers and removed (n=474).

*Protein-level processing.* Proteins were summarized as the sum of all unique peptide intensities corresponding to that protein, and sample loading normalization was performed across all three experiments to account for inter-experimental technical variability. The peptides selected at the MS2 level for any given protein is in part random. This stochasticity means that proteins are typically quantified by different peptides in each experiment. Thus, although SPQC samples should yield identical protein measurements in each of the three experiments, they exhibit variability due to their quantification by different peptides. To account for this protein-level bias, we utilized the internal reference scaling (IRS) approach described by Plubell et al., 2017100. IRS normalization scales the geometric average of all SPQC measurements across all experiments to be equal. In brief, each protein is multiplied by a scaling factor adjusting its reference values to be equal to the geometric mean of all SPQC samples from the three experiments. This normalization step effectively standardizes protein measurements between different mass spectrometry experiments.

The final normalization step was to perform sample pool normalization using SPQC samples as a reference. Sample pool normalization scales the mean of all biological replicates to be equal to the mean of all SPQC replicates in a manner similar to IRS normalization. This normalization step, sometimes referred to as global internal standard normalization, accounts for batch effects between experiments, and reflects the fact that after technical normalization, the mean of biological replicates should be equal to the mean of SPQC replicates as they are effectively equivalent.

Before assessing protein differential abundance, we removed irreproducible quantified proteins. Across all 42 biological replicates, we observed that a small number of proteins had potential outlier measurements that were either several orders of magnitude greater or less than the median of its replicates. In order to identify and remove these proteins, we assessed the reproducibility of protein measurements within a fraction in the same manner used to identify and filter QC outlier peptides. A small number of proteins were identified as outliers if the average log ratio of their 3 replicates was more than 4 standard deviations away from the mean of its intensity bin (n=349). Proteins with any missing QC data (n=1041), proteins identified by a single peptide (n=758), or proteins that were sparsely quantified (present in less than 50% of all samples; n=49) were removed. In total, we retained **5,897** of the original **7,521** proteins in the final dataset.

*Differential protein abundance.* Differential protein abundance was assessed using the final normalized protein data for intrafraction comparisons between WT and MUT groups using a general linear model as implemented by the edgeR R library (edgeR::glmFIT)101. This approach is appropriate for over-dispersed, negative binomially distributed count data with a small number of replicates101,102 (for its application to proteomics see Plubell et al., 2017100). P-values were corrected using the Benjamini Hochberg procedure within edgeR. An FDR threshold of 0.1 was set for significance.

*Adjusted protein abundance.* For a select number of proteins, we tested the hypothesis that the abundance of proteins in the WT group was significantly greater than the mean of the MUT group. We adjusted normalized protein abundances for fraction differences by fitting the data with an additive linear model with fraction as blocking factor as implemented by the removeBatchEffect algorithm from the R limma package103. Differences between WT and MUT groups were then assessed using the adjusted data and a one-sided student’s t-test with Bonferroni p-value correction in R.

**Electron Microscopy**

Adult (7mo) WT and MUT SWIPP1019R mice were deeply anesthetized with isoflurane and then transcardially perfused with warmed heparinized saline (25U/ml heparin) for 4 minutes, followed by ice-cold 0.15M cacodylate buffer pH 7.4 containing 2.5% glutaraldehyde (Electron Microscopy Sciences #16320), 3% paraformaldehyde, and 2mM CaCl2 for 15 minutes. Brain samples were dissected and stored on ice in the same fixative for 2 hours before washing in 0.1M sodium cacodylate buffer (3 changes for 15 minutes each). Samples were then post-fixed in 1.0% OsO4 in 0.1 M Sodium cacodylate buffer for 1 hour on a rotator. Samples were then washed in 3, 15-minute changes of 0.1M sodium cacodylate. Samples were then placed into *en bloc* stain (1% uranyl acetate) overnight at 4°C. Subsequently they were dehydrated in a series of ascending acetone concentrations including 50%, 70%, 95%, and 100% for three cycles with 15 minutes incubation at each concentration change. Samples were then placed in a 50:50 mixture of Epon:acetone overnight on a rotator. This solution was then replaced twice with 100% epoxy resin (epon) for at least 2 hours at room temperature on a rotator. Samples were embedded with 100% epon resin in beem capsules for 48 hours at 60°C. Samples were ultrathin sectioned to 60-70nm on a Reichert Ultracut E ultramicrotome. Harvested grids were then stained with 2% uranyl acetate in 50% ethanol for 30 minutes and Sato’s lead stain for 1 min. Micrographs were acquired using a Phillips CM12 electron microscope operating at 80Kv, at 1700x magnification. Micrographs were analyzed in Adobe Photoshop 2019, using the “magic wand” tool to demarcate and measure the area of electron-dense and electron-lucent regions of interest (ROIs). Statistical analyses of ROI measurements were performed in Prism8 software. The experimenter was blinded to genotype for image acquisition and analysis.

**Behavioral Assays**

Behavioral tests were performed on age-matched WT and homozygous SWIPP1019R mutant littermates. Male and female mice were used in all experiments. Testing was performed at two time points: P42-55 days old as a young adult age, and 5.5 months old as mid-adulthood, so that we could compare disease progression in this mouse model to human patients3. The sequence of behavioral testing was: light-dark testing (to measure anxiety-like behavior), open field activity (to measure exploratory activity and anxiety-like behavior), Y maze (to measure working memory), object novelty recognition (to measure short-term and long-term object recognition memory), treadscan (to assess gait), and steady-speed rotarod (to assess motor control and strength) for 40-55 day old mice. Testing was performed over 1.5 weeks, interspersed with rest days for acclimation. This sequence was repeated with the same cohort at 5.5-6 months old, with three additional measures added to the end of testing: fear conditioning (to assess associative fear memory), a hearing test (to measure tone response), and a shock threshold test (to assess somatosensation). Of note, a separate, second cohort of mice was evaluated for fear conditioning, hearing, and shock threshold testing at adolescence. After each trial, equipment was cleaned with Labsan to remove residual odors. The experimenter was blinded to genotype for all behavioral analyses.

**Light-Dark**

Light-dark testing was performed in a mouse shuttle-box (Med Associates) composed of two adjoined boxes: a dark chamber (under 5 lux), and a light chamber (600 lux), divided by a door controlled by a computer program. Mice were acclimated to low light (50lux) for 1 hour prior to testing to eliminate desensitization to the light box. Mice were placed in the dark chamber before each trial and testing began by triggered door opening. The mouse was then given full access to both chambers for a 5 min trial. Total distance traveled, time spent in each chamber, and number of transitions between chambers were detected by beam breaks and analyzed by Med Associates software.

