Genetic Disruption of WASHC4 Drives Endo-lysosomal Dysfunction and Cognitive-Movement Impairments in Mice and Humans

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SUMMARY

Mutation of the WASH complex subunit, SWIP, is implicated in human intellectual disability, but the cellular etiology of this association is unknown. We identify the neuronal WASH complex proteome, revealing a network of endosomal proteins. To uncover how dysfunction of endosomal SWIP leads to disease, we generate a mouse model of the human $WASHC4^{c.3056C>G}$ mutation. Quantitative spatial proteomics of SWIP^{P1019R} mouse brain confirms that this mutation destabilizes the WASH complex and reveals significant perturbations in both endosomal and lysosomal pathways. Cellular and histological analyses confirm that SWIP^{P1019R} results in endo-lysosomal disruption and uncover indicators of neurodegeneration. We find that SWIP^{P1019R} not only impacts cognition, but also causes significant progressive motor deficits in mice. Remarkably, a retrospective analysis of SWIP^{P1019R} patients confirms this deficit in humans. Combined, these findings support the model that WASH complex destabilization, resulting from SWIP^{P1019R}, drives cognitive and motor impairments via endo-lysosomal dysfunction in the brain.

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 this vesicular relay system by coordinating protein sorting between multiple cellular compartments, including surface receptor endocytosis and recycling, as well as degradative shunting to the lysosome. How endosomal trafficking is modulated in neurons remains a vital area of research due to the unique degree of spatial segregation between organelles in neurons, and its strong implication in neurodevelopmental and neurodegenerative diseases.

In non-neuronal cells, an evolutionarily conserved complex, the Wiskott-Aldrich Syndrome protein and SCAR Homology (WASH) complex, coordinates endosomal trafficking (Derivery and Gautreau, 2010; Linardopoulou et al., 2007). WASH is composed of five core protein components: WASHC1 (aka WASH1), WASHC2 (aka FAM21), WASHC3 (aka CCDC53), WASHC4 (aka SWIP), and WASHC5 (aka Strumpellin) (encoded by genes *Washc1-Washc5*, respectively), which are broadly expressed in multiple organ systems (Alekhina et al., 2017; Kustermann et al., 2018; McNally et al., 2017; Simonetti and Cullen, 2019; Thul et al., 2017). 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 scission (Gomez and Billadeau, 2009; Lee et al., 2016; Phillips-Krawczak et al., 2015; Piotrowski et al., 2013; Simonetti and Cullen, 2019). It also interacts with at least three main cargo adaptor complexes — the Retromer, Retriever, and COMMD/CCDC22/CCDC93 (CCC) complexes — all of which associate with distinct

65 sorting nexins to select specific cargo and enable their trafficking to other cellular

66 locations (Binda et al., 2019; Farfán et al., 2013; McNally et al., 2017; Phillips-Krawczak

67 et al., 2015; Seaman and Freeman, 2014; Singla et al., 2019). Loss of the WASH complex

68 in non-neuronal cells has detrimental effects on endosomal structure and function, as its

69 loss results in aberrant endosomal tubule elongation and cargo mislocalization (Bartuzi

70 et al., 2016; Derivery et al., 2009; Gomez and Billadeau, 2009; Gomez et al., 2012;

71 Phillips-Krawczak et al., 2015; Piotrowski et al., 2013).

72 Consistent with the association between the endosomal trafficking system and

73 pathology, dominant missense mutations in *WASHC5* (protein: Strumpellin) are

74 associated with hereditary spastic paraplegia (SPG8) (De Bot et al., 2013; Valdmanis et

75 al., 2007), and autosomal recessive point mutations in *WASHC4* (protein: SWIP) and

76 *WASHC5* are associated with syndromic and non-syndromic intellectual disabilities

77 (Assoum et al., 2020; Elliott et al., 2013; Ropers et al., 2011). In particular, an autosomal

78 recessive mutation in *WASHC4* (c.3056C>G; p.Pro1019Arg) was identified in a cohort of

79 children with non-syndromic intellectual disability (Ropers et al., 2011). Cell lines derived

80 from these patients exhibited decreased abundance of WASH proteins, leading the

81 authors to hypothesize that the observed cognitive deficits in SWIP[P1019R] patients resulted

82 from disruption of neuronal WASH signaling (Ropers et al., 2011). However, whether this

83 mutation leads to perturbations in neuronal endosomal integrity, or how this might lead to

84 cellular changes associated with disease, are unknown.

85 Here we report the analysis of neuronal WASH and its molecular role in disease

86 pathogenesis. We use *in vivo* proximity proteomics (iBioID) to uncover the neuronal

87 WASH proteome and demonstrate that it is highly enriched for components of endosomal

88   trafficking. We then generate a mouse model of the human *WASHC4^{c.3056c>g}* mutation

89   (SWIP^{P1019R}) (Ropers et al., 2011) to discover how this mutation may alter neuronal

90   trafficking pathways and test whether it leads to phenotypes congruent with human

91   patients. Using an adapted, spatial proteomics approach, (Geladaki et al., 2019) coupled

92   with a systems-level analysis of protein covariation networks, we find strong evidence for

93   substantial disruption of neuronal endosomal and lysosomal pathways *in vivo*. Cellular

94   analyses confirm a significant impact on neuronal endo-lysosomal trafficking *in vitro* and

95   *in vivo*, with evidence of lipofuscin accumulation and progressive apoptosis activation,

96   molecular phenotypes that are indicative of neurodegenerative pathology. Behavioral

97   analyses of SWIP^{P1019R} mice at adolescence and adulthood confirm a role of WASH in

98   cognitive processes, and reveal profound, progressive motor dysfunction. Importantly,

99   retrospective examination of SWIP^{P1019R} patient data confirms motor dysfunction

100   coincident with cognitive impairments in humans. Our results establish that impaired

101   WASH complex function leads to altered neuronal endo-lysosomal function, which

102   manifests behaviorally as cognitive and movement impairments.

103

104   **RESULTS**

105   **Identification of the WASH complex proteome *in vivo* confirms a neuronal role in**

106   **endosomal trafficking.** While multiple mutations within the WASH complex have been

107   identified in humans (Assoum et al., 2020; Elliott et al., 2013; Ropers et al., 2011;

108   Valdmanis et al., 2007), how these mutations lead to neurological dysfunction remains

109   unknown (Figure 1A). Given that previous work in non-neuronal cultured cells and non-

110   mammalian organisms have established that the WASH complex functions in endosomal

111 trafficking, we first aimed to confirm whether this role was conserved in the mouse

112 nervous system (Alekhina et al., 2017; Billadeau et al., 2010; Derivery et al., 2009; Gomez

113 and Billadeau, 2009; Gomez et al., 2012). To discover the likely molecular functions of

114 the neuronal WASH complex, we utilized an *in vivo* BioID (iBioID) paradigm developed in

115 our laboratory to identify the WASH complex proteome from brain tissue (Uezu et al.,

116 2016). BioID probes were generated by fusing a component of the WASH complex,

117 WASH1 (gene *Washc1*), with the promiscuous biotin ligase, BioID2 (WASH1-BioID2,

118 Figure 1B), or by expressing BioID2 alone (negative control, solubleBioID2) under the

119 neuron-specific, human Synapsin-1 promoter. We injected adenoviruses (AAV)

120 expressing these constructs into the cortex of wild-type postnatal day zero (P0) mice

121 (Figure 1B). Two weeks post-injection, we administered daily subcutaneous biotin for

122 seven days to biotinylate *in vivo* substrates. The viruses displayed efficient expression

123 and activity in brain tissue, as evidenced by colocalization of the WASH1-BioID2 viral

124 epitope (HA) and biotinylated proteins (Streptavidin) (Figures 1C-F). For label-free

125 quantitative high-mass accuracy LC-MS/MS analyses, whole brain samples were

126 collected at P22, snap-frozen, and processed as previously described (Uezu et al., 2016).

127 A total of 2,311 proteins were identified across all three experimental replicates, which

128 were further analyzed for those with significant enrichment in WASH1-BioID2 samples

129 over solubleBioID2 negative controls (Table S1).

130   The resulting neuronal WASH proteome included 174 proteins that were

131 significantly enriched (Fold-change ≥ 3.0, Benjamini-Hochberg P-Adjust < 0.1, Figure

132 1G). Of these proteins, we identified all five WASH complex components (Figure 1H), as

133 well as 13 previously reported WASH complex interactors (Figure 1I) (McNally et al.,

134   2017; Phillips-Krawczak et al., 2015; Simonetti and Cullen, 2019; Singla et al., 2019),

135   which provided strong validity for our proteomic approach and analyses. Additional

136   bioinformatic analyses of the neuronal WASH proteome identified a network of proteins

137   implicated in vesicular trafficking, including 23 proteins enriched for endosomal functions

138   (Figure 1J) and 24 proteins enriched for endocytic functions (Figure 1K). Among these

139   endosomal and endocytic proteins were components of the recently identified endosomal

140   sorting complexes, CCC (CCDC93 and COMMD9) and Retriever (VPS35L) (Phillips-

141   Krawczak et al., 2015; Singla et al., 2019), as well as multiple sorting nexins important for

142   recruitment of trafficking regulators to the endosome and cargo selection, such as SNX1-

143   3, and SNX16 (Kvainickas et al., 2017; Maruzs et al., 2015; Simonetti et al., 2017). These

144   data demonstrated that the WASH complex interacts with many of the same proteins in

145   neurons as it does in yeast, amoebae, flies, and mammalian cell lines. Furthermore, there

146   were 32 proteins enriched for cytoskeletal regulatory functions (Figure 1L), including

147   actin-modulatory molecules such as the Arp2/3 complex subunit ARPC5, which is

148   consistent with WASH's role in activating this complex to stimulate actin polymerization

149   at endosomes for vesicular scission (Billadeau et al., 2010; Derivery et al., 2009). The

150   WASH1-BioID2 isolated complex also contained 28 proteins known to localize to the

151   excitatory post-synapse (Figure 1M). This included many scaffolding proteins, such as

152   SHANK2-3 and DLGAP2-4 (Chen et al., 2011; Mao et al., 2015; Monteiro and Feng, 2017;

153   Wan et al., 2011), as well as modulators of synaptic receptors such as SYNGAP1 and

154   SHISA6 (Barnett et al., 2006; Clement et al., 2012; Kim et al., 2003; Klaassen et al.,

155   2016), which was consistent with the idea that vesicular trafficking plays an important part

156 in synaptic function and regulation. Taken together, these results support a major

157 endosomal trafficking role of the WASH complex in mouse brain.

158

159 **SWIP^{P1019R} does not incorporate into the WASH complex, reducing its stability and**

160 **levels *in vivo*.** To determine how disruption of the WASH complex may lead to disease,

161 we generated a mouse model of a human missense mutation found in children with

162 intellectual disability, *WASHC4^{c.3056c>g}* (encoding protein SWIP^{P1019R}) (Ropers et al.,

163 2011). Due to the sequence homology of human and mouse *Washc4* genes, we were

164 able to introduce the same point mutation in exon 29 of murine *Washc4* using CRISPR

165 (Derivery and Gautreau, 2010; Ropers et al., 2011). This C>G point mutation results in a

166 Proline>Arginine substitution at position 1019 of SWIP's amino acid sequence (Figure

167 2A), a region thought to be critical for its binding to another WASH component,

168 Strumpellin (Jia et al., 2010; Ropers et al., 2011). Western blot analysis of brain lysate

169 from adult homozygous SWIP^{P1019R} mutant mice (referred to from here on as MUT mice)

170 displayed significantly decreased expression of two WASH complex members, WASH1

171 and Strumpellin (Figure 2B). These results phenocopied data from the human patients

172 (Ropers et al., 2011) and suggested that the WASH complex is unstable in the presence

173 of this SWIP point mutation *in vivo*. To test whether this mutation disrupted interactions

174 between WASH complex subunits, we compared the ability of wild-type SWIP (WT) and

175 SWIP^{P1019R} (MUT) to co-immunoprecipitate with WASH1 and Strumpellin in HEK cells.

176 Compared to WT, MUT SWIP co-immunoprecipitated significantly less WASH1 and

177 Strumpellin (IP: 41.4% and 54.8% of WT SWIP, respectively), suggesting that the

178 SWIP^{P1019R} mutation hinders WASH complex formation (Figure S1). Together these data

179 support the notion that SWIP^{P1019R} is a damaging mutation that not only impairs its

180 function, but also results in significant reductions of the WASH complex as a whole.

181

182 **Spatial proteomics and unbiased network covariation analysis reveal significant**

183 **disruptions in the endo-lysosomal pathway of SWIP^{P1019R} mutant mouse brain.**

184 Next, we aimed to understand the molecular impact of the SWIP^{P1019R} mutation on the

185 subcellular organization of the brain proteome. We employed multiplex tandem-mass-tag

186 (TMT) proteomics to quantify proteins in subcellular brain fractions. In addition to

187 evaluating differential protein abundance between WT and SWIP^{P1019R} MUT brain, we

188 analyzed network-level changes in groups of covarying proteins to better understand

189 WASH's function and explore the cellular mechanisms by which SWIP^{P1019R} causes

190 disease.

191   We performed a 16-plex TMT spatial proteomics experiment by isolating seven

192 different cellular fractions from adult SWIP^{P1019R} MUT and WT mouse brain (Geladaki et

193 al., 2019; Hallett et al., 2008). Brains from 10-month-old mice were gently homogenized

194 to release intact organelles, followed by successive centrifugation steps to enrich

195 subcellular compartments into different fractions based on their density (Figure 2C)

196 (Geladaki et al., 2019). Seven WT and seven MUT fractions (each prepared from one

197 brain, 14 samples total) were labeled with unique isobaric tandem-mass tags and

198 concatenated. We also included two sample pooled quality controls (SPQCs), which

199 allowed us to assess experimental variability and perform normalization between

200 experiments. By performing this experiment in triplicate, deep coverage of the mouse

201 brain proteome was obtained—across all 48 samples we quantified 86,551 peptides,

202   corresponding to 7,488 proteins. After data pre-processing, normalization, and filtering

203   we retained 5,897 reproducibly quantified proteins in the final dataset (Table S2).