**Open Field**

Mice were monitored individually using VersaMax software (Accuscan Instruments) for 1h in a square open field arena (21x21cm) under 350 lux illumination. Locomotion (distance traveled), rearing (vertical beam breaks), time in region (center or edge/corner), and stereotypic activities (repetitive beam breaks <1s) were captured in 5 min time bins.

**Y-maze**

Working memory was evaluated by measuring spontaneous alternations in a 3-arm Y-maze under indirect illumination (80-90 lux). A mouse was placed in the center of the maze and allowed to freely explore all arms, each of which had different visual cues for spatial recognition. Trials were 5 min in length, with video data and analyses captured by EthoVision XT 11.0 software (Noldus Information Technology). Entry to an arm was define as the mouse being >1 body length into a given arm. An alternation was defined as three successive entries into each of the different arms. Total % alternation was calculated as the total number of alternations/the total number of arm entries minus 2 x100.

**Novel Object Recognition**

One hour before testing, mice were individually exposed to the testing arena (a 48 x 22 x 18cm white opaque arena) for 10min under 80-100lux illumination without any objects. The test consisted of three phases: training (day 1), short-term memory test (STM, day 1), and long-term memory test (LTM, day 2). For the training phase, two identical objects were placed 10 cm apart, against opposing walls of the arena. A mouse was placed in the center of the arena and given full access to explore both objects for 5 min and then returned to its home cage. For STM testing, one of the training objects remained (the now familiar object), and a novel object replaced one of the training objects (similar in size, different shape). The mouse was returned to the arena 30 minutes after the training task and allowed to explore freely for 5 mins. For LTM testing, the novel object was replaced with another object, and the familiar object remained unchanged. The LTM test was also 5 min in duration, conducted 24hr after the training task. Behavior was scored using Ethovision 11.0 XT software (Noldus) and analyzed by a blind observer. Object contact was defined as the mouse’s nose within 1 cm of the object. We analyzed both number of nose contacts with each object and duration of contacts. Preference scores were calculated as (duration contactnovel - duration contactfamiliar) / total duration contactnovel+familiar. Positive scores signified a preference for the novel object; whereas, negative scores denoted a preference for the familiar object, and scores approaching zero indicated no preference.

**Treadscan**

A TreadScan forced locomotion treadmill system (CleversSys Inc, Reston, Virginia) was used for gait recording and analysis. Each mouse was recorded walking on a transparent treadmill at 45 days old, and again at 5.5 months old. Mice were acclimated to the treadmill chamber for 1 minute before the start of recording to eliminate exploratory behavior confounding normal gait. Trials were 20 seconds in length, with mice walking at speeds between 13.83 and 16.53 cm/sec (P45 WT average 15.74 cm/s; P45 MUT average 15.80 cm/s; 5.5mo WT average 15.77 cm/s; 5.5mo MUT average 15.85 cm/s). A high-speed digital camera attached to the treadmill captured limb movement at a frame rate of 100 frames/second. We used TreadScan software (CleversSys) and representative WT and MUT videos to generate footprint templates, which were then used to identify individual paw profiles for each limb. Parameters such as stance time, swing time, step length, track width, and limb coupling were recorded for the entire 20 sec duration for each animal. Output gait tracking was verified manually by a blinded experimenter to ensure consistent limb tracking throughout the duration of each video.

**Steady Speed Rotarod**

A 5-lane rotarod (Med Associates) was used for steady-speed motor analysis. The rod was run at a steady speed of 32rpm for four, 5-minute trials, with a 40-minute inter-trial interval. We recorded mouse latency to fall by infrared beam break, or manually for any mouse that completed two or more rotations on the rod without walking. Mice were randomized across lanes for each trial.

**Fear Conditioning**

Animals were examined in contextual and cued fear conditioning as described by Rodriguiz and Wetsel104. Two separate cohorts of mice were used testing the two age groups. A three-day testing paradigm was used to assess memory: conditioning on day 1, context testing 24-hr post-conditioning on day 2, and cued tone testing 48hr post-conditioning on day 3. All testing was conducted in fear conditioning chambers (Med Associates). In the conditioning phase, mice were first acclimated to the test chamber for two minutes under ~100 lux lumination. Then a 2900Hz, 80dB tone (conditioned stimulus, CS) played for 30 sec, which terminated with a paired 0.4mA, 2 sec scrambled foot shock (unconditioned stimulus, US). Mice were removed from the chamber and returned to their home cage 30 sec later. In the context testing phase, mice were placed in the same conditioning chamber and monitored for freezing behavior for a 5 min trial period, in the absence of the CS and US. For cued tone testing, the chambers were modified to different dimensions and shapes, contained different floor and wall textures, and lighting was adjusted to 50 lux. Mice acclimated to the chamber for 2 min, and then the CS was presented continuously for 3 min. Contextual and cued fear memory was assessed by freezing behavior, captured by automated video software (CleversSys Inc).

**Hearing Test**

We tested mouse hearing using a startle platform (Med Associates, St. Albans, Vermont) connected to Startle Pro Software in a sound-proof chamber. Mice were placed in a ventilated restraint cylinder connected to the startle response detection system to measure startle to each acoustic stimulus. After two minutes of acclimation, mice were assessed for an acoustic startle response to seven different tone frequencies, 2kHz, 3kHz, 4kHz, 8kHz, 12kHz, 16kHz, and 20kHz that were randomly presented three times each at four different decibels, 80, 100, 105, and 110dB, for a total of 84 trials. A random inter-trial interval of 15-60 seconds (average 30sec) was used to prevent anticipation of a stimulus. An animal’s reaction to the tone was recorded as startle reactivity in the first 100msec of the stimulus presentation, which was transduced through the platform’s load cell and expressed in arbitrary units (AU).

**Startle Response (Somatosensation)**

Mouse somatosensation was tested by placing mice in a startle chamber (Med Associates) connected to Startle Pro Software. Mice were placed atop a multi-bar cradle within a ventilated plexiglass restraint cylinder, which allows for horizontal movement within the chamber, but not upright rearing. After two minutes of acclimation, each mouse was exposed to 10 different scrambled shock intensities, ranging from 0 to 0.6mA with randomized inter-trial intervals of 20-90 seconds. Each animal’s startle reactivity during the first 100 msec of the shock was transduced through the platform’s load cell and recorded as area under the curve (AUC) in arbitrary units (AU).

**Quantification and Statistics**

Experimental conditions, number of replicates, and statistical tests used are stated in each figure legend. Each experiment was replicated at least three times (or on at least 3 separate animals) to assure rigor and reproducibility. Both male and female age-matched mice were used for all experiments, with data pooled from both sexes. Data compilation and statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, CA), using a significance level of alpha=0.05. Prism provides exact p values unless p<0.0001. All data are reported as mean ± SEM. Each data set was tested for normal distribution using a D’Agostino-Person normality test to determine whether parametric (unpaired Student’s t-test, one-way ANOVA, two-way ANOVA) or non-parametric (Mann-Whitney, Kruskal-Wallis) tests should be used. Parametric assumptions were confirmed with the Shapiro-Wilk test (normality) and Levine’s test (error variance homogeneity) for ANOVA with repeated measures testing.

**Code Availability**

All data and code to reproduce the proteomics analyses are available online at <https://github.com/twesleyb/SwipProteomics>.

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**Figure Legends:**

**Fig. 1 *In vivo* BioID reveals WASH complex proteome in mouse brain. a**,The WASH complex is composed of five subunits, *Washc1* (WASH1), *Washc2* (Fam21), *Washc3* (ccdc53), *Washc4* (SWIP), and *Washc5* (Strumpellin). Human mutations in these components are associated with spastic paraplegia4,40,105,Ritscher-Schinzel Syndrome41, and intellectual disability3,6. **b**, A BioID2 probe was attached to the c-terminus of WASH1 and expressed under the human synapsin-1 (hSyn1) promoter in an AAV construct for *in vivo* BioID (iBioID). **c**, iBioID probes (WASH1-BioID2-HA, or negative control solubleBioID2-HA) were injected into wild-type mouse brain at P0 and allowed to express for two weeks. Subcutaneous biotin injections (24 mg/kg) were administered over seven days for biotinylation, and then brains were harvested for isolation and purification of biotinylated proteins. LC-MS/MS identified proteins significantly enriched in all three replicates of WASH1-BioID2 samples over soluble-BioID2 controls. **d**,Representative image of WASH1-BioID2-HA expression in mouse cortex (Cx). Individual panels show nuclei (DAPI, blue), AAV construct HA epitope (green), and biotinylated proteins (Streptavidin, red). Merged image shows colocalization of HA and Streptavidin (yellow). Scale bar, 50 µm. **e**, iBioID identified known and unknown proteins interactors of the WASH complex in murine neurons. Nodes are named according to gene name, size represents protein abundance fold-enrichment over negative control (range: 3 to 181.7), solid grey lines delineate iBioID interactions between the Washc1 probe (seen in yellow at the center) and identified proteins, dashed lines indicate known protein-protein interactions from HitPredict database97. Clustergrams of: **f**,all five WASH complex proteins identified by iBioID; **g**,previously reported WASH interactors (13/176), including the CCC complex; **h**, endosomal trafficking proteins (23/176 proteins); **i**,endocytic proteins (24/176); **j**,proteins involved in cytoskeletal regulation (32/176), including Arp2/3 subunit Arpc5; and **k**, synaptic proteins (28/176). Clustergrams were annotated by hand and cross-referenced with Metascape98 GO enrichment of WASH1 proteome constituents over all proteins identified in the BioID experiment.

**Fig. 2 Subcellular fractionation reveals significant disruption in endo-lysosomal protein levels in SWIPP1019R mutant brains. a**,Mouse model of the human SWIPP1019Rmissense mutation created using CRISPR. A C>G point mutation was introduced into exon29 of murine *Washc4*, leading to a P1019R amino acid substitution. **b**, Representative western blot of whole brain lysate from adult mice with wild-type (WT, *Washc4*C/C) and homozygous (MUT, *Washc4*G/G) mutations, probed for two WASH complex components, Strumpellin and WASH1 (predicted kDa: 134 and 72, respectively), and loading control, B-tubulin (55kDa). Graphs of protein band intensities relative to WT are seen below: n=3 mice per genotype, Strumpellin (WT 100.0 ± 5.2%, MUT 3.5 ± 0.7%, t2.1=18.44, p=0.0024), WASH1 (WT 100.0 ± 3.8%, MUT 1.1 ± 0.4%, t2.1=25.92, p=0.0013), and B-tubulin (WT 100.0 ± 8.2%, MUT 94.1 ± 4.1%, U=4, p>0.99). **c**, Spatial proteomics experimental design. Brains were extracted and gently homogenized from one WT and one MUT mouse (10mo). The lysates were passed through a ball-bearing homogenizer to release organelles, and then centrifuged at progressively higher speeds to separate cellular compartments. 7 of these organellar pellets from each animal, along with two quality control samples (QC), were labeled with unique TMT tags and concatenated for simultaneous LC-MS/MS analysis. This experiment was repeated three times (3 WT and 3 MUT brains total). **d**,Protein module 19 (M19) contains subunits of the WASH, CCC, and Retriever complexes. Node size denotes relative abundance in MUT brain, purple color denotes proteins with altered abundance in MUT brain relative to WT, black node boarder denotes proteins identified in WASH proteome (Fig 1). Lines indicate known protein-protein interactions from HitPredict database97, with strength of interaction correlated to darkness of line color. **e**, Difference in M19 total normalized protein abundance, across the seven fractions analyzed (WT 13.2 ± XX, MUT 12.9 ± XX, p=0.0007). **f**, Module 2 (M2) containing multiple proteins with increased abundance in MUT brain compared to WT, including lysosomal proteins Ctss, Ctsl, Grn, Ids, Man2b1. Proteins with altered abundance in MUT brain are denoted in pink. **g**, Difference in M2 total normalized protein abundance, across the seven fractions analyzed (WT 13.75 ± XX, MUT 13.85 ± XX, p=0.0006). \*\*\*p<0.001, one-sided student’s t-test with Bonferroni correction (e,g)