204   We used generalized linear models (GLMs) to assess differential protein

205   abundance for intra-fraction comparisons between WT and MUT genotypes, and for

206   overall comparisons between WT and MUT groups, adjusted for baseline differences in

207   subcellular fraction. In the first analysis, there were 85 proteins with significantly altered

208   abundance in at least one of the 7 subcellular fractions (Benjamini-Hochberg P-Adjust <

209   0.1, Table S2 and Figure S2A). Five proteins were differentially abundant between WT

210   and MUT in all 7 fractions, including four WASH proteins and RAB21A—a known WASH

211   interactor that functions in early endosomal trafficking (WASHC1, WASHC2, WASHC4,

212   WASHC5, Figure 2E) (Olmo et al.; Simpson et al., 2004). The abundance of the remaining

213   WASH complex protein, WASHC3, was found to be very low and was not retained in the

214   final dataset due to its sparse quantification. These data affirm that the SWIP^{P1019R}

215   mutation destabilizes the WASH complex. Next, to evaluate global differences between

216   WT and MUT brain, we analyzed the average effect of genotype on protein abundance

217   across all fractions. At this level, there were 687 differentially abundant proteins between

218   WT and MUT brain (Bonferroni P-Adjust < 0.05) (Table S2). We then aimed to place these

219   differentially abundant proteins into a more meaningful biological context using a

220   systems-based approach.

221   For network-based analyses, we clustered the protein covariation network defined

222   by pairwise correlations between all 5,897 proteins. Our data-driven, quality-based

223   approach used Network Enhancement (Wang et al., 2018) to remove biological noise

224   from the covariation network and employed the Leiden algorithm (Traag et al., 2019) to

225 identify optimal partitions of the graph. We enforced module quality by permutation testing

226 (Ritchie et al., 2016) to ensure that identified modules exhibited a non-random topology.

227 Clustering of the protein covariation graph identified 255 modules of proteins that strongly

228 covaried together (see Methods for complete description of clustering approach).

229 To test for module-level differences between WT and MUT brain, we summarized

230 modules for each biological replicate (a single subcellular fraction prepared from either a

231 WT or MUT mouse) as the sum of their proteins, and extended our GLM framework to

232 identify changes in module abundance (adjusted for fraction differences) between

233 genotypes. 37 of the 255 modules exhibited significant differences in WT versus MUT

234 brain (Bonferroni P-Adjust < 0.05; Table S3). Of note, the module containing the WASH

235 complex, M19, was predicted to have endosomal function by annotation of protein

236 function, and was enriched for proteins identified by WASH1-BioID2 (hypergeometric test

237 P-Adjust < 0.05, bold node edges, Figure 2D). Similar to the WASH iBioID proteome (Fig

238 1), M19 contained components of the CCC (CCDC22, CCDC93, COMMD1-3, COMMD7,

239 and COMMD9) and Retriever sorting complexes (VPS26C and VPS35L), but not the

240 Retromer sorting complex, suggesting that in the brain, the WASH complex may not

241 interact as closely with Retromer as it does in other cells (Figure 2D). Across all fractions,

242 the abundance of M19 was significantly lower in MUT brain compared to WT, providing

243 evidence that the SWIP^{P1019R} mutation reduces the stability of this protein subnetwork

244 and impairs its function (Figure 2F-G).

245 In contrast to the observed decrease in abundance of M19, an endosomal module,

246 we observed two modules, M213 and M2, which contained numerous key lysosomal

247 components and exhibited increased abundance in MUT brain. M213 (Figure 3B)

11

contained the lysosomal protease Cathepsin B (CTSB) as well as two key lysosomal

hydrolases GLB1 and MAN2B. M2 (Figure 3D) contained several lysosomal hydrolases

(e.g. GNS, GLA, and MAN2B1) as well as two Cathepsins (CTSS and CTSL). Notably,

M2 contains the lysosomal glycoprotein progranulin (GRN), a protein whose normal

function is integral to proper lysosome function and whose loss is widely linked with

neurodegenerative pathologies. M2 also contained the hydrolase and lysosomal storage

disorder gene, IDS—whose loss causes a metabolic disorder, Hunter's Syndrome, whose

severe presentation includes a host of neurological symptoms. The overall increase in

abundance of modules M2 and M213 (Figure 3C and 3E) may reflect an increase in flux

through degradative lysosomal pathways in SWIP[P1019R] brain (Figures 3B-3E). In support

of this hypothesis, other critical lysosomal proteins such as HEXB, were also increased

in MUT samples (Figure S2), indicating that disruption of the WASH complex increases

demand for lysosomal substrate degradation (Elia et al., 2019; Kuil et al., 2019; Mok et

al., 2003; Tiribuzi et al., 2011). Module 2 (Figure 3D) also included membrane proteins

ITGA5, a known WASH interactor, and the ATPase ATP13A2, a cation transporter whose

loss causes Kufor-Rakeb syndrome, a Parkinson's disease, as well as excreted proteins

such as the metallopeptidase MMP17 that function in degradation and reorganization of

the extracellular matrix in normal and pathological conditions. The increased abundance

of these proteins in M2 may reflect their lysosomal degradation (Zech et al., 2011). Taken

together these changes seem to reflect a pathological condition characterized by changes

in lysosome metabolism and altered interactions with endosomal and cellular trafficking

machinery.

270   In addition to endo-lysosomal changes, network alterations were evident for an

271   endoplasmic reticulum (ER) module (M83), supporting a shift in the proteostasis of mutant

272   neurons (Figure S3C). Notably, within the ER module, M83, there was increased

273   abundance of chaperones (e.g. HSPA5, PDIA3, PDIA4, PDIA6, and DNAJC3) that are

274   commonly engaged in presence of misfolded proteins (Bartels et al., 2019; Kim et al.,

275   2020; Montibeller and de Belleroche, 2018; Synofzik et al., 2014; Wang et al., 2016). This

276   elevation of ER stress modulators can be indicative of neurodegenerative states, in which

277   the unfolded protein response (UPR) is activated to resolve misfolded species (Garcia-

278   Huerta et al., 2016; Hetz and Saxena, 2017). Taken together, these data demonstrate

279   that the WASH complex functions in close proximity to endosomal compartments and that

280   its loss from these locations likely alters not only endosomal trafficking, but also lysosomal

281   function, with increased stress on cellular homeostasis.

282   Besides these endo-lysosomal and homeostatic changes, we also observed two

283   synaptic modules (M35 and M143) that were also reduced in MUT brain (Figure S3D-E).

284   These included mostly excitatory post-synaptic proteins such as SHISA6 (also identified

285   in WASH1-BioID2, Figure 1), HOMER2, and DLG4, consistent with endosomal WASH

286   influencing synaptic regulation. Decreased abundance of these modules indicates that

287   loss of the WASH complex may result in failure of these proteins to be properly trafficked

288   to the synapse.

289

290   **Mutant neurons display structural abnormalities in endo-lysosomal compartments**

291   ***in vitro.*** Combined, the proteomics data strongly suggested that endo-lysosomal

292   pathways are altered in adult SWIP^{P1019R} mutant mouse brain. Next, we 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, Figure 4A), then fixed and stained for established markers of early endosomes (Early Endosome Antigen 1, EEA1; Figures 4B and 4C) and lysosomes (Cathepsin D, CathD; Figures 4D and 4E). Reconstructed three-dimensional volumes of EEA1 and Cathepsin D puncta revealed that MUT neurons display larger EEA1+ somatic puncta than WT neurons (Figures 4G and 4J), but no difference in the total number of EEA1+ puncta (Figure 4F). This finding is consistent with a loss-of-function mutation, as loss of WASH activity prevents cargo scission from endosomes and leads to cargo accumulation (Bartuzi et al., 2016; Gomez et al., 2012). On the other hand, MUT neurons exhibited significantly less Cathepsin D+ puncta than WT neurons (Figure 4H), but the remaining puncta were significantly larger than those of WT neurons (Figures 4I and 4K). These data support the finding that the SWIP^{P1019R} mutation results in both molecular and morphological abnormalities in the endo-lysosomal pathway.

SWIP^{P1019R} mutant brains exhibit markers of abnormal endo-lysosomal structures and cell death *in vivo*. As there is strong evidence that dysfunctional endo-lysosomal trafficking and elevated ER stress are associated with neurodegenerative disorders, adolescent (P42) and adult (10 month-old, 10mo) WT and MUT brain tissue were analyzed for the presence of cleaved caspase-3, a marker of apoptotic pathway activation, in three brain regions (Boatright and Salvesen, 2003; Porter and Jänicke, 1999). Very little cleaved caspase-3 staining was present in WT and MUT mice at adolescence (Figures 5A, 5B, and S4). However, at 10mo, the MUT motor cortices

316 displayed significantly greater cleaved capsase-3 staining compared to age-matched

317 WT littermate controls (Figures 5D-5F), but did not exhibit any significant difference in the

318 number of total cells (Figures 5G-5K). Furthermore, this difference appeared to be

319 selective for the motor cortex, as we did not observe significant differences in cleaved

320 caspase-3 staining at either age for striatal or cerebellar regions (Figures S4). These data

321 suggested that neurons of the motor cortex were particularly susceptible to disruption of

322 the endo-lysosomal pathway, perhaps because long-range corticospinal projections

323 require high fidelity of trafficking pathways (Blackstone et al., 2011; Slosarek et al., 2018;

324 Wang et al., 2014).

325   To further examine the morphology of primary motor cortex neurons at a

326 subcellular resolution, samples from age-matched 7-month-old WT and MUT mice (7mo,

327 3 animals each) were imaged by transmission electron microscopy (TEM). Strikingly, we

328 observed large electron-dense inclusions in the cell bodies of MUT neurons (arrows,

329 Figure 5M; pseudo-colored region, 5O). These dense structures were associated

330 electron-lucent lipid-like inclusions (asterisk, Figure 5O), and were consistent with

331 lipofuscin accumulation at lysosomal residual bodies (Poët et al., 2006; Valdez et al.,

332 2017; Yoshikawa et al., 2002). Lipofuscin is a by-product of lysosomal breakdown of

333 lipids, proteins, and carbohydrates, which naturally accumulates over time in non-dividing

334 cells such as neurons (Höhn and Grune, 2013; Moreno-García et al., 2018; TERMAN and

335 BRUNK, 1998). However, excessive lipofuscin accumulation is thought to be detrimental

336 to cellular homeostasis by inhibiting lysosomal function and promoting oxidative stress,

337 often leading to cell death (Brunk and Terman, 2002; Powell et al., 2005). As a result,

338 elevated lipofuscin is considered a biomarker of neurodegenerative disorders, including

339 Alzheimer's disease, Parkinson's disease, and Neuronal Ceroid Lipofuscinoses (Moreno-

340 García et al., 2018). Therefore, the marked increase in lipofuscin area and number seen

341 in MUT electron micrographs (Figures 5P and 5Q, respectively) is consistent with an

342 increase in lysosomal breakdown of cellular material. Together these data indicate that

343 SWIP^{P1019R} results in pathological lysosomal function that could lead to

344 neurodegeneration.

345

346 **SWIP^{P1019R} mutant mice display persistent deficits in cued fear memory recall.** To

347 observe the functional consequences of the SWIP^{P1019R} mutation, we next studied WT

348 and MUT mouse behavior. Given that children with homozygous SWIP^{P1019R} point

349 mutations display intellectual disability (Ropers et al., 2011) and SWIP^{P1019R} mutant mice

350 exhibit endo-lysosomal disruptions implicated in neurodegenerative processes, behavior

351 was assessed at two ages: adolescence (P40-50), and mid-late adulthood (5.5-6.5 mo).

352 Interestingly, MUT mice performed equivalently to WT mice in episodic and working

353 memory paradigms, including novel object recognition and Y-maze alternations (Figure

354 S5). However, in a fear conditioning task, MUT mice displayed a significant deficit in cued

355 fear memory (Figure 6). This task tests the ability of a mouse to associate an aversive

356 event (a mild electric footshock) with a paired tone (Figure 6A). Freezing behavior of mice

357 during tone presentation is attributed to hippocampal or amygdala-based fear memory

358 processes (Goosens and Maren, 2001; Maren and Holt, 2000; Vazdarjanova and

359 McGaugh, 1998). Forty-eight hours after exposure to the paired tone and footshock, MUT

360 mice showed a significant decrease in conditioned freezing to tone presentation

361 compared to their WT littermates (Figures 6B and 6C). To ensure that this difference was

362   not due to altered sensory capacities of MUT mice, we measured the startle response of

363   mice to both electric foot shock and presented tones. In line with intact sensation, MUT

364   mice responded comparably to WT mice in these tests (Figure S6). These data

365   demonstrate that although MUT mice perceive footshock sensations and auditory cues,

366   it is their memory of these paired events that is significantly impaired. Additionally, this

367   deficit in fear response was evident at both adolescence and adulthood (top panels, and

368   bottom panels, respectively, Figures 6B and 6C). These changes are consistent with the

369   hypothesis that SWIP^{P109R} is the cause of cognitive impairments in humans.

370

371   **SWIP^{P1019R} mutant mice exhibit surprising motor deficits that are confirmed in**

372   **human patients.** Because SWIP^{P1019R} results in endo-lysosomal pathology consistent

373   with neurodegenerative disorders in the motor cortex, we next analyzed motor function of

374   the mice over time. First, we tested the ability of WT and MUT mice to remain on a rotating

375   rod for five minutes (Rotarod, Figures 7A-7C). At both adolescence and adulthood, the

376   MUT mice performed markedly worse than WT littermate controls (Fig 7C). Mouse

377   performance was not significantly different across trials, which suggested that this

378   difference in retention time was not due to progressive fatigue, but more likely due to an

379   overall difference in motor control (Mann and Chesselet, 2015).

380   To study the animals' movement at a finer scale, the gait of WT and MUT mice

381   was also analyzed using a TreadScan system containing a high-speed camera coupled

382   to a transparent treadmill (Figure 7D) (Beare et al., 2009). Interestingly, while the gait

383   parameters of mice were largely indistinguishable across genotypes at adolescence, a

384   striking difference was seen when the same mice were aged to adulthood (Figures 7E-

385 7G). In particular, MUT mice took slower (Figure 7E), longer strides (Figure 7F), stepping

386 closer to the midline of their body (track width, Figure S7), and their gait symmetry was

387 altered so that their strides were no longer perfectly out of phase (out of phase=0.5, Figure

388 7G). While these differences were most pronounced in the rear limbs (as depicted in

389 Figure 7E-7G), the same trends were present in front limbs (Figure S7). These findings

390 demonstrate that SWIP^{P1019R} results in a progressive motor function decline that was

391 detectable by the rotarod task at adolescence, but which became more prominent with

392 age, as both gait and strength functions deteriorated.

393   These marked motor findings prompted us to re-evaluate the original reports of

394 human SWIP^{P1019R} patients (Ropers et al., 2011). While developmental delay or learning

395 difficulties were the primary impetus for medical evaluation, all patients also exhibited

396 motor symptoms (mean age = 10.4 years old, Figure 7H). The patients' movements were

397 described as "clumsy" with notable fine motor difficulties, dysmetria, dysdiadochokinesia,

398 and mild dysarthria on clinical exam (Figure 7H). Recent communication with the parents

399 of these patients, who are now an average of 21 years old, revealed no notable symptom

400 exacerbation. It is therefore possible that the SWIP^{P1019R} mouse model either exhibits

401 differences from human patients or may predict future disease progression for these

402 individuals, given that we observed significant worsening at 5-6 months old in mice (which

403 is thought to be equivalent to ~30-35 years old in humans)(Dutta and Sengupta, 2016;

404 Zhang et al., 2019).

405

406 **DISSCUSSION**

407   Taken together, the data presented here support a mechanistic model whereby

408   SWIP^{P1019R} causes a loss of WASH complex function, resulting in endo-lysosomal

409   disruption and accumulation of neurodegenerative markers, such as upregulation of

410   unfolded protein response modulators and lysosomal enzymes, as well as build-up of

411   lipofuscin and cleaved caspase-3 over time. To our knowledge, this study provides the

412   first mechanistic evidence of WASH complex impairment having direct and indirect

413   organellar effects that lead to cognitive deficits and progressive motor impairments

414   (Figure 8).

415   Using *in vivo* proximity-based proteomics in wild-type mouse brain, we identify that

416   the WASH complex interacts with the CCC (COMMD9 and CCDC93) and Retriever

417   (VPS35L) cargo selective complexes (Bartuzi et al., 2016; Singla et al., 2019).

418   Interestingly, we did not find significant enrichment of the Retromer sorting complex, a

419   well-known WASH interactor, suggesting that it may play a minor role in neuronal WASH-

420   mediated cargo sorting (Figure 1). These data are supported by our TMT proteomics and

421   covariation network analyses of SWIP^{P1019R} mutant brain, which clustered the WASH,

422   CCC, and Retriever complexes together in M19, but not the Retromer complex (Figure

423   2). Systems-level protein covariation analyses also revealed that disruption of these

424   WASH-CCC-Retriever interactions may have multiple downstream effects on the

425   endosomal machinery, since endosomal modules displayed significant changes in

426   SWIP^{P1019R} brain (including M19, Figure 2, as well as M14, Figure S3B), with

427   corresponding decreases in the abundance of endosomal proteins including Retromer

428   subunits (VPS29 and VPS35), associated sorting nexins (e.g. SNX17 and SNX27),

429   known WASH interactors (e.g. RAB21 and FKBP15), and cargos (e.g. LRP1 and ITGA3)

430   (Figures S2 and S3) (Farfán et al., 2013; Fedoseienko et al., 2018; Halff et al., 2019;

431   Harbour et al., 2012; McNally et al., 2017; Olmo et al.; Pan et al., 2010; Ye et al., 2020;

432   Zimprich et al., 2011). While previous studies have indicated that Retromer and CCC

433   influence endosomal localization of WASH (Harbour et al., 2012; Phillips-Krawczak et al.,

434   2015; Singla et al., 2019), our findings of altered endosomal networks containing

435   decreased Retromer, Retriever, and CCC protein levels in SWIP[P1019R] mutant brain point

436   to a possible feedback mechanism wherein WASH impacts the protein abundance and/or

437   stability of these interactors. Future studies defining the hierarchical interplay between

438   the WASH, Retromer, Retriever, and CCC complexes in neurons could provide clarity on

439   how these mechanisms are organized.

440   In addition to highlighting the neuronal roles of WASH in CCC- and Retriever-

441   mediated endosomal sorting, our proteomics approach also identified protein modules

442   with increased abundance in SWIP[P1019R] mutant brain. The proteins in these modules fell

443   into two interesting categories: lysosomal enzymes and proteins involved in the

444   endoplasmic reticulum (ER) stress response. Of note, some of the lysosomal enzymes

445   with elevated levels in MUT brain (GRN, M2; GNS, M213; and HEXB, M6; Figures 3 and

446   S2) are also implicated in lysosomal storage disorders, where they generally have

447   decreased, rather than increased, function or expression (Mok et al., 2003; Ward et al.,

448   2017; Zampieri et al., 2009). We speculate that loss of WASH function in our mutant

449   mouse model may lead to increased accumulation of cargo and associated machinery at

450   early endosomes (as seen in Figure 4, enlarged EEA1^{+} puncta), eventually overburdening

451   the vesicles and triggering transition to late endosomes for subsequent fusion with

452   degradative lysosomes (Figure 8). This would effectively increase delivery of endosomal

453　substrates to the lysosome compared to baseline, resulting in enlarged, overloaded

454　lysosomal structures, and elevated demand for degradative enzymes. For example, since

455　mutant neurons display increased lysosomal module protein abundance (Figure 3), and

456　larger lysosomal structures (Figures 4 and 5), they may require higher quantities of

457　progranulin (GRN, Figure 3; M2) for sufficient lysosomal acidification (Tanaka et al.,

458　2017).

459　　　　Our findings that SWIP^{P1019R} results in reduced WASH complex stability and

460　function, which may ultimately drive lysosomal dysfunction, are supported by studies in

461　non-mammalian cells. For example, expression of a dominant-negative form of WASH1

462　in amoebae impairs recycling of lysosomal V-ATPases (Carnell et al., 2011) and loss of

463　WASH in *Drosophila* plasmocytes affects lysosomal acidification (Gomez et al., 2012;

464　Nagel et al., 2017; Zech et al., 2011). Moreover, mouse embryonic fibroblasts lacking

465　WASH1 display abnormal lysosomal morphologies, akin to the structures we observed in

466　cultured SWIP^{P1019R} MUT neurons (Gomez et al., 2012).

467　　　　In addition to lysosomal dysfunction, endoplasmic reticulum (ER) stress is

468　commonly observed in neurodegenerative states, where accumulation of misfolded

469　proteins disrupts cellular proteostasis (Cai et al., 2016; Hetz and Saxena, 2017;

470　Montibeller and de Belleroche, 2018). This cellular strain triggers the adaptive unfolded

471　protein response (UPR), which attempts to restore cellular homeostasis by increasing the

472　cell's capacity to retain misfolded proteins within the ER, remedy misfolded substrates,

473　and trigger degradation of persistently misfolded species. Involved in this process are ER

474　chaperones that we identified as increased in SWIP^{P1019R} mutant brain including BiP

475　(HSPA5), calreticulin (CALR), calnexin (CANX), and the protein disulfide isomerase

476   family members (PDIA1, PDIA4, PDIA6) (M83; Figure S3) (Garcia-Huerta et al., 2016).

477   Many of these proteins were identified in the ER protein module found to be significantly

478   altered in MUT mouse brain (M83), supporting a network-level change in the ER stress

479   response (Figure S3C). One notable exception to this trend was endoplasmin (HSP90B1,

480   M136), which exhibited significantly decreased abundance in SWIP^{P1019R} mutant brain

481   (Table S2). This is surprising given that endoplasmin has been shown to coordinate with

482   BiP in protein folding (Sun et al., 2019), however it may highlight a possible compensatory

483   mechanism. Additionally, prolonged UPR can stimulate autophagic pathways in neurons,

484   where misfolded substrates are delivered to the lysosome for degradation (Cai et al.,

485   2016). These data highlight a relationship between ER and endo-lysosomal disturbances

486   as an exciting avenue for future research.

487   It has become clear that preservation of the endo-lysosomal system is critical to

488   neuronal function, as mutations in mediators of this process are implicated in neurological

489   diseases such as Parkinson's disease, Huntington's disease, Alzheimer's disease,

490   Frontotemporal Dementia, Neuronal Ceroid Lipofuscinoses (NCLs), and Hereditary

491   Spastic Paraplegia (Baker et al., 2006; Connor-Robson et al., 2019; Edvardson et al.,

492   2012; Follett et al., 2019; Harold et al., 2009; Mukherjee et al., 2019; Pal et al., 2006;

493   Quadri et al., 2013; Seshadri et al., 2010; Tachibana et al., 2019; Valdmanis et al., 2007).

494   These genetic links to predominantly neurodegenerative conditions have supported the

495   proposition that loss of endo-lysosomal integrity can have compounding effects over time

496   and contribute to progressive disease pathologies. In particular, NCLs are lysosomal

497   storage disorders primarily found in children, with heterogenous presentations and

498   multigenic causations (Mukherjee et al., 2019). The majority of genes implicated in NCLs

499   affect lysosomal enzymatic function or transport of proteins to the lysosome (Mukherjee

500   et al., 2019; Poët et al., 2006; Ramirez-Montealegre and Pearce, 2005; Yoshikawa et al.,

501   2002). Most patients present with marked neurological impairments, such as learning

502   disabilities, motor abnormalities, vision loss, and seizures, and have the unifying feature

503   of lysosomal lipofuscin accumulation upon pathological examination (Mukherjee et al.,

504   2019). While the human SWIP^{P1019R} mutation has not been classified as an NCL (Ropers

505   et al., 2011), findings from our mutant mouse model suggest that loss of WASH complex

506   function leads to phenotypes bearing strong resemblance to NCLs, including lipofuscin

507   accumulation (Figures 4-7). As a result, our mouse model could provide the opportunity

508   to study these pathologies at a mechanistic level, while also enabling preclinical

509   development of treatments for their human counterparts.

510   Currently there is an urgent need for greater mechanistic investigations of

511   neurodegenerative disorders, particularly in the domain of endo-lysosomal trafficking.

512   Despite the continual increase in identification of human disease-associated genes, our

513   molecular understanding of how their protein equivalents function and contribute to

514   pathogenesis remains limited. Here we employ a systems-level analysis of proteomic

515   datasets to uncover biological perturbations linked to SWIP^{P1019R}. We demonstrate the

516   power of combining *in vivo* proteomics and systems network analyses with *in vitro* and *in*

517   *vivo* functional studies to uncover relationships between genetic mutations and molecular

518   disease pathologies. Applying this platform to study organellar dysfunction in other

519   neurodegenerative disorders with protein trafficking defects may uncover convergent

520   disease pathways.

521

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.

DECLARATION OF INTERESTS

545   The authors declare no competing interests.

546

547   **FIGURE TITLES AND LEGENDS**

548   **Figure 1. Identification of the WASH complex proteome *in vivo* confirms a neuronal**

549   **role in endosomal trafficking**

550   (A) The WASH complex is composed of five subunits, *Washc1* (WASH1), *Washc2*

551   (FAM21), *Washc3* (CCDC53), *Washc4* (SWIP), and *Washc5* (Strumpellin). Human

552   mutations in these components are associated with spastic paraplegia (De Bot et al.,

553   2013; Jahic et al., 2015; Valdmanis et al., 2007), Ritscher-Schinzel Syndrome (Elliott et

554   al., 2013), and intellectual disability (Assoum et al., 2020; Ropers et al., 2011).

555   (B) A BioID2 probe was attached to the c-terminus of WASH1 and expressed under the

556   human synapsin-1 (hSyn1) promoter in an AAV construct for *in vivo* BioID (iBioID). iBioID

557   probes (WASH1-BioID2-HA, or negative control solubleBioID2-HA) were injected into

558   wild-type mouse brain at P0 and allowed to express for two weeks. Subcutaneous biotin

559   injections (24 mg/kg) were administered over seven days for biotinylation, and then brains

560   were harvested for isolation and purification of biotinylated proteins. LC-MS/MS identified

561   proteins significantly enriched in all three replicates of WASH1-BioID2 samples over

562   soluble-BioID2 controls.

563   (C) Representative image of WASH1-BioID2-HA expression in a mouse coronal brain

564   section (Cx = cortex, Hipp = hippocampus, Thal = thalmus). Scale bar, 1 mm.