**Fig. 3 Cortical neurons from SWIP mutant mice display endo-lysosomal abnormalities *in vitro*. a**,Experimental design. Cortices were dissected from P0 pups and neurons were dissociated and cultured on glass coverslips for 15 days. Cultures were fixed, stained, and imaged using confocal microscopy. 3D puncta volumes were reconstructed from z-stack images using Imaris software. **b** (WT) **c** (MUT),Representative 3D reconstructions of DIV15 neurons stained for EEA1 (yellow) and MAP2 (magenta). **f**, Graph of the average number of EEA1+ volumes per soma in each image (WT 95.0 ± 5.5, n=24 neurons; MUT 103.7 ± 3.7, n=24 neurons; t40.2=1.314, p=0.1961). **g**, Graph of the average EEA1+ volume size per soma shows larger EEA1+ volumes in MUT neurons (WT 0.15 ± 0.01 µm3, n=24 neurons; MUT 0.30 ± 0.02 µm3, n=24 neurons; U=50, p<0.0001). **d** (WT) **e** (MUT), Representative 3D reconstructions of DIV15 neurons stained for Cathepsin D (cyan) and MAP2 (magenta). **h**,Graph of the average number of Cathepsin D+ volumes per soma illustrates less Cathepsin D+ volumes in MUT neurons (WT 30.4 ± 1.4, n=42; MUT 17.2 ± 0.9, n=42; t71=7.943, p<0.0001). **i,** Graph of the average Cathepsin D+ volume size per soma demonstrates larger Cathepsin D+ volumes in MUT neurons (WT 0.54 ± 0.02 µm3, n=42; MUT 0.69 ± 0.04 µm3, n=42; t63=3.701, p=0.0005). **j** and **k**,Histograms of EEA1+ (j) and CathD+ (k) volumes illustrate differences in size distributions between MUT and WT neurons. Analyses included at least three separate culture preparations. Scale bars, 5 µm. Data reported as mean ± SEM, error bars are SEM. \*\*\*p<0.001, \*\*\*\*p<0.0001, two-tailed t-tests or Mann-Whitney U test (g)

**Fig. 4 ­­The SWIPP1019Rmutationis associated with increased cell death and abnormal endo-lysosomal structures *in vivo*. a** (WT) and **b** (MUT),Representative images of adolescent (P42) motor cortex stained with cleaved caspase-3 (CC3, green) and DAPI (blue). **c**,Anatomical representation of mouse brain with motor cortex highlighted in blue, adapted from Allen Brain Atlas106.  **d** (WT) and **e** (MUT),Representative images of adult (10 mo) motor cortex stained with CC3 (green) and DAPI (blue). **f,** Graph depicting the % of DAPI+ nuclei that are positive for CC3 per image. No difference is seen at P42, but the amount of CC3+ nuclei is significantly higher in aged MUT mice (P42 WT 6.97 ± 0.80%, P42 MUT 5.26 ± 0.90%, 10mo WT 25.38 ± 2.05%, 10mo MUT 44.01 ± 1.90%, H=74.12, p<0.0001). **g**, **h**, **i**, and **j** are the DAPI co-stained images for **a**, **b**, **d**, and **e**, respectively. **k**, Graph depicting the average number of DAPI+ nuclei per image at two different ages shows no significant difference between genotypes (P42 WT 40.96± 1.86, P42 MUT 47.21 ±1.87, 10mo WT 41.79 ±2.00, 10mo MUT 38.04 ± 1.84, F1,92=0.4356, p=0.5109). n=24 images per condition taken from 4 different mice for **f** and **k**. Scale bar for CC3 and DAPI, 15 µm. **l** (WT) and **m** (MUT), Representative transmission electron microscopy (TEM) images taken of somas from adult (7mo) motor cortex.  delineates electron-dense lipofuscin material. **n,** Graph of areas of electron-dense regions of interest (ROI) shows increased ROI size in MUT neurons (WT 2.4x105 ± 2.8x104 nm2, n=50 ROIs; MUT 8.2x105 ± 9.7 x104 nm2, n=75 ROIs; U=636, p<0.0001). **o,** Graph of the average number of presumptive lysosomes with associated electron-dense material reveals increased number in MUT samples (WT 3.14 ± 0.72 ROIs, n=14 images; MUT 10.86 ± 1.42 ROIs, n=14 images; U=17, p<0.0001). Forn and o,images were taken from multiple TEM grids, prepared from n=3 animals per genotype.Scale bar for TEM, 1 µm. Data reported as mean ± SEM, error bars are SEM. \*\*\*p<0.001, \*\*\*\*p<0.0001, Kruskal-Wallis test (f), two-way ANOVA (k), Mann-Whitney U test (n-o)

**Fig. 5 SWIPP1019Rmutant mice display persistent deficits in cued fear memory recall. a,** Experimental fear conditioning paradigm. After acclimation to a conditioning chamber, mice received a mild aversive 0.4mA footshock paired with a 2900Hz tone. 48 hours later, the mice were placed in a chamber with different tactile and visual cues. The mice acclimated for two minutes and then the 2900Hz tone was played (no footshock) and freezing behavior was assessed. **b,** Line graphs of WT and MUT freezing response during cued tone memory recall. Data represented as average freezing per genotype in 30 s time bins. The tone is presented after t= 120 s, and remains on for 120 seconds (blue shading). Two different cohorts of mice were used for age groups P42 (top) and 6.5mo (bottom). Two-way ANOVA analysis of average freezing during Pre-Tone and Tone periods reveal a Genotype x Time effect at P42 (WT n=10, MUT n=10, F1,18=4.944, p=0.0392) and 6.5mo (WT n=13, MUT n=11, F1,22= 13.61, p=0.0013). **c,** Graphs showing the average %time freezing per animal before and during tone presentation. Top: freezing is reduced by 20% in MUT adolescent mice compared to WT littermates (Pre-tone WT 16.5 ± 2.2%, n=10; Pre-tone MUT 13.0 ± 1.8%, n=10; t36=0.8569, p=0.6366; Tone WT 52.8 ± 3.8%, n=10; Tone MUT 38.0 ± 3.6%, n=10; t36=3.539, p=0.0023), Bottom: freezing is reduced by over 30% in MUT adult mice compared to WT littermates (Pre-tone WT 21.1 ± 2.7%, n=13; Pre-tone MUT 23.7 ± 3.8%, n=11; t44=0.4675, p=0.8721; Tone WT 69.7 ± 4.3%, n=13; Tone MUT 53.1 ± 5.2%, n=11; t44=2.921, p=0.0109). Data reported as mean ± SEM, error bars are SEM. \*p<0.05, \*\*p<0.01, two-way ANOVAs (b) and Sidak’s post-hoc analyses (c)