565   (D) Representative image of WASH1-BioID2-HA expression in mouse cortex (inset from

566   C). Individual panels show nuclei (DAPI, blue), AAV construct HA epitope (green), and

567   biotinylated proteins (Streptavidin, red). Merged image shows colocalization of HA and

568   Streptavidin (yellow). Scale bar, 50 μm.

569   (E) Representative image of WASH1-BioID2-HA expression in mouse hippocampus

570   (inset from C). Scale bar, 50 μm.

571   (F) Representative image of WASH1-BioID2-HA expression in mouse thalamus (inset

572   from C). Scale bar, 50 μm.

573   (G) iBioID identified known and unknown proteins interactors of the WASH complex in

574   murine neurons. Nodes size represents protein abundance fold-enrichment over negative

575   control (range: 3 to 181.7), solid grey edges delineate iBioID interactions between the

576   WASHC1 probe (seen in yellow at the center) and identified proteins, dashed edges

577   indicate known protein-protein interactions from HitPredict database (López et al., 2015).

578   (H-I) Clustergrams of:

579   (H) All five WASH complex proteins identified by iBioID.

580   (I) Previously reported WASH interactors (13/174), including the CCC and Retriever

581   complexes.

582   (J) Endosomal trafficking proteins (23/174 proteins).

583   (K) Endocytic proteins (24/174).

584   (L) Proteins involved in cytoskeletal regulation (32/174), including Arp2/3 subunit ARPC5.

585   (M) Synaptic proteins (28/174). Clustergrams were annotated by hand and cross-

586   referenced with Metascape (Zhou et al., 2019) GO enrichment of WASH1 proteome

587   constituents over all proteins identified in the BioID experiment.

588

Figure 2. SWIP^P1019R does not incorporate into the WASH complex, reducing its stability and levels *in vivo*

(A) Mouse model of the human SWIP^P1019R missense 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 and quantification of WASH components, Strumpellin and WASH1 (predicted sizes in kDa: 134 and 72, respectively), as well as loading control β-Tubulin (55kDa) from whole adult whole brain lysate prepared from SWIP WT (*Washc4^C/C*) and SWIP homozygous MUT (*Washc4^G/G*) mice. Bar plots show quantification of band intensities relative to WT (n=3 mice per genotype). Strumpellin (WT 100.0 ± 5.2%, MUT 3.5 ± 0.7%, $t_{2.1}$=18.44, p=0.0024) and WASH1 (WT 100.0 ± 3.8%, MUT 1.1 ± 0.4%, $t_{2.1}$=25.92, p=0.0013) were significantly decreased. Equivalent amounts of protein were analyzed in each condition (β-Tubulin: WT 100.0 ± 8.2%, MUT 94.1 ± 4.1%, U=4, p>0.99).

(C) Spatial TMT proteomics experimental design. 7 subcellular fractions were prepared from one WT and one MUT mouse (10mo). These samples, as well as two pooled quality control (QC) samples, 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 its weighted degree centrality (~importance in module), purple color indicates proteins with altered abundance in MUT brain relative to WT, black node border denotes proteins identified in the WASH1-BioID proteome (Figure 1), black

612   edges indicate known protein-protein interactions, and grey-red edges denote the relative

613   strength of protein covariation within a module (gray = weak, red = strong).

614   (E) Difference in normalized protein abundance for four WASH proteins found in M19

615   (SWIP: WT 6.28 ± 0.41, MUT 4.89 ± 0.28, p=3.98x10^{-28}; WASH1: WT 6.41 ± 0.47, MUT

616   4.65 ± 0.62, p=7.92x10^{-18}; FAM21: WT 6.29 ± 0.48, MUT 5.29 ± 0.49, p=3.27x10^{-16};

617   Strumpellin: WT 7.85 ± 0.52, MUT 6.59 ± 0.53, p=9.06x10^{-25}) and one control (Tubulin

618   4a: WT 8.57 ± 0.52, MUT 8.52 ± 0.58, p>0.99) across all three experimental replicates

619   (n=3 independent experiments), presented as $\log_2$(adjusted protein intensities).

620   (F) Normalized average intensities for every protein within M19 across all seven

621   subcellular fractions analyzed. Teal lines delineate protein levels in WT samples, purple

622   lines delineate protein levels in MUT samples (averaged across three experimental

623   replicates). Bolded lines demarcate the fitted intensity values for WT and MUT proteins

624   (n=3 independent experiments).

625   (G) Difference in M19 abundance, adjusted for fraction differences and presented as

626    $\log_2$(adjusted module abundance) (WT 13.21 ± 0.003, MUT 12.99 ± 0.003, p=0.0007; n=3

627   independent experiments).

628

629   **Figure 3. Disruption of lysosomal protein networks in SWIP^{P1019R} mutant brain**

630   (A) Simplified schematic of the endo-lysosomal pathway in neurons. (B) Network graph

631   of module 213 (M213). M213 contains multiple lysosomal proteins that are increased in

632   MUT brain. Network attributes: Node size denotes its weighted degree centrality

633   (~importance in module), blue color indicates proteins with altered abundance in MUT

634   brain relative to WT, black edges indicate known protein-protein interactions, and grey-

red edges denote the relative strength of protein covariation within a module (gray = weak, red = strong).

(C) The overall effect of genotype on M213 abundance (WT 12.17 ± 0.002, MUT 12.33 ± 0.002, p=0.0037).

(D) Module 2 (M2) contains multiple lysosomal proteins with increased abundance in MUT brain compared to WT, including CTSS, CTSL, GRN, IDS, MAN2B1. Network attributes depicted: same as in A.

(E) Difference in M2 module abundance, adjusted for fraction differences for all seven of the brain fractions analyzed, and presented as $\log_2$(adjusted module intensity) (WT 13.74 ± 0.001, MUT 13.85 ± 0.0009, p=0.0006). ** p<0.01, ***p<0.001, empirical Bayes quasi-likelihood F-test with Bonferroni correction (C, E).

Figure 4. SWIP^{P1019R} mutant neurons display structural abnormalities in endo-lysosomal compartments *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) Representative 3D reconstruction of WT DIV15 neuron stained for EEA1 (yellow) and MAP2 (magenta).

(C) Representative 3D reconstruction of MUT DIV15 neuron stained for EEA1 (yellow) and MAP2 (magenta).

657   (D) Representative 3D reconstruction of DIV15 WT neuron stained for Cathepsin D (cyan)

658   and MAP2 (magenta).

659   (E) Representative 3D reconstruction of DIV15 MUT neuron stained for Cathepsin D

660   (cyan) and MAP2 (magenta).

661   (F) Graph of the average number of EEA1+ volumes per soma in each image (WT 95.0

662   ± 5.5, n=24 neurons; MUT 103.7 ± 3.7, n=24 neurons; $t_{40.2}$=1.314, p=0.1961).

663   (G) Graph of the average EEA1+ volume size per soma shows larger EEA1+ volumes in

664   MUT neurons (WT 0.15 ± 0.01 μm^3, n=24 neurons; MUT 0.30 ± 0.02 μm^3, n=24 neurons;

665   U=50, p<0.0001).

666   (H) Graph of the average number of Cathepsin D+ volumes per soma illustrates less

667   Cathepsin D+ volumes in MUT neurons (WT 30.4 ± 1.4, n=42; MUT 17.2 ± 0.9, n=42;

668   t_{71}=7.943, p<0.0001).

669   (I) Graph of the average Cathepsin D+ volume size per soma demonstrates larger

670   Cathepsin D+ volumes in MUT neurons (WT 0.54 ± 0.02 μm^3, n=42; MUT 0.69 ± 0.04

671   μm^3, n=42; t_{63}=3.701, p=0.0005).

672   (J) Histogram of EEA1+ volumes illustrate differences in size distributions between MUT

673   and WT neurons.

674   (K) Histogram of CathD+ volumes show differences in size distributions between MUT

675   and WT neurons. Analyses included at least three separate culture preparations. Scale

676   bars, 5 μm (B-E). Data reported as mean ± SEM, error bars are SEM. ***p<0.001,

677   ****p<0.0001, two-tailed t-tests or Mann-Whitney U test (G).

678

Figure 5. SWIP^{P1019R} mutant brains exhibit markers of abnormal endo-lysosomal structures and cell death *in vivo*

(A-B) Representative images of adolescent (P42) WT and MUT motor cortex stained with cleaved caspase-3 (CC3, green).

(C) Anatomical representation of mouse brain with motor cortex highlighted in blue, adapted from Allen Brain Atlas (Oh et al., 2014).

(D-E) Representative images of adult (10 mo) WT and MUT motor cortex stained with CC3 (green).

(H) Graph depicting the normalized percentage 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). We observed no difference in number of nuclei per image between genotypes.

(F, G, I, and J) DAPI co-stained images for (A, B, D, and E, respectively). Scale bar for (A-J), 15 µm.

(K) Representative transmission electron microscopy (TEM) image taken of soma from adult (7mo) WT motor cortex. Arrowheads delineate electron-dense lipofuscin material, Nuc = nucleus.

(L) Representative transmission electron microscopy (TEM) image taken of soma from adult (7mo) MUT motor cortex.

(M) Inset from (K) highlights lysosomal structure in WT soma. Pseudo-colored region depicts lipofuscin area, demarcated as L.

(N) Inset from (L) highlights large lipofuscin deposit in MUT soma (L, pseudo-colored region) with electron-dense and electron-lucent lipid-like (asterisk) components.

(O) Graph of areas of electron-dense regions of interest (ROI) shows increased ROI size in MUT neurons (WT $2.4x10^5 \pm 2.8x10^4$ nm^2, n=50 ROIs; MUT $8.2x10^5 \pm 9.7 x10^4$ nm^2, n=75 ROIs; U=636, p<0.0001).

(P) 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). For (N) and (O), images were taken from multiple TEM grids, prepared from n=3 animals per genotype. Scale bar for all TEM images, 1 μm. Data reported as mean ± SEM, error bars are SEM. ***p<0.001, ****p<0.0001, Kruskal-Wallis test (F), Mann-Whitney U test (O-P).

Figure 6. SWIP^{P1019R} mutant 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 (Tone ON). Two different cohorts of mice were used for age groups P42 (top) and 6.5mo (bottom). Two-way ANOVA analysis of

724   average freezing during Pre-Tone and Tone periods reveal a Genotype x Time effect at

725   P42 (WT n=10, MUT n=10, $F_{1,18}$=4.944, p=0.0392) and 6.5mo (WT n=13, MUT n=11,

726   $F_{1,22}$= 13.61, p=0.0013).

727   (C) Graphs showing the average %time freezing per animal before and during tone

728   presentation. Top: freezing is reduced by 20% in MUT adolescent mice compared to WT

729   littermates (Pre-tone WT 16.5 ± 2.2%, n=10; Pre-tone MUT 13.0 ± 1.8%, n=10;

730   t_{36}=0.8569, p=0.6366; Tone WT 52.8 ± 3.8%, n=10; Tone MUT 38.0 ± 3.6%, n=10;

731   t_{36}=3.539, p=0.0023), Bottom: freezing is reduced by over 30% in MUT adult mice

732   compared to WT littermates (Pre-tone WT 21.1 ± 2.7%, n=13; Pre-tone MUT 23.7 ± 3.8%,

733   n=11; t_{44}=0.4675, p=0.8721; Tone WT 69.7 ± 4.3%, n=13; Tone MUT 53.1 ± 5.2%, n=11;

734   t_{44}=2.921, p=0.0109). Data reported as mean ± SEM, error bars are SEM. *p<0.05,

735   **p<0.01, two-way ANOVAs (B) and Sidak's post-hoc analyses (C).

736

737   **Figure 7. SWIP^{P1019R} mutant mice exhibit surprising motor deficits that are**

738   **confirmed in human patients**

739   (A) Rotarod experimental setup. Mice walked atop a rod rotating at 32rpm for 5 minutes,

740   and the duration of time they remained on the rod before falling was recorded.

741   (B) Line graph of average duration animals remained on the rod per genotype across four

742   trials, with an inter-trial interval of 40 minutes. The same cohort of animals was tested at

743   two different ages, P45 (top) and 5.5 months (bottom). Genotype had a significant effect

744   on task performance at both ages (top, P45: genotype effect, $F_{1,25}$=7.821, p=0.0098.

745   bottom, 5.5mo: genotype effect, $F_{1,23}$= 7.573, p=0.0114).

746   (C) Graphs showing the average duration each animal remained on the rod across trials.

747   At both ages, the MUT mice exhibited an almost 50% reduction in their ability to remain

748   on the rod (Top, P45: WT 169.9 ± 25.7 s, MUT 83.8 ± 15.9 s, U=35, p=0.0054. Bottom,

749   5.5mo: WT 135.9 ± 20.9 s, MUT 66.7 ± 9.5 s, t_{18}=3.011, p=0.0075).

750   (D) TreadScan task. Mice walked on a treadmill for 20 s while their gate was captured

751   with a high-speed camera. Diagrams of gait parameters measured in (E-G) are shown

752   below the TreadScan apparatus.

753   (E) Average swing time per stride for hindlimbs. At P45 (top), there is no significant

754   difference in rear swing time (WT 156.2 ± 22.4 ms, MUT 132.3 ± 19.6 ms, U=83,

755   p=0.7203). At 5.5mo (bottom), MUT mice display significantly longer rear swing time (WT

756   140 ± 6.2 ms, MUT 252.0 ± 21.6 ms, t_{12}=4.988, p=0.0003).

757   (F) Average stride length for hindlimbs. At P45 (top), there is no significant difference in

758   stride length (WT 62.3 ± 2.0 mm, MUT 60.5 ± 2.1 mm, U=75, p=0.4583). At 5.5mo

759   (bottom), MUT mice take significantly longer strides with their hindlimbs (WT 60.8 ± 0.8

760   mm, MUT 73.6 ± 2.7 mm, $t_{11.7}$=4.547, p=0.0007).

761   (G) Average homologous coupling for front and rear limbs. Homologous coupling is 0.5

762   when the left and right feet are completely out of phase. At P45 (top), WT and MUT mice

763   exhibit normal homologous coupling (WT 0.48 ± 0.005, MUT 0.48 ± 0.004, U=76.5,

764   p=0.4920). At 5.5 mo (bottom), MUT mice display decreased homologous coupling,

765   suggestive of abnormal gait symmetry (WT 0.48 ± 0.003, MUT 0.46 ± 0.004, $t_{18.8}$=3.715,

766   p=0.0015). At P45: n=14 WT, n=13 MUT; At 5.5mo: n=14 WT, n=11 MUT.

767   (H) Table of motor findings in clinical exam of human patients with homozygous

768   SWIP[P1019R] mutation. All patients exhibit motor dysfunction (+ = symptom present). Data

769 reported as mean ± SEM, error bars are SEM. *p<0.05, **p<0.01, ***p<0.001,

770 ****p<0.0001, two-way repeated measure ANOVAs (B), Mann-Whitney U tests and two-

771 tailed t-tests (C-G).

772

773 **Figure 8. Model of neuronal endo-lysosomal pathology in SWIP^{P1019R} mutant mice**

774 (A) Wild-type WASH function in mouse brain. Under normal conditions, the WASH

775 complex interacts with many endosomal proteins and cytoskeletal regulators, such as the

776 Arp2/3 complex. These interactions enable restructuring of the endosome surface (actin

777 in gray) and allow for cargo segregation and scission of vesicles. Substrates are

778 transported to the late endosome for lysosomal degradation, to the Golgi network for

779 modification, or to the cell surface for recycling.

780 (B) Loss of WASH function leads to increased lysosomal degradation in mouse brain.

781 Destabilization of the WASH complex leads to enlarged endosomes and lysosomes, with

782 increased substrate accumulation at the lysosome. This suggests an increase in flux

783 through the endo-lysosomal pathway, possibly as a result of mis-localized endosomal

784 substrates.

785 (C) Wild-type mice exhibit normal motor function.

786 (D) SWIP^{P1019R} mutant mice display progressive motor dysfunction in association with

787 these subcellular alterations.

788

789 **STAR★METHODS**

790 Detailed methods are provided in the online version of this paper and include the following:

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826

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828

829 **RESOURCE AVAILABILITY**

830 **Lead Contact**

831 Further information and requests for resources and reagents should be directed to and

832 will be fulfilled by the Lead Contact, Scott Soderling (scott.soderling@duke.edu).

833 **Materials Availability**

834 Plasmids generated by this study are available upon request from corresponding author

835 Scott H. Soderling (scott.soderling@duke.edu).

836 **Data and Code Availability**

837   The data and source code used in this study are available online at

838   https://github.com/twesleyb/SwipProteomics.

839

840   **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

841   **Animals**

842   We generated *Washc4* mutant (SWIP^{P1019R}) mice in collaboration with the Duke

843   Transgenic Core Facility to mimic the *de novo* human variant at amino acid 1019 of

844   human *WASHc4.* A CRISPR-induced CCT>CGT point mutation was introduced into exon

845   29 of *Washc4*. 50ng/µl of the sgRNA (5'-ttgagaatactcacaagaggagg-3'), 100ng/µl Cas9

846   mRNA, and 100ng/µl of a repair oligo containing the C>G mutation were injected into the

847   cytoplasm of B6SJLF1/J mouse embryos (Jax #100012) (see Supplemental Table 4 for

848   the sequence of the repair oligo). Mice with germline transmission were then backcrossed

849   into a C57BL/ 6J background (Jax #000664). At least 5 backcrosses were obtained before

850   animals were used for behavior. We bred heterozygous SWIP^{P1019R} mice together to

851   obtain age-matched mutant and wild-type genotypes for cell culture and behavioral

852   experiments. Genetic sequencing was used to screen for germline transmission of the

853   C>G point mutation (*FOR:* 5'-tgcttgtagatgtttttcct-3', *REV*: 5'-gttaacatgatcctatggcg-3'). All

854   mice were housed in the Duke University's Division of Laboratory Animal Resources or

855   Behavioral Core facilities at 2-5 animals/cage on a 14:10h light:dark cycle. All

856   experiments were conducted with a protocol approved by the Duke University Institutional

857   Animal Care and Use Committee in accordance with NIH guidelines.

858   **Cell Culture**

859   HEK293T cells (ATCC #CRL-11268) were purchased from the Duke Cell Culture

860   facility. and were tested for mycoplasma contamination. HEK239T cells were used for

861   coimmunoprecipitation experiments and preparation of AAV viruses.

862   **Primary Neuronal Culture**

863   Primary neuronal cultures were prepared from P0 mouse cortex. P0 mouse pups

864   were rapidly decapitated and cortices were dissected and kept individually in 5ml

865   Hibernate A (Thermo #A1247501) supplemented with 2% B27(Thermo #17504044) at

866   4ºC overnight to allow for individual animal genotyping before plating. Neurons were then

867   treated with Papain (Worthington #LS003120) and DNAse (VWR #V0335)-supplemented

868   Hibernate A for 18min at 37ºC and washed twice with plating medium (plating medium:

869   Neurobasal A (Thermo #10888022) supplemented with 10% horse serum, 2% B-27, and

870   1% GlutaMAX (Thermo #35050061)), and triturated before plating at 250,000 cells/well

871   on poly-L-lysine-treated coverslips (Sigma #P2636) in 24-well plates. Plating medium was

872   replaced with growth medium (Neurobasal A, 2% B-27, 1% GlutaMAX) 2 hours later. Cell

873   media was supplemented and treated with AraC at DIV5 (5uM final concentration/well).

874   Half-media changes were then performed every 4 days.

875   **Human Subjects**

876   We retrospectively analyzed clinical findings from seven children with homozygous

877   *WASHC4*[c.3056C>G] mutations (obtained by Dr. Rajab in 2010 at the Royal Hospital, Muscat,

878   Oman). The original report of these human subjects and consent for data use can be

879   found in (Ropers et al., 2011).

880

881   **METHOD DETAILS**

Plasmid DNA 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 al(Kim et al., 2016). An hSyn1-solubleBioID2-HA construct was created similarly, by removing Actin Chromobody from the above construct. Oligonucleotide sequences are reported in Supplementary Table 4. Sequences of the plasmid DNA constructs are available online (see Key Resources Table).

AAV Viral Preparation

AAV preparations were performed as described previously(Uezu et al., 2016). The day before transfection, HEK293T cells were plated at a density of 1.5×10^7 cells per $15cm^2$ plate in DMEM media with 10% fetal bovine serum and 1% Pen/Strep (Thermo #11965-092, Sigma #F4135, Thermo #15140-122). Six HEK293T $15cm^2$ 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

905 mixed in OptiMEM with PEI-MAX (final concentration 80µg/ml, Polysciences #24765).

906 2ml of this solution were then added dropwise to each of the 6 HEK293T 15cm^2 plates.

907 Eight hours later, the media was replaced with 20ml DMEM+10%FBS. 72 hours post-

908 transfection, cells were scraped and collected in the media, pooled, and centrifuged at

909 1,500rpm for 5min at RT. The final pellet from the 6 cell plates was resuspended in 5ml

910 of cell lysis buffer (15 mM NaCl, 5 mM Tris-HCl, pH 8.5), and freeze-thawed three times

911 using an ethanol/dry ice bath. The lysate was then treated with 50U/ml of Benzonase

912 (Novagen #70664), for 30min in a 37ºC water bath, vortexed, and then centrifuged at

913 4,500rpm for 30min at 4ºC. The resulting supernatant containing AAV particles was added

914 to the top of an iodixanol gradient (15%, 25%, 40%, 60% top to bottom) in an Optiseal

915 tube (Beckman Coulter #361625). The gradient was then centrifuged using a Beckman

916 Ti-70 rotor in a Beckman XL-90 ultracentrifuge at 67,000rpm for 70min, 18ºC. The purified

917 viral solution was extracted from the 40%/60% iodixanol interface using a syringe, and

918 placed into an Amicon 100kDa filter unit (#UFC910024). The viral solution was washed

919 in this filter 3 times with 1X ice-cold PBS by adding 5ml of PBS and centrifuging at

920 4,900rpm for 45min at 4ºC to obtain a final volume of approximately 200µl of concentrated

921 virus that was aliquoted into 5-10µl aliquots and stored at -80ºC until use.

922 **Immunocytochemistry**

923 Primary antibodies: Rabbit anti-EEA1 (Cell Signaling Technology #C45B10,

924 1:500), Rat anti-CathepsinD (Novus #204712, 1:250), Guinea Pig anti-MAP2 (Synaptic

925 Systems #188004, 1:500)

926 Secondary antibodies: Goat anti-Rabbit Alexa Fluor 568 (Invitrogen #A11036,

927 1:1000), Goat anti-Guinea Pig Alexa Fluor 488 (Invitrogen #A11073, 1:1000), Goat anti-

928 Rat Alexa Fluor 488 (Invitrogen #A11006, 1:1000), Goat anti-Guinea Pig Alexa Fluor 555

929 (Invitrogen #A21435, 1:1000)

930   At DIV15, neurons were fixed for 15 minutes using ice-cold 4%PFA/4% sucrose in

931 1X PBS, pH 7.4 (for EEA1 staining), or 30 minutes with 50% Bouin's solution/4% sucrose

932 (for CathepsinD staining, Sigma #HT10132), pH 7.4(Cheng et al., 2018). Fixed neurons

933 were washed with 1X PBS, then permeabilized with 0.25% TritonX-100 in PBS for 8

934 minutes at room temperature (RT), and blocked with 5%normal goat serum/0.2%Triton-

935 X100 in PBS (blocking buffer) for 1 hour at RT with gentle rocking. For EEA1/MAP2

936 staining, samples were incubated with primary antibodies diluted in blocking buffer at RT

937 for 1 hour. For CathepsinD/MAP2 staining, samples were incubated with primary

938 antibodies diluted in blocking buffer overnight at 4ºC. For both conditions, samples were

939 washed three times with 1X PBS, and incubated for 30min at RT with secondary

940 antibodies, protected from light. After secondary antibody staining, coverslips were

941 washed three times with 1X PBS, and mounted with FluoroSave mounting solution

942 (Sigma #345789). See antibody section for primary and secondary antibody

943 concentrations.

944 **Immunohistochemistry**

945   Primary antibodies: Rabbit anti-Cleaved Caspase-3 (Cell Signaling Technology

946 #9661, 1:2000), Mouse anti-Calbindin (Sigma #C9848, 1:2000), Rat anti-HA 3F10 (Sigma

947 #12158167001, 1:500)

948   Secondary antibodies: Donkey anti-Rabbit Alexa Fluor 488 (Invitrogen #A21206,

949 1:2000), Goat anti-Mouse Alexa Fluor 594 (Invitrogen #A11032, 1:2000), Goat anti-Rat

950 Alexa Fluor 488 (Invitrogen #A11006, 1:5000), Streptavidin Alexa Fluor 594 conjugate

951 (Invitrogen #S32356, 1:5000), 4′,6-diamidino-2-phenylindole (DAPI, Sigma #D9542,

952 1:1000 for 10min at RT)

953   Mice were deeply anesthetized with isoflurane and then transcardially perfused

954 with ice-cold heparinized PBS (25U/ml) by gravity flow. After clearing of liver and lungs

955 (~2min), perfusate was switched to ice-cold 4% PFA in 1X PBS (pH 7.4) for 15 minutes.

956 Brains were dissected, post-fixed in 4%PFA overnight at 4ºC, and then cryoprotected in

957 30% sucrose/1X PBS for 48hr at 4ºC. Fixed brains were then mounted in OTC (Sakura

958 TissueTek #4583) and stored at -20ºC until cryosectioning. Every third sagittal section

959 (30 µm thickness) was collected from the motor cortex and striatal regions. Free-floating

960 sections were then permeabilized with 1%TritonX-100 in 1X PBS at RT for 2 hr, and

961 blocked in 1X blocking solution (Abcam #126587) diluted in 0.2%TritonX-100 in 1X PBS

962 for 1hr at RT. Sections were then incubated in primary antibodies diluted in the 1X

963 blocking solution for two overnights at 4ºC. After three washes with 0.2%TritonX-100 in

964 1X PBS, the sections were then incubated in secondary antibodies diluted in 1X blocking

965 buffer for one overnight at 4ºC. Sections were then washed four times with 0.2%TritonX-

966 100 in 1X PBS at RT, and mounted onto coverslips with FluoroSave mounting solution

967 (Sigma #345789).