**Fig. 6** **SWIPP1019Rmutant mice exhibit progressive motor deficits. a**,Rotarod experimental setup. Mice walked atop a rod rotating at 32rpm for 5 minutes, and the duration of time they remained on the rod before falling was recorded. **b**,Line graph of average duration animals remained on the rod per genotype across four trials, with an inter-trial interval of 40 minutes. The same cohort of animals was tested at two different ages, P45 (top) and 5.5 months (bottom). Genotype had a significant effect on task performance at both ages (top, P45: genotype effect, F1,25=7.821, p=0.0098. bottom, 5.5mo: genotype effect, F1,23= 7.573, p=0.0114). **c**,Graphs showing the average duration each animal remained on the rod across trials. At both ages, the MUT mice exhibited an almost 50% reduction in their ability to remain on the rod (Top, P45: WT 169.9 ± 25.7 s, MUT 83.8 ± 15.9 s, U=35, p=0.0054. Bottom, 5.5mo: WT 135.9 ± 20.9 s, MUT 66.7 ± 9.5 s, t18=3.011, p=0.0075). **d**,Treadscan task. Mice walked on a treadmill for 20 s while their gate was captured with a high-speed camera. Diagrams of gait parameters measured in **e**-**g** are shown below the Treadscan apparatus. **e**,Average swing time per stride for hindlimbs. At P45 (top), there is no significant difference in rear swing time (WT 156.2 ± 22.4 ms, MUT 132.3 ± 19.6 ms, U=83, p=0.7203). At 5.5mo (bottom), MUT mice display significantly longer rear swing time (WT 140 ± 6.2 ms, MUT 252.0 ± 21.6 ms, t12=4.988, p=0.0003). **f**,Average stride length for hindlimbs. At P45 (top), there is no significant difference in stride length (WT 62.3 ± 2.0 mm, MUT 60.5 ± 2.1 mm, U=75, p=0.4583). At 5.5mo (bottom), MUT mice take significantly longer strides with their hindlimbs (WT 60.8 ± 0.8 mm, MUT 73.6 ± 2.7 mm, t11.7=4.547, p=0.0007). **g**,Average homologous coupling for front and rear limbs. Homologous coupling is 0.5 when the left and right feet are completely out of phase. At P45 (top), WT and MUT mice exhibit normal homologous coupling (WT 0.48 ± 0.005, MUT 0.48 ± 0.004, U=76.5, p=0.4920). At 5.5 mo (bottom), MUT mice display decreased homologous coupling, suggestive of abnormal gait symmetry (WT 0.48 ± 0.003, MUT 0.46 ± 0.004, t18.8=3.715, p=0.0015). At P45: n=14 WT, n=13 MUT; At 5.5mo: n=14 WT, n=11 MUT. **h**, Table of motor findings in clinical exam of human patients with homozygous SWIPP1019R mutation. All patients exhibit motor dysfunction (+=symptom present). Data reported as mean ± SEM, error bars are SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, two-way repeated measure ANOVAs (b), Mann-Whitney U tests and two-tailed t-tests (c-g)

**Fig. 7 Model of neuronal endo-lysosomal pathology in SWIPP1019Rmutant mice. a**,Wild-type WASH function in mouse brain. Under normal conditions, the WASH complex interacts with many endosomal proteins and cytoskeletal regulators, such as the Arp2/3 complex. These interactions enable restructuring of the endosome surface (actin in gray) and allow for cargo segregation and scission of vesicles. Substrates are transported to the late endosome for lysosomal degradation, to the Golgi network for modification, or to the cell surface for recycling. **b**, Loss of WASH function leads to increased lysosomal degradation in mouse brain. Destabilization of the WASH complex leads to enlarged endosomes and lysosomes, with increased substrate accumulation at the lysosome. This suggests an increase in flux through the endo-lysosomal pathway, possibly as a result of mis-localized endosomal substrates. **c**, Wild-type mice exhibit normal motor function. **d**, SWIPP1019Rmutant mice display progressive motor dysfunction in association with these subcellular alterations.

**Extended Data Fig. 1 iBioID constructs display efficient expression in wild-type mouse brain. a**,Experimental design. AAV constructs were injected into the brain of P0 pups. After two weeks of expression, mice were given daily subcutaneous biotin injections (24mg/kg) for seven days to biotinylate nearby substrates. Mice were then sacrificed by terminal perfusion, and brains were processed for cryosectioning, immunostaining, and imaging. **b**,Example of a coronal section from an AAV-hSyn1-Wash1-BioID2-HA-injected brain, showing the immunostaining of nuclei (DAPI, blue), the HA viral epitope (green), and biotinylated substrates (streptavidin, red). Scale bar 1mm. **c** (Cortical, Cx), **d** (Hippocampal, Hipp), and **e** (Thalamic, Thal), expression of DAPI, HA, and Biotin for the insets delineated in b. Co-localization of the HA viral epitope and biotinylated substrates are seen as yellow in the merged images on the right. Scale bar for c-e, 50 µm.

**Extended Data Fig. 2 Overexpression of SWIPP1019R decreases WASH complex binding in cultured cells. a**,Schematic showing overexpression of WT or MUT SWIPP1019R in HEK293T cells followed by immunoprecipitation. **b**,Western blots of input (5%, left) and immunoprecipitated (IP, right) protein. Two samples per condition were run on two separate gels, n=4 separate experiments. **c**,Quantification of bnormalized to WT. Strumpellin (WT 100.0 ± 6.8%, MUT 54.8 ± 8.0%, t5.9=4.290, p=0.0054), WASH1 (WT 100.0 ± 7.3%, MUT 41.4 ± 4.4%, t4.9=6.902, p=0.0011), HA (WT 100.0 ± 4.1%, MUT 107.8 ± 4.1%, t6.0=1.344, p=0.2275). Data reported as mean ± SEM, error bars are SEM. \*\*p<0.01, two-tailed t-tests

**Extended Data Fig. 3 SWIPP1019R MUT brain displays significant alterations in protein abundance compared to WT. a**, Interactome of altered proteins. Nodes reflect gene name, light gray lines delineate proteins identified as different in MUT compared to WT (ΔWashc4 in center), dark dashed lines indicate known protein-protein interactions from HitPredict database97. Color reflects cellular function seen in c. **b**,Difference in normalized protein abundance of four WASH proteins (SWIP: WT 6.28, MUT 4.89, std error 0.036, p=1.71x10-32; WASH1: WT 6.28, MUT 5.28, std error 0.056, p=3.38x10-19; Fam21: WT 7.85, MUT 6.58, std error 0.040, p=2.10x10-27; Strumpellin: WT 6.41, MUT 4.59, std error 0.092, p=6.19x10-16) and one control (Tubulin 4a: WT 8.57, MUT 8.52, std error 0.032, p=0.362) across all samples, presented as log2(adjusted intensities). **c**, Cellular function of proteins in a, as reflected in published literature. % reflects the percentage of proteins in a category out of the total 85 altered proteins. Clustergrams of: **d**,proteins with increased (red) or decreased (blue) abundance in MUT brains compared to WT; **e**,known protein components of the WASH complex and their previously reported interactors; **f**,proteins with lysosomal function; **g**, proteins identified in the WASH1-BioID2 proteome (Fig 1); **h**, proteins with links to intellectual disability (I.D.) or neurodegeneration (Degen.). Data reported as mean ± SEM, error bars are SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, one-sided student’s t-test with Bonferroni correction (b)