968 **Western Blotting**

969   Primary antibodies: Rabbit anti-Strumpellin (Santa Cruz #sc-87442, 1:500), Rabbit

970 anti-WASH1 c-terminal (Sigma #SAB4200373, 1:500), Mouse anti-Beta Tubulin III

971 (Sigma #T8660, 1:10,000), Mouse anti-HA (BioLegend #MMS-101P, 1:5000)

972   Secondary antibodies: Donkey anti-Rabbit-HRP (GE Life Sciences #NA934,

973 1:5,000), Goat anti-mouse-HRP (GE Life Sciences #NA931, 1:5000)

974   Ten micrograms of each sample were electrophoresed through a 12-well, 4-20%

975   SDS-PAGE gel (Bio-Rad #4561096) at 100V for 1hr at RT, transferred onto a

976   nitrocellulose membrane (GE Life Sciences #GE10600002) at 100V for 70min at RT on

977   ice, and blocked with 5% nonfat dry milk in TRIS-buffered saline containing 0.05% Tween-

978   20 (TBST, pH 7.4). Gels were saved for Coomassie staining at RT for 30 min. Membranes

979   were probed with one primary antibody at a time for 24hr at 4ºC, then washed four times

980   with TBST at RT before incubating with the corresponding species-specific secondary

981   antibody at RT for 1hr. Membranes were washed with TBST, and then enhanced

982   chemiluminescence (ECL) substrate was added (Thermo Fischer #32109). Membranes

983   were exposed to autoradiography films and scanned with an Epson 1670 at 600dpi. We

984   probed each membrane with one antibody at a time, then stripped the membrane with

985   stripping buffer (Thermo Fischer #21059) for 10min at RT, and then blocked for 1hr at RT

986   before probing with the next antibody. Order of probes: Strumpellin, then β-Tubulin, then

987   WASH1. We determined the optical density of the bands using Image J software (NIH).

988   Data obtained from three independent experiments were plotted and statistically analyzed

989   using GraphPad Prism (version 8) software.

990   **Immunoprecipitation**

991   HEK293T cells were transfected with pmCAG-SWIP-WT-HA or pmCAG-SWIP-

992   MUT-HA constructs for three days, as previously described(Mason et al., 2011). Cells

993   were lysed with lysis buffer (25mM HEPES, 150mM NaCl, 1mM EDTA, 1% NonidetP-40,

994   pH 7.4) containing protease inhibitors (5mM NaF, 1mM orthovanadate, 1mM AEBSF, and

995   2 μg/mL leupeptin/pepstatin) and centrifuged at 1,700g for 5 min. Collected supernatant

996   was incubated with 30μl of pre-washed anti-HA agarose beads (Sigma #A2095) on a

997   sample rotator (15 rpm) for 2 hrs at 4ºC. Beads were then washed 3 times with lysis

998   buffer, and sample buffer was added before subjecting to immunoblotting as described

999   above. The protein-transferred membrane was probed individually for WASH1,

1000   Strumpellin, and HA. Data were collected from four separate preparations of WT and MUT

1001   conditions.

1002   **Electron Microscopy**

1003   Adult (7mo) WT and MUT SWIP^{P1019R} mice were deeply anesthetized with

1004   isoflurane and then transcardially perfused with warmed heparinized saline (25U/ml

1005   heparin) for 4 minutes, followed by ice-cold 0.15M cacodylate buffer pH 7.4 containing

1006   2.5% glutaraldehyde (Electron Microscopy Sciences #16320), 3% paraformaldehyde,

1007   and 2mM CaCl$_2$ for 15 minutes. Brain samples were dissected and stored on ice in the

1008   same fixative for 2 hours before washing in 0.1M sodium cacodylate buffer (3 changes

1009   for 15 minutes each). Samples were then post-fixed in 1.0% OsO$_4$ in 0.1 M Sodium

1010   cacodylate buffer for 1 hour on a rotator. Samples were then washed in 3, 15-minute

1011   changes of 0.1M sodium cacodylate. Samples were then placed into *en bloc* stain (1%

1012   uranyl acetate) overnight at 4°C. Subsequently, samples were dehydrated in a series of

1013   ascending acetone concentrations including 50%, 70%, 95%, and 100% for three cycles

1014   with 15 minutes incubation at each concentration change. Samples were then placed in

1015   a 50:50 mixture of epoxy resin (Epon) and acetone overnight on a rotator. This solution

1016   was then replaced twice with 100% fresh Epon for at least 2 hours at room temperature

1017   on a rotator. Samples were embedded with 100% Epon resin in BEEM capsules (Ted

1018   Pella) for 48 hours at 60°C. Samples were ultrathin sectioned to 60-70nm on a Reichert

1019   Ultracut E ultramicrotome. Harvested grids were then stained with 2% uranyl acetate in

1020 50% ethanol for 30 minutes and Sato's lead stain for 1 min. Micrographs were acquired

1021 using a Phillips CM12 electron microscope operating at 80Kv, at 1700x magnification.

1022 Micrographs were analyzed in Adobe Photoshop 2019, using the "magic wand" tool to

1023 demarcate and measure the area of electron-dense and electron-lucent regions of

1024 interest (ROIs). Statistical analyses of ROI measurements were performed in GraphPad

1025 Prism (version 8) software. The experimenter was blinded to genotype for image

1026 acquisition and analysis.

1027 **iBioID Sample Preparation**

1028 AAV2/9 viral probes, hSyn1-WASH1-BioID2-HA or hSyn1-solubleBioID2-HA, were

1029 injected into wild-type CD1 mouse brains using a Hamilton syringe (#7635-01) at age P0-

1030 P1 to ensure viral spread throughout the forebrain(Glascock et al., 2011). 15 days post-

1031 viral injection, biotin was subcutaneously administered at 24mg/kg for seven consecutive

1032 days for biotinylation of proteins in proximity to BioID2 probes. Whole brains were

1033 extracted on the final day of biotin injections, snap frozen, and stored in liquid nitrogen

1034 until protein purification. Seven brains were used for protein purification of each probe,

1035 and each purification was performed three times independently (21 brains total for

1036 WASH1-BioID2, 21 for solubleBioID2).

1037 We performed all homogenization and protein purification on ice. A 2ml Dounce

1038 homogenizer was used to individually homogenize each brain in a 1:1 solution of Lysis-

1039 R:2X-RIPA buffer solution with protease inhibitors (Roche cOmplete tablets

1040 #11836153001). Each sample was sonicated three times for 7 seconds and then

1041 centrifuged at 5000g for 5min at 4ºC. Samples were transferred to Beckman Coulter 1.5ml

1042 tubes (#344059), and then spun at 45,000rpm in a Beckman Coulter tabletop

1043   ultracentrifuge (TLA-55 rotor) for 1hr at 4ºC. SDS was added to supernatants (final 1%)

1044   and samples were then boiled for 5min at 95ºC. We next combined supernatants from

1045   the same condition together (WASH1-BioID2 vs. solubleBioID2) in 15ml conical tubes to

1046   rotate with 30µl high-capacity NeutrAvidin beads overnight at 4ºC (Thermo #29204).

1047   The following day, all steps were performed under a hood with keratin-free

1048   reagents. Samples were spun down at 6000rpm, 4ºC for 5min to pellet the beads and

1049   remove supernatant. The pelleted beads then went through a series of washes, each for

1050   10 min at RT with 500ul of solvent, and then spun down on a tabletop centrifuge to pellet

1051   the beads for the next wash. The washes were as follows: 2% SDS twice, 1% TritonX100-

1052   1%deoxycholate-25mM $LiCl_2$ once, 1M NaCL twice, 50mM Ammonium Bicarbonate

1053   (Ambic) five times. Beads were then mixed 1:1 with a 2X Laemmli sample buffer that

1054   contained 3mM biotin/50mM Ambic, boiled for 5 mins at 95ºC, vortexed three times, and

1055   then biotinylated protein supernatants were stored at -80ºC until LC-MS/MS.

1056   **LC-MS/MS for iBioID**

1057   We gave the Duke Proteomics and Metabolomics Shared Resource (DPMSR) six

1058   eluents from streptavidin resins (3 x WASH1-BioID2, 3 x solubleBioID2), stored on dry

1059   ice. Samples were reduced with 10 mM dithiolthreitol for 30 min at 80ºC and alkylated

1060   with 20 mM iodoacetamide for 30 min at room temperature. Next, samples were

1061   supplemented with a final concentration of 1.2% phosphoric acid and 256 µL of S-Trap

1062   (Protifi) binding buffer (90% MeOH/100mM TEAB). Proteins were trapped on the S-Trap,

1063   digested using 20 ng/µl sequencing grade trypsin (Promega) for 1 hr at 47ºC, and eluted

1064   using 50 mM TEAB, followed by 0.2% FA, and lastly using 50% ACN/0.2% FA. All

1065   samples were then lyophilized to dryness and resuspended in 20 µL 1%TFA/2%

1066   acetonitrile containing 25 fmol/μL yeast alcohol dehydrogenase (UniProtKB P00330;

1067   ADH_YEAST). From each sample, 3 μL was removed to create a pooled QC sample

1068   (SPQC) which was run analyzed in technical triplicate throughout the acquisition period.

1069   Quantitative LC/MS/MS was performed on 2 μL of each sample, using a

1070   nanoAcquity UPLC system (Waters) coupled to a Thermo QExactive HF-X high resolution

1071   accurate mass tandem mass spectrometer (Thermo) via a nanoelectrospray ionization

1072   source. Briefly, the sample was first trapped on a Symmetry C18 20 mm × 180 μm

1073   trapping column (5 μl/min at 99.9/0.1 v/v water/acetonitrile), after which the analytical

1074   separation was performed using a 1.8 μm Acquity HSS T3 C18 75 μm × 250 mm column

1075   (Waters) with a 90-min linear gradient of 5 to 30% acetonitrile with 0.1% formic acid at a

1076   flow rate of 400 nanoliters/minute (nL/min) with a column temperature of 55ºC. Data

1077   collection on the QExactive HF-X mass spectrometer was performed in a data-dependent

1078   acquisition (DDA) mode of acquisition with a r=120,000 (@ m/z 200) full MS scan from

1079   m/z 375 – 1600 with a target AGC value of $3e^6$ ions followed by 30 MS/MS scans at

1080   r=15,000 (@ m/z 200) at a target AGC value of $5e^4$ ions and 45 ms. A 20s dynamic

1081   exclusion was employed to increase depth of coverage. The total analysis cycle time for

1082   each sample injection was approximately 2 hours.

1083   **LOPIT-DC Subcellular Fractionation**

1084   We performed three independent fractionation experiments with one adult SWIP

1085   mutant brain and one WT mouse brain fractionated in each experiment. Each mouse was

1086   sacrificed by isoflurane inhalation and its brain was immediately extracted and placed into

1087   a 2ml Dounce homogenizer on ice with 1ml isotonic TEVP homogenization buffer (320mM

1088   sucrose, 10mM Tris base, 1mM EDTA, 1mM EGTA, 5mM NaF, pH7.4(Hallett et al.,

1089   2008)). A cOmplete mini protease inhibitor cocktail tablet (Sigma #11836170001) was

1090   added to a 50ml TEVP buffer aliquot immediately before use. Brains were homogenized

1091   for 15 passes with a Dounce homogenizer to break the tissue, and then this lysate was

1092   brought up to a 5ml volume with additional TEVP buffer. Lysates were then passed

1093   through a 0.5ml ball-bearing homogenizer for two passes (14 µm ball, Isobiotec) to

1094   release organelles. Final brain lysate volumes were approximately 7.5ml each. Lysates

1095   were then divided into replicate microfuge tubes (Beckman Coulter #357448) to perform

1096   differential centrifugation, following Geladaki et. al's LOPIT-DC protocol(Geladaki et al.,

1097   2019). Centrifugation was carried out at 4ºC in a tabletop Eppendorf 5424 centrifuge for

1098   spins at 200g, 1,000g, 3,000g, 5,000g, 9,000g, 12,000g, and 15,000g. To isolate the final

1099   three fractions, a tabletop Beckman TLA-100 ultracentrifuge with a TLA-55 rotor was used

1100   at 4ºC with speeds of: 30,000g, 79,000g, and 120,000g, respectively. Samples were kept

1101   on ice at all times and pellets were stored at -80ºC. Pellets from seven fractions (5,000g-

1102   120,000g) were used for proteomic analyses.

1103   **16-plex TMT LC-MS/MS**

1104   The Duke Proteomics and Metabolomics Shared Resource (DPMSR) processed

1105   and prepared fraction pellets from all 42 frozen samples simultaneously (7 fractions per

1106   brain from 3 WT and 3 MUT brains). Due to volume constraints, each sample was split

1107   into 3 tubes, for a total of 126 samples, which were processed in the following manner:

1108   100µL of 8M Urea was added to the first aliquot then probe sonicated for 5 seconds with

1109   an energy setting of 30%. This volume was then transferred to the second and then third

1110   aliquot after sonication in the same manner. All tubes were centrifuged at 10,000g and

1111   any residual volume from tubes 1 and 2 were added to tube 3. Protein concentrations

1112   were determined by BCA on the supernatant in duplicate (5 µL each assay). Total protein

1113   concentrations for each replicate ranged from 1.1 mg/mL to 7.8 mg/mL with total protein

1114   quantities ranging from 108.3 to 740.81 µg. 60 µg of each sample was removed and

1115   normalized to 52.6µL with 8M Urea and 14.6µL 20% SDS. Samples were reduced with

1116   10 mM dithiolthreitol for 30 min at 80ºC and alkylated with 20 mM iodoacetamide for 30

1117   min at room temperature. Next, they were supplemented with 7.4 µL of 12% phosphoric

1118   acid, and 574 µL of S-Trap (Protifi) binding buffer (90% MeOH/100mM TEAB). Proteins

1119   were trapped on the S-Trap, digested using 20 ng/µl sequencing grade trypsin (Promega)

1120   for 1 hr at 47ºC, and eluted using 50 mM TEAB, followed by 0.2% FA, and lastly using

1121   50% ACN/0.2% FA. All samples were then lyophilized to dryness.

1122   Each sample was resuspended in 120 µL 200 mM triethylammonium bicarbonate,

1123   pH 8.0 (TEAB). From each sample, 20µL was removed and combined to form a pooled

1124   quality control sample (SPQC). Fresh TMTPro reagent (0.5 mg for each 16-plex reagent)

1125   was resuspended in 20 µL 100% acetonitrile (ACN) and was added to each sample.