**Extended Data Fig. 4 Multiple protein networks display significant alterations in MUT brain compared to WT. a**, All proteins identified by TMT proteomics plotted based on two of their principle components (PC1, PC2). Each protein is color-coded by its corresponding module. PVE = percent variance explained. Clustergrams of: **b**, Module 14 (M14) containing endosomal proteins; **c**, Module 211 (M211) containing lysosomal proteins; **d**, Modules 84 (M84) containing endoplasmic reticulum (ER) proteins; **e-f**, Modules 143 and 35 (M143, M35) containing synaptic proteins. **g**, Summary plots of total protein abundance in each of the seven analyzed brain fractions for each module, presented as log2(adjusted intensities). All modules display significant differences between WT and MUT samples (M14: WT 13.9, MUT 13.8, std error XX, p=0.0198; M211: WT 12.2, MUT 12.3, std error XX, p=0.0037; M84: WT 14.4, MUT 14.7, std error XX, p= 0.0083; M143: WT 14.54, MUT 14.45, std error XX, p= 0.0143; M35: WT 14.8, MUT 14.1, std error XX, p= 0.0029). Data reported as mean ± SEM, error bars are SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, one-sided student’s t-test with Bonferroni correction (b)

**Extended Data Fig. 5 There is no significant difference in striatal cell death between WT and MUT mice. a** (WT) and **b** (MUT),Representative images of adolescent (P42) striatum stained with cleaved caspase-3 (CC3, green). **c**,Anatomical representation of mouse brain with striatum highlighted in blue, adapted from Allen Brain Atlas106.  **d** (WT) and **e** (MUT),Representative images of adult (10 mo) motor cortex stained with CC3 (green). **f**,Graph depicting the % of DAPI+ nuclei that are positive for CC3 per image. No difference is seen between genotypes at either age (P42 WT 3.70 ± 0.99%, P42 MUT 1.95 ± 0.49%, 10mo WT 16.77 ± 2.09%, 10mo MUT 24.86 ± 2.17%, H=61.87, p<0.0001). **g**, **h**, **i**, and **j** are the DAPI (blue) co-stained images for a, b, d, and e, respectively. **k**, Graph depicting the average number of DAPI+ nuclei per image at two different age points for WT and MUT mice shows no significant difference between genotypes (P42 WT 53.13± 1.48, P42 MUT 47.79 ±1.82, 10mo WT 45.84 ±2.50, 10mo MUT 47.36 ± 2.65, H=7.517, p=0.0571). n=24 images per condition for f and k, taken from 4 different mice for each condition. Scale bar for CC3 and DAPI, 15 µm. Data reported as mean ± SEM, error bars are SEM. Kruskal-Wallis non-parametric tests.

**Extended Data Fig. 6 There is no significant difference in cerebellar structure between WT and MUT mice. a** (WT) and **b** (MUT),Representative images of adolescent (P42) cerebellum stained with cleaved caspase-3 (CC3, green). **c**,Anatomical representation of mouse cerebellum, adapted from Allen Brain Atlas106. Blue region highlights area used for imaging.  **d** (WT) and **e** (MUT),Representative images of adult (10 mo) cerebellum stained with CC3 (green). No significant CC3 staining is observed at either age. **f**,Graph depicting the number of Calbindin+ somas per image, a marker for Purkinje cells. No difference is seen between genotypes at either age (P42 WT 20.50 ± 0.53, P42 MUT 20.67 ± 0.59, 10mo WT 21.42 ± 0.85, 10mo MUT 22.63 ± 0.74, H=4.891, p=0.1799). **g**, **h**, **i**, and **j** are the Calbindin+ (red) co-stained images for a, b, d, and e, respectively. **k**, **l**, **m** and **n** are the DAPI+ (nuclei, blue) merged images for g, h i, and j, respectively. Scale bars are 50 µm. Data obtained from four animals per condition, and reported as mean ± standard error of the mean (SEM), with error bars as SEM. Kruskal-Wallis test (f)

**Extended Data Fig. 7 SWIPP1019R mutant mice do not display deficits in spatial working memory. a**,Y-maze paradigm. Mice were placed in the center of the maze and allowed to explore all three arms freely for five minutes. Each arm had distinct visual cues. **b**,Graphs depicting the percent alternations achieved for each mouse at adolescence (top, WT 51.48 ± 2.47%, MUT 50.81 ± 2.19%, t24=0.2036, p=0.8404) and adulthood (bottom, WT 56.12 ±1.53%, MUT 57.93 ± 2.56%, t18=0.6074, p=0.5511) reveals no difference between genotypes. **c,** Graphs of the number of direct revisits mice made to the arm they just explored reveal no difference between genotypes at adolescence (top, WT 2.64 ± 0.50, MUT 2.42 ± 0.42, t24=0.3482, p=0.7308) or adulthood (bottom, WT 1.36 ± 0.33, MUT 1.42 ± 0.36, t24=0.1231, p=0.9031). **d**, Similar to c, there were no differences in the number of indirect revisits (ex: arm A🡪 arm B🡪 arm A) between genotypes at either adolescence (top, WT 10.21 ± 1.03, MUT 12.00 ± 1.48, U=69, p=0.4515) or adulthood (bottom, WT 12.86 ± 1.26, MUT 15.17 ± 1.43, t23=1.211, p=0.2380). **e**, There were no significant differences in total distance travelled, suggesting that motor function did not affect Y-maze performance (P45 WT 2401 ± 98.9 cm, P45 MUT 2406 ± 121.0 cm, t22=0.03281, p=0.9741; 5mo WT 2761 ± 111.6 cm, 5mo MUT 3124 ± 191.1 cm, t18=1.638, p=0.1189). For all measures, WT n=14, MUT n=12. Data reported as mean ± SEM, error bars are SEM. Two-tailed t-tests or Mann-Whitney U tests (d)

**Extended Data Fig. 8 SWIPP1019R mutant mice do not exhibit deficits in novel object recognition. a**,Novel object task. Mice first performed a 5-minute trial in which they were placed in an arena with two identical objects and allowed to explore freely, while their behavior was tracked with video software. Mice were returned to their home cage, and then re-introduced to the arena a half an hour later, where one of the objects had been replaced with a novel object, and their behavior was again tracked. Twenty-four hours later the same test was performed, but the novel object was replaced with another new object. **b**, Graphs depicting animals’ preference for the novel object during the three phases of the task, training (Train), short-term memory (STM), and long-term memory (LTM). No significant difference in object preference is seen between genotypes for any phase (Train P44 WT -0.092 ± 0.065, Train P44 MUT -0.123 ± 0.072, STM P44 WT 0.488 ± 0.076, STM P44 MUT 0.315 ± 0.094, LTM P45 WT 0.479 ± 0.046, LTM P45 MUT 0.373 ± 0.076, F1,22=1.840, p=0.1887; Train 5mo WT -0.066 ± 0.053, Train 5mo MUT -0.130 ± 0.089, STM 5mo WT 0.416 ± 0.060, STM 5mo MUT 0.274 ± 0.096, LTM 5mo WT 0.316 ± 0.059, LTM 5mo MUT 0.306 ± 0.050, F1,22=0.9735, p=0.3345). **c**, Graphs depicting the total amount of time (in seconds) animals spent exploring both objects in each phase of the task. No significant difference in exploration time was observed across genotypes at either age, suggesting that genotype does not hinder object exploration (Train P44 WT 38.10 ± 2.45 s, Train P44 MUT 50.04 ± 5.44 s, STM P44 WT 55.02 ± 4.31 s, STM P44 MUT 63.60 ± 4.50 s, LTM P45 WT 46.75 ± 3.34 s, LTM P45 MUT 68.08 ± 7.54 s, F1,22=7.373, p=0.0126; Train 5mo WT 39.10 ± 3.62 s, Train 5mo MUT 51.15 ± 4.91 s, STM 5mo WT 44.75 ± 4.87 s, STM 5mo MUT 49.56 ± 6.16 s, LTM 5mo WT 39.02 ± 5.29 s, LTM 5mo MUT 55.15 ± 7.34 s, F1,22=2.936, p=0.1007). For both ages, WT n=13, MUT n=11. Data reported as mean ± SEM, error bars are SEM. Two-way ANOVAs

**Extended Data Fig. 9 SWIPP1019R mutant mice do not have significant deficits in contextual fear memory recall, auditory perception, or tactile sensation. a**, Experimental fear conditioning scheme. After acclimation, mice received a mild aversive 0.4mA footshock paired with a 2900Hz tone in a conditioning chamber. 24 hours later, the mice were placed back in the same chamber to assess freezing behavior (without footshock or tone). **b**, Experimental startle response setup used to assess hearing and somatosensation. Mice were placed in a plexiglass tube atop a load cell that measured startle movements in response to stimuli. **c**, Line graphs of WT and MUT freezing response during the contextual memory recall task. Data represented as average freezing per genotype in 30 second time bins. The task was performed with two different cohorts for the different ages, P42 (top) and 6.5mo (bottom). Top: no significant difference in freezing at P42 (Two-way repeated measure ANOVA, Genotype effect, F1,18= 0.088, p=0.7698. Sidak’s post-hoc analysis, 30 s p=0.8388, 60 s p=0.9990, 90 s p=0.9964, 120 s p=0.3281), Bottom: no significant difference in freezing at 6.5mo (Two-way repeated measure ANOVA, Genotype effect, F1,22= 3.723, p=0.0667. Sidak’s post-hoc analysis, 30 s p=0.8977, 60 s p=0.1636, 90 s p=0.9979, 120 s p=0.0037). **d**,Graphs showing the average total freezing time per animal during context exposure. Top: no significant difference is seen between WT and MUT mice at P42 (WT 34.01 ± 6.32%, MUT 36.99 ± 7.81%, t17=2.985, p=0.7699). Bottom: no significant different is seen between genotypes at 6.5mo (WT 43.94 ± 6.00%, MUT 28.73 ± 4.80%, t21.6=1.980, p=0.0606). **e**,Graphs of individual animals’ startle response to a 3000Hz tone played at 80dB. MUT mice were not significantly more reactive to the tone than WT at P50 (WT 25.96 ± 4.95, MUT 40.68 ± 5.05, U=35, p=0.2799), or at 6.5mo (WT 14.07 ± 3.27, MUT 14.85 ± 1.49, U=47, p=0.2768). **f**, Graphs of individual animals’ startle response to a 0.4mA footshock. No significant difference observed between genotypes at either age (P55 WT 1527 ± 215.7, P55 MUT 1996 ± 51.0, U=28.50, p=0.0542; 6.5mo WT 1545 ± 179.5, 6.5mo MUT 1817 ± 119.1, U=47, p=0.2360). Startle response reported in arbitrary units (A.U.). For all adolescent measures: WT n=10, MUT n=10. For adult freezing measures: WT n=13, MUT n=11. For adult startle responses: WT n=13, MUT n=10. Data reported as mean ± SEM, error bars are SEM.\*p<0.05, \*\*p<0.01, two-way repeated measure ANOVAs (c), two-tailed t-tests (d), and Mann-Whitney U tests (e-f)

**Extended Data Fig. 10 Progressive gait changes in SWIPP1019Rmutant mice are not restricted to rear limbs. a**,Graph of average swing time per stride for front limbs. At P45 (top), there is no significant difference in front swing time(WT 177.5 ± 10.9 ms, MUT 175.3 ± 8.7 ms, t24=0.1569, p=0.8766). At 5.5 mo (bottom), MUT mice take significantly longer to swing their forelimbs (WT 178.6 ± 6.2 ms, MUT 206.3 ± 7.2 ms, t21.4=2.927, p=0.0079). **b**,Graph of average stride length for front limbs. At P45 (top), there is no difference in WT and MUT stride length (WT 57.0 ± 0.9 mm, MUT 59.2 ± 1.4 mm, U=68, p=0.2800). At 5.5mo (bottom), MUT mice take significantly longer strides with their forelimbs (WT 60.0 ± 0.6 mm, MUT 63.7 ± 0.9 mm, t17=3.545, p=0.0024). **c**,Graph of average homolateral coupling, the fraction of a reference foot’s stride when its ipsilateral foot starts its stride. At P45, there is no significant difference in homolateral coupling (WT 0.48 ± 0.005, MUT 0.48 ± 0.004, U=90, p=0.9713), but at 5.5mo, MUT mice display decreased homolateral coupling (WT 0.48 ± 0.002, MUT 0.45 ± 0.005, t13.5=3.469, p=0.0039). **d**,Graph of average track width between front limbs. At P45 (top), there is a significantly narrower front track width in MUT compared to WT (WT 16.99 ± 0.15 mm, MUT 15.12 ± 0.33 mm, t17=5.192, p<0.0001). This difference persists into adulthood at 5.5mo (WT 19.36 ± 0.23 mm, MUT 16.74 ± 0.46 mm, t15=5.055, p=0.0001). **e**,Graph of average track width between rear limbs. At P45 (top), there is no difference in WT and MUT rear track widths (WT 29.58 ± 0.51 mm, MUT 28.77 ± 0.36 mm, t23=1.292, p=0.2091). At 5.5mo (bottom), mutants display significantly narrower rear track widths (WT 32.59 ± 0.34 mm, MUT 30.01 ± 0.46 mm, t19.4=4.502, p=0.0002). For P45 measures: WT n=14, MUT n=13; for 5.5mo measures: WT n=14, MUT n=11. Data reported as mean ± SEM, error bars are SEM. \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, two-tailed t-tests or Mann-Whitney U tests