1126   Samples were incubated for 1 hour at RT. After the 1-hour reaction, 5 µL of 5%

1127   hydroxylamine was added and incubated for 15 minutes at room temperature to quench

1128   the reaction. Each 16-plex TMT experiment consisted of the WT and MUT fractions from

1129   one mouse, as well as the 2 SPQC samples. Samples corresponding to each experiment

1130   were concatenated and lyophilized to dryness.

1131   Samples were resuspended in 800µL 0.1% formic acid. 400µg was fractionated

1132   into 48 unique high pH reversed-phase fractions using pH 9.0 20 mM Ammonium formate

1133   as mobile phase A and neat acetonitrile as mobile phase B. The column used was a 2.1

1134   mm x 50 mm XBridge C18 (Waters) and fractionation was performed on an Agilent 1100

1135 HPLC with G1364C fraction collector. Throughout the method, the flow rate was 0.4

1136 mL/min and the column temperature was 55ºC. The gradient method was set as follows:

1137 0 min, 3%B; 1 min, 7% B; 50 min, 50%B; 51 min, 90% B; 55 min, 90% B; 56 min, 3% B;

1138 70 min, 3% B. 48 fractions were collected in equal time segments from 0 to 52 minutes,

1139 then concatenated into 12 unique samples using every 12th fraction. For instance,

1140 fraction 1, 13, 25, and 37 were combined, fraction 2, 14, 26, and 38 were combined, etc.

1141 Fractions were frozen and lyophilized overnight. Samples were resuspended in 66 μL

1142 1%TFA/2% acetonitrile prior to LC-MS analysis.

1143 Quantitative LC/MS/MS was performed on 2 μL (1 μg) of each sample, using a

1144 nanoAcquity UPLC system (Waters) coupled to a Thermo Orbitrap Fusion Lumos high

1145 resolution accurate mass tandem mass spectrometer (Thermo) equipped with a FAIMS

1146 Pro ion-mobility device via a nanoelectrospray ionization source. Briefly, the sample was

1147 first trapped on a Symmetry C18 20 mm × 180 μm trapping column (5 μl/min at 99.9/0.1

1148 v/v water/acetonitrile), after which the analytical separation was performed using a 1.8

1149 μm Acquity HSS T3 C18 75 μm × 250 mm column (Waters) with a 90-min linear gradient

1150 of 5 to 30% acetonitrile with 0.1% formic acid at a flow rate of 400 nanoliters/minute

1151 (nL/min) with a column temperature of 55ºC. Data collection on the Fusion Lumos mass

1152 spectrometer was performed for three different compensation voltages (CV: -40v, -60v, -

1153 80v). Within each CV, a data-dependent acquisition (DDA) mode of acquisition with a

1154 r=120,000 (@ m/z 200) full MS scan from m/z 375 – 1600 with a target AGC value of $4e^5$

1155 ions was performed. MS/MS scans were acquired in the Orbitrap at r=50,000 (@ m/z 200)

1156 from m/z 100 with a target AGC value of $1e^5$ and max fill time of 105 ms. The total cycle

1157 time for each CV was 1s, with total cycle times of 3 sec between like full MS scans. A 45s

1158 dynamic exclusion was employed to increase depth of coverage. The total analysis cycle

1159 time for each sample injection was approximately 2 hours.

1160 Following UPLC-MS/MS analyses, data were imported into Proteome Discoverer

1161 2.4 (Thermo Scientific). The MS/MS data were searched against a SwissProt Mouse

1162 database (downloaded November 2019) plus additional common contaminant proteins,

1163 including yeast alcohol dehydrogenase (ADH), bovine casein, bovine serum albumin, as

1164 well as an equal number of reversed-sequence "decoys" for FDR determination. Mascot

1165 Distiller and Mascot Server (v 2.5, Matrix Sciences) were utilized to produce fragment ion

1166 spectra and to perform the database searches. Database search parameters included

1167 fixed modification on Cys (carbamidomethyl) and variable modification on Met (oxidation),

1168 Asn/Gln (deamindation), Lys (TMTPro) and peptide N-termini (TMTPro). Data were

1169 searched at 5 ppm precursor and 0.02 product mass accuracy with full trypsin enzyme

1170 rules. Reporter ion intensities were calculated using the Reporter Ions Quantifier

1171 algorithm in Proteome Discoverer. Percolator node in Proteome Discoverer was used to

1172 annotate the data at a maximum 1% protein FDR.

1173 **Mouse Behavioral Assays**

1174 Behavioral tests were performed on age-matched WT and homozygous

1175 SWIP^{P1019R} mutant littermates. Male and female mice were used in all experiments.

1176 Testing was performed at two time points: P42-55 days old as a young adult age, and 5.5

1177 months old as mid-adulthood, so that we could compare disease progression in this

1178 mouse model to human patients(Ropers et al., 2011). The sequence of behavioral testing

1179 was: Y-maze (to measure working memory), object novelty recognition (to measure short-

1180 term and long-term object recognition memory), TreadScan (to assess gait), and steady-

1181 speed rotarod (to assess motor control and strength) for 40-55 day old mice. Testing was

1182 performed over 1.5 weeks, interspersed with rest days for acclimation. This sequence

1183 was repeated with the same cohort at 5.5-6 months old, with three additional measures

1184 added to the end of testing: fear conditioning (to assess associative fear memory), a

1185 hearing test (to measure tone response), and a shock threshold test (to assess

1186 somatosensation). Of note, a separate, second cohort of mice was evaluated for fear

1187 conditioning, hearing, and shock threshold testing at adolescence. After each trial,

1188 equipment was cleaned with Labsan to remove residual odors. The experimenter was

1189 blinded to genotype for all behavioral analyses.

1190 **Y-maze**

1191 Working memory was evaluated by measuring spontaneous alternations in a 3-

1192 arm Y-maze under indirect illumination (80-90 lux). A mouse was placed in the center of

1193 the maze and allowed to freely explore all arms, each of which had different visual cues

1194 for spatial recognition. Trials were 5 min in length, with video data and analyses captured

1195 by EthoVision XT 11.0 software (Noldus Information Technology). Entry to an arm was

1196 define as the mouse being >1 body length into a given arm. An alternation was defined

1197 as three successive entries into each of the different arms. Total % alternation was

1198 calculated as the total number of alternations/the total number of arm entries minus 2

1199 x100.

1200 **Novel Object Recognition**

1201 One hour before testing, mice were individually exposed to the testing arena (a 48

1202 x 22 x 18cm white opaque arena) for 10min under 80-100lux illumination without any

1203 objects. The test consisted of three phases: training (day 1), short-term memory test

1204 (STM, day 1), and long-term memory test (LTM, day 2). For the training phase, two

1205 identical objects were placed 10 cm apart, against opposing walls of the arena. A mouse

1206 was placed in the center of the arena and given full access to explore both objects for 5

1207 min and then returned to its home cage. For STM testing, one of the training objects

1208 remained (the now familiar object), and a novel object replaced one of the training objects

1209 (similar in size, different shape). The mouse was returned to the arena 30 minutes after

1210 the training task and allowed to explore freely for 5 mins. For LTM testing, the novel object

1211 was replaced with another object, and the familiar object remained unchanged. The LTM

1212 test was also 5 min in duration, conducted 24hr after the training task. Behavior was

1213 scored using Ethovision 11.0 XT software (Noldus) and analyzed by a blind observer.

1214 Object contact was defined as the mouse's nose within 1 cm of the object. We analyzed

1215 both number of nose contacts with each object and duration of contacts. Preference

1216 scores were calculated as (duration $contact_{novel}$ - duration $contact_{familiar}$) / total duration

1217 $contact_{novel+familiar}$. Positive scores signified a preference for the novel object; whereas,

1218 negative scores denoted a preference for the familiar object, and scores approaching

1219 zero indicated no preference.

1220 **TreadScan**

1221 A TreadScan forced locomotion treadmill system (CleversSys Inc, Reston,

1222 Virginia) was used for gait recording and analysis. Each mouse was recorded walking on

1223 a transparent treadmill at 45 days old, and again at 5.5 months old. Mice were acclimated

1224 to the treadmill chamber for 1 minute before the start of recording to eliminate exploratory

1225 behavior confounding normal gait. Trials were 20 seconds in length, with mice walking at

1226 speeds between 13.83 and 16.53 cm/sec (P45 WT average 15.74 cm/s; P45 MUT

1227 average 15.80 cm/s; 5.5mo WT average 15.77 cm/s; 5.5mo MUT average 15.85 cm/s).

1228 A high-speed digital camera attached to the treadmill captured limb movement at a frame

1229 rate of 100 frames/second. We used TreadScan software (CleversSys) and

1230 representative WT and MUT videos to generate footprint templates, which were then used

1231 to identify individual paw profiles for each limb. Parameters such as stance time, swing

1232 time, step length, track width, and limb coupling were recorded for the entire 20 sec

1233 duration for each animal. Output gait tracking was verified manually by a blinded

1234 experimenter to ensure consistent limb tracking throughout the duration of each video.

1235 **Steady Speed Rotarod**

1236 A 5-lane rotarod (Med Associates, St. Albans, VT) was used for steady-speed

1237 motor analysis. The rod was run at a steady speed of 32rpm for four, 5-minute trials, with

1238 a 40-minute inter-trial interval. We recorded mouse latency to fall by infrared beam break,

1239 or manually for any mouse that completed two or more rotations on the rod without

1240 walking. Mice were randomized across lanes for each trial.

1241 **Fear Conditioning**

1242 Animals were examined in contextual and cued fear conditioning as described by

1243 Rodriguiz and Wetsel(Rodriguiz and Wetsel, 2006). Two separate cohorts of mice were

1244 used testing the two age groups. A three-day testing paradigm was used to assess

1245 memory: conditioning on day 1, context testing 24-hr post-conditioning on day 2, and cued

1246 tone testing 48hr post-conditioning on day 3. All testing was conducted in fear

1247 conditioning chambers (Med Associates). In the conditioning phase, mice were first

1248 acclimated to the test chamber for two minutes under ~100 lux illumination. Then a

1249 2900Hz, 80dB tone (conditioned stimulus, CS) played for 30 sec, which terminated with

1250   a paired 0.4mA, 2 sec scrambled foot shock (unconditioned stimulus, US). Mice were

1251   removed from the chamber and returned to their home cage 30 sec later. In the context

1252   testing phase, mice were placed in the same conditioning chamber and monitored for

1253   freezing behavior for a 5 min trial period, in the absence of the CS and US. For cued tone

1254   testing, the chambers were modified to different dimensions and shapes, contained

1255   different floor and wall textures, and lighting was adjusted to 50 lux. Mice acclimated to

1256   the chamber for 2 min, and then the CS was presented continuously for 3 min. Contextual

1257   and cued fear memory was assessed by freezing behavior, captured by automated video

1258   software (CleversSys).

1259   **Hearing Test**

1260   We tested mouse hearing using a startle platform (Med Associates) connected to

1261   Startle Pro Software in a sound-proof chamber. Mice were placed in a ventilated restraint

1262   cylinder connected to the startle response detection system to measure startle to each

1263   acoustic stimulus. After two minutes of acclimation, mice were assessed for an acoustic

1264   startle response to seven different tone frequencies, 2kHz, 3kHz, 4kHz, 8kHz, 12kHz,

1265   16kHz, and 20kHz that were randomly presented three times each at four different

1266   decibels, 80, 100, 105, and 110dB, for a total of 84 trials. A random inter-trial interval of

1267   15-60 seconds (average 30sec) was used to prevent anticipation of a stimulus. An

1268   animal's reaction to the tone was recorded as startle reactivity in the first 100msec of the

1269   stimulus presentation, which was transduced through the platform's load cell and

1270   expressed in arbitrary units (AU).

1271   **Startle Response (Somatosensation)**

1272   Mouse somatosensation was tested by placing mice in a startle chamber (Med

1273   Associates) connected to Startle Pro Software. Mice were placed atop a multi-bar cradle

1274   within a ventilated plexiglass restraint cylinder, which allows for horizontal movement

1275   within the chamber, but not upright rearing. After two minutes of acclimation, each mouse

1276   was exposed to 10 different scrambled shock intensities, ranging from 0 to 0.6mA with

1277   randomized inter-trial intervals of 20-90 seconds. Each animal's startle reactivity during

1278   the first 100 msec of the shock was transduced through the platform's load cell and

1279   recorded as area under the curve (AUC) in arbitrary units (AU).

1280

1281   **QUANTIFICATION AND STATISTICAL ANALYSIS**

1282   Experimental conditions, number of replicates, and statistical tests used are stated

1283   in each figure legend. Each experiment was replicated at least three times (or on at least

1284   3 separate animals) to assure rigor and reproducibility. Both male and female age-

1285   matched mice were used for all experiments, with data pooled from both sexes. Data

1286   compilation and statistical analyses for all non-proteomic data were performed using

1287   GraphPad Prism (version 8, GraphPad Software, CA), using a significance level of

1288   alpha=0.05. Prism provides exact p values unless $p < 0.0001$. All data are reported as

1289   mean ± SEM. Each data set was tested for normal distribution using a D'Agostino-Person

1290   normality test to determine whether parametric (unpaired Student's t-test, one-way

1291   ANOVA, two-way ANOVA) or non-parametric (Mann-Whitney, Kruskal-Wallis) tests

1292   should be used. Parametric assumptions were confirmed with the Shapiro-Wilk test

1293   (normality) and Levine's test (error variance homogeneity) for ANOVA with repeated

1294 measures testing. The analysis of iBioID and TMT proteomics data are described below.