**Extended Data Fig. 11 SWIPP1019Rmutant mice display selective anxiety-like behaviors with age. a**,Open field task. Mice were placed in the center of an empty arena enclosed by four plexiglass walls and allowed to explore freely for half an hour. **b**, Graphs of the total time mice spent in the center of the arena reveals no difference between genotypes at adolescence (WT 404.9 ± 34.1 s, MUT 525.7 ± 52.6 s, t21=1.926, p=0.0678) or adulthood (WT 705.9 ± 59.1 s, MUT 574.0 ± 62.8 s, t25=1.528, p=0.1391). **c,** Graphs of the total time mice spent performing stereotypic anxiety-like movements (such as repetitive grooming) reveal no difference between genotypes (Adolescent: WT 149.8 ± 9.1 s, MUT 145.8 ± 9.2 s, t25=0.3099, p=0.7592. Adult: WT 157.5 ± 7.6 s, MUT 152.3 ± 4.9 s, t22=0.5716, p=0.5734). **d**, Graphs of the total distance mice traveled reveal no significant differences between genotypes at adolescence (WT 1238 ± 96.0 cm, MUT 1543 ± 234.4 cm, U=83, p=0.7203), but a trend towards hyperactivity in adult MUT mice (WT 1250 ± 86.5 cm, MUT 2080 ± 324.4 cm, U=57, p=0.1048), suggesting anxiety-like features. **e**, Diagram of the light-dark task. Each mouse was placed in the dark box and allowed to explore either side of the chamber for five minutes. The number of crosses and total activity of each mouse was measured by infrared beam breaks. **f**, Graphs of the percentage of total trial time that mice spent in the light box. No difference is observed at adolescence (WT 40.87 ± 2.37%, MUT 32.34 ± 4.96%, t17=1.551, p=0.1390), but adult MUT mice spend significantly less time than WT mice in the light box (WT 38.89 ± 1.87%, MUT 17.81 ± 3.91%, t17=4.860, p=0.0001), suggesting an increase in anxiety-like behavior. **g**, Graphs of the length of time elapsed before mice make their first transition from the dark box to the light box reveal no difference at adolescence (WT 12.79 ± 1.70 s, MUT 12.46 ± 4.09 s, U=51.50, p=0.0558), but a large increase in latency for some adult MUT mice (WT 10.17 ± 0.99 s, MUT 69.41 ± 30.20 s, U=64, p=0.1974), consistent with anxious behavior. **h**, Graphs of the total number of beam breaks by each mouse reveal no differences between genotypes at a young age (WT 360.5 ± 25.9, MUT 418.7 ± 24.3, t25=1.639, p=0.1137), but a significant increase in beam breaks of adult MUT mice (WT 322.1 ± 25.9, MUT 450.9 ± 35.4, t22=2.938, p=0.0075), possibly reflecting stereotypic or repetitive behaviors from increased anxiety. For all measures: WT n=14, MUT n=13. Data reported as mean ± SEM, error bars are SEM. \*\*p<0.01, \*\*\*p<0.001, two-tailed t-tests or Mann-Whitney U tests

**Extended Data Fig. 12 Motor dysfunction may reflect genotypic differences in grip strength or weight. a**,Depiction of grip strength test. Animals were permitted to use either all four limbs (4-limb) or just front limbs (front limb) to resist against light tension applied to their tail, and the strength of their resistive tug was measured in grams. Hind limb strength was calculated by subtracting front limb from 4-limb measurements. Animals performed three trials for each measurement. For all adolescent measurements: WT n=14, MUT n=13. For all adult measurements: WT n=14, MUT n=11 **b**, Average 4-limb grip strength per animal across trials at adolescence (P42-45, top) and adulthood (5.5mo, bottom). MUT mice demonstrate significantly weaker 4-limb grip strength at both ages (P42 WT 156.7 ± 4.5 g, P42 MUT 130.4 ± 3.2 g, U=15.50, p<0.0001; 5.5mo WT 180.8 ± 4.0 g, 5.5mo MUT 137.5 ± 4.9 g, t21=6.830, p<0.0001). **c**,Average front grip strength per animal across trials (adolescence, top; adult, bottom). MUT mice display significantly weaker front limb grip strength at both time points (P42 WT 75.6 ± 2.2 g, P42 MUT 66.0 ± 1.6 g, t23.5=3.473, p=0.0020; 5.5mo WT 84.8 ± 2.6 g, 5.5mo MUT 74.9 ± 1.8 g, t22=3.092, p=0.0053). **d**,Average calculated rear grip strength per animal across trials (adolescence, top; adult, bottom). MUT mice display significantly weaker rear limb strength at both ages (P42 WT 81.1 ± 3.4 g, P42 MUT 64.4 ± 3.4 g, t25=3.514, p=0.0017; 5.5mo WT 96.0 ± 3.5 g, 5.5mo MUT 62.6 ± 3.7 g, t22=6.578, p<0.0001). **e**, Animal weight schematic. For adolescent measurements: WT n=14, MUT n=13. For adult measurements: WT n=13, MUT n=11 **f**,Weights of animals in grams (adolescence, top; adulthood, bottom). While a difference in weight was seen between genotypes at adolescence (P45 WT 19.6 ± 0.5 g, P45 MUT 15.3 ± 1.2, t12=3.345, p=0.0058), this difference did not persist into adulthood (5.5mo WT 28.9 ± 1.4 g, 5.5mo MUT 25.4 ± 1.5 g, t24.4=1.924, p=0.0661). Data reported as mean ± SEM, error bars are SEM. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001, two-tailed t-tests or Mann-Whitney U tests