1295 All proteomic data and analysis scripts are available online (see Resource Availability).

1296 **Imaris 3D reconstruction**

1297 For EEA1+ and CathepsinD+ puncta analyses, coverslips were imaged on a Zeiss

1298 LSM 710 confocal microscope. Images were sampled at a resolution of 1024 x 1024

1299 pixels with a dwell time of 0.45µsec using a 63x/1.4 oil immersion objective, 2.0 times

1300 digital zoom, and a z-step size of 0.37 µm. Images were saved as ".lsm" formatted files,

1301 and quantification was performed on a POGO Velocity workstation in the Duke Light

1302 Microscopy Core Facility using Imaris 9.2.0 software (Bitplane, South Windsor, CT). For

1303 analyses, we first used the "surface" tool to make a solid fill surface of the MAP2-stained

1304 neuronal soma and dendrites, with the background subtraction option enabled. We

1305 selected a threshold that demarcated the neuron structure accurately while excluding

1306 background. For EEA1 puncta analyses, a 600 x 800 µm selection box was placed around

1307 the soma in each image and surfaces were created for EEA1 puncta within the selection

1308 box. Similarly, for CathepsinD puncta analyses, a 600 x 600 µm selection box was placed

1309 around the soma(s) in each image for surface creation. The same threshold settings were

1310 used across all images, and individual surface data from each soma were exported for

1311 aggregate analyses. The experimenter was blinded to sample conditions for both image

1312 acquisition and analysis.

1313 **Cleaved Caspase-3 Image Analysis**

1314 Z-stack images were acquired on a Zeiss 710 LSM confocal microscope. Images

1315 were sampled at a resolution of 1024 x 1024 pixels with a dwell time of 1.58µsec, using

1316 a 63x/1.4 oil immersion objective (for cortex and striatum) or 20x/0.8 dry objective

1317 (cerebellum), a 1.0 times digital zoom, and a z-step size of 0.67 µm. Images were saved

1318 as ".lsm" formatted files, and then converted into maximum intensity projections (MIP)

1319 using Zen 2.3 SP1 software. Quantification of CC3 colocalization with DAPI was

1320 performed on the MIPs using the Particle Analyzer function in FIJI ImageJ software. The

1321 experimenter was blind to sample conditions for both image acquisition and analysis.

1322 **iBioID Quantitative Analysis**

1323 Following UPLC-MS/MS analyses, data was imported into Proteome Discoverer

1324 2.2 (Thermo Scientific Inc.), and aligned based on the accurate mass and retention time

1325 of detected ions ("features") using Minora Feature Detector algorithm in Proteome

1326 Discoverer. Relative peptide abundance was calculated based on area-under-the-curve

1327 (AUC) of the selected ion chromatograms of the aligned features across all runs. The

1328 MS/MS data was searched against the SwissProt *Mus musculus* database (downloaded

1329 in April 2018) with additional proteins, including yeast ADH1, bovine serum albumin, as

1330 well as an equal number of reversed-sequence "decoys" for false discovery rate (FDR)

1331 determination. Mascot Distiller and Mascot Server (v 2.5, Matrix Sciences) were utilized

1332 to produce fragment ion spectra and to perform the database searches. Database search

1333 parameters included fixed modification on Cys (carbamidomethyl), variable modifications

1334 on Meth (oxidation) and Asn and Gln (deamidation), and were searched at 5 ppm

1335 precursor and 0.02 Da product mass accuracy with full trypsin enzymatic rules. Peptide

1336 Validator and Protein FDR Validator nodes in Proteome Discoverer were used to annotate

1337 the data at a maximum 1% protein FDR.

1338 Protein intensities were exported from Proteome Discoverer and processed using

1339 custom R scripts. Carboxylases and keratins, as well as 315 mitochondrial proteins(Calvo

1340　et al., 2016), were removed from the identified proteins as known contaminants. Next, we

1341　performed sample loading normalization to account for technical variation between the 9

1342　individual MS runs. This is done by multiplying intensities from each MS run by a scaling

1343　factor, such that the average of all total run intensities are equal. As QC samples were

1344　created by pooling equivalent aliquots of peptides from each biological replicate, the

1345　average of all biological replicates should be equal to the average of all technical SPQC

1346　replicates. We performed sample pool normalization to SPQC samples to standardize

1347　protein measurements across all samples and correct for batch effects between MS

1348　analyses. Sample pool normalization adjusts the protein-wise mean of all biological

1349　replicates to be equal to the mean of all SPQC replicates. Finally, proteins that were

1350　identified by a single peptide, and/or identified in less than 50% of samples were removed.

1351　Any remaining missing values were inferred to be missing not at random due to the left

1352　shifted distribution of proteins with missing values and imputed using the k-nearest

1353　neighbors algorithm using the impute.knn function in the R package impute

1354　(impute::impute.knn). Normalized protein data was analyzed using edgeR, an R package

1355　for the analysis of differential expression/abundance that models count data using a

1356　binomial distribution methodology. Differential enrichment of proteins in the WASH1-

1357　BioID2 pull-down relative to the solubleBioID2 control pull-down were evaluated with an

1358　exact test as implemented by the edgeR::exactTest function. To consider a protein

1359　enriched in the WASH interactome, we required that a protein exhibit a fold change

1360　greater than 3 over the negative control with an exact test Benjamini Hochberg adjusted

1361　p-value (FDR) less than 0.1. With these criteria, 174 proteins were identified as WASH1

1362   interactome proteins. Raw peptide and final normalized protein data as well as the

1363   statistical results can be found in Supplementary Table 1.

1364   Proteins that function together often interact directly. We compiled experimentally-

1365   determined protein-protein interactions (PPIs) among the WASH1 interactome from the

1366   HitPredict database(López et al., 2015) using a custom R package, getPPIs, (available

1367   online at twesleyb/getPPIs). We report PPIs among the WASH1 interactome in

1368   Supplemental Table 1.

1369   Bioinformatic GO analysis was conducted by manual annotation of identified

1370   proteins and confirmed with Metascape analysis(Zhou et al., 2019) of WASH1-BioID2

1371   enriched proteins using the 2,311 proteins identified in the mass spec analysis as

1372   background.

1373   Raw peptide intensities were exported from Proteome Discover for downstream

1374   analysis and processing in R. Following database searching, protein scoring using the

1375   Protein FDR Validator algorithm, and removal of contaminant species, the dataset

1376   retained 86,551 peptides corresponding to the identification of 7,488 unique proteins.

1377   These data, as well as statistical results can be found in Supplemental Table 2.

1378   **TMT Proteomics Quantitative Analysis**

1379   Peptide level data from the spatial proteomics analysis of SWIP^{P1019R} MUT and

1380   MUT brain were exported from Proteome Discoverer (version 2.4) and analyzed using

1381   custom R and Python scripts. Peptides from contaminant and non-mouse proteins were

1382   removed. First, we performed sample loading normalization, normalizing the total ion

1383   intensity for each TMT channel within an experiment to be equal. Sample loading

1384   normalization corrects for small differences in the amount of sample analyzed and

1385 labeling reaction efficiency differences between individual TMT channels within an

1386 experiment.

1387 We found that in each TMT experiment there were a small number of missing

1388 values (mean percent missing = 1.6 +/- 0.17%). Missing values were inferred to be

1389 missing at random based on the overlapping distributions of peptides with missing values

1390 and peptides without missing values. We imputed these missing values using the k-

1391 nearest neighbor algorithm (impute::impute.knn). Missing values for SPQC samples were

1392 not imputed. Peptides with any missing SPQC data were removed.

1393 Following sample loading normalization, SPQC replicates within each experiment

1394 should yield identical measurements. As peptides with irreproducible QC measurements

1395 are unlikely to be quantitatively robust, and their inclusion may bias downstream

1396 processing (see IRS normalization below), we sought to remove them. To assess intra-

1397 batch variability, we utilized the method described by Ping et al., 2019(Ping et al., 2018).

1398 Briefly, peptides were binned into 5 groups based on the average intensity of the two

1399 SPQC replicates. For each pair of SPQC measurements, the log ratio of SPQC intensities

1400 was calculated. To identify outlier QC peptides, we plotted the distribution of these log

1401 ratios for each bin. Peptides with ratios that were more than four standard deviations away

1402 from the mean of its intensity bin were considered outliers and removed (total number of

1403 SPQC outlier peptides removed = 474).

1404 Proteins were summarized as the sum of all unique peptide intensities

1405 corresponding to a unique UniProtKB Accession identifier, and sample loading

1406 normalization was performed across all three experiments to account for inter-

1407 experimental technical variability. In a TMT experiment, the peptides selected for MS2

1408   fragmentation for any given protein is partially random, especially at lower signal-to-noise

1409   peptides. This stochasticity means that proteins are typically quantified by different

1410   peptides in each experiment. Thus, although SPQC samples should yield identical protein

1411   measurements in each of the three experiments (as it is the same sample analyzed in

1412   each experiment), the observed protein measurements exhibit variability due to their

1413   quantification by different peptides. To account for this protein-level bias, we utilized the

1414   internal reference scaling (IRS) approach described by Plubell et al., 2017(Plubell et al.,

1415   2017). IRS normalization scales the protein-wise geometric average of all SPQC

1416   measurements across all experiments to be equal, and simultaneously adjusts biological

1417   replicates. In brief, each protein is multiplied by a scaling factor which adjusts its intra-

1418   experimental SPQC values to be equal to the geometric mean of all SPQC values for the

1419   three   experiments.   This   normalization   step   effectively   standardizes   protein

1420   measurements between different mass spectrometry experiments.

1421   The final normalization step was to perform sample pool normalization using

1422   SPQC samples as a reference. This normalization step, sometimes referred to as global

1423   internal standard normalization, accounts for batch effects between experiments, and

1424   reflects the fact that after technical normalization, the mean of biological replicates should

1425   be equal to the mean of SPQC replicates.

1426   Before assessing protein differential abundance, we removed irreproducible

1427   proteins. This included proteins that were quantified in less than 50% of all samples,

1428   proteins that were identified by a single peptide, and proteins that had missing SPQC

1429   values. Across all 42 biological replicates, we observed that a small number of proteins

1430   had potential outlier measurements that were either several orders of magnitude greater

1431   or less than the mean of its replicates. In order to identify and remove these proteins, we

1432   assessed the reproducibility of protein measurements within a fraction in the same

1433   manner used to identify and filter SPQC outlier peptides. A small number of proteins were

1434   identified as outliers if the average log ratio of their 3 technical replicates was more than

1435   4 standard deviations away from the mean of its intensity bin (n=349). In total, we retained

1436   5,897 of the original 7,488 proteins in the final dataset.

1437   Differential protein abundance was assessed using the final normalized protein

1438   data for intrafraction comparisons between WT and MUT groups using a general linear

1439   model as implemented by the edgeR::glmQLFit and edgeR::glmQLFTest functions(MD

1440   et al., 2009). Although this approach was originally devised for analysis of single-cell

1441   RNA-sequencing data, this approach is also appropriate for proteomics count data which

1442   is over-dispersed, negative binomially distributed, and often only includes a small number

1443   of replicates (for an example of edgeR's application to proteomics see Plubell *et al*.,

1444   2017(Plubell et al., 2017))(McCarthy et al., 2012; MD et al., 2009). For intrafraction

1445   comparisons, P-values were corrected using the Benjamini Hochberg procedure within

1446   edgeR. An FDR threshold of 0.1 was set for significance for intrafraction comparisons.

1447   We utilized edgeR's flexible GLM framework to test the hypothesis that the

1448   abundance of proteins in the WT group was significantly different from that in the MUT

1449   group irrespective of fraction differences (Supplementary Table 2). For WT vs. MUT

1450   contrasts, we considered proteins with an FDR < 0.05 significant (n=687). For plotting,

1451   we adjusted normalized protein abundances for fraction differences by fitting the data with

1452   an additive linear model with fraction as a blocking factor, as implemented by the

1453   removeBatchEffect algorithm from the R limma package(Ritchie et al., 2015).

1454   To construct a protein covariation graph, we assessed the pairwise covariation

1455 (correlation) between all 5,897 proteins using the biweight midcorrelation

1456 (WGCNA::bicor) statistic, a robust alternative to Pearson's correlation. The resulting

1457 complete, signed, weighted, and symmetric adjacency matrix was then re-weighted using

1458 Network Enhancement. Network enhancement removes noise from the graph, and

1459 facilitates downstream community detection(Wang et al., 2018).

1460   The enhanced adjacency matrix was clustered using the Leiden algorithm(Traag

1461 et al., 2019), a recent extension and improvement of the well-known Louvain

1462 algorithm(Mucha et al., 2010). The Leiden algorithm functions to optimize the partition of

1463 a graph into modules by maximizing a quality statistic. We utilized the 'Surprise' quality

1464 statistic(Traag et al., 2015) to identify optimal partitions of the protein covariation graph.

1465 To facilitate biological inferences drawn from the network's organization, we recursively

1466 split 27 modules that contained more than 100 nodes and removed modules that were

1467 smaller than 5 proteins. Initial clustering of the network resulted in the identification of 324

1468 modules.

1469   To reduce the likelihood of identifying false positive modules, we enforced module

1470 quality using a permutation procedure (NetRep::modulePreservation)(Ritchie et al., 2016)

1471 and removed modules with any insignificant permutation statistics (Bonferroni P-Adjust >

1472 0.05). The following statistics were used to enforce module quality: 'avg.weight' (average

1473 edge weight), 'avg.cor' (average bicor correlation R^2), and 'avg.contrib' (quantifies how

1474 similar an individual protein's abundance profile is to the summary of its module). Proteins

1475 which were assigned to modules with insignificant module quality statistics were not

1476 considered clustered as the observed quality of their module does not differ from random.

After filtering, approximately 85% of all proteins were assigned a cluster. The median

percent variance explained by the first principle component of a module (a measure of

module cohesiveness) was high (59.8%). After removal of low-quality modules, the

analysis retained 255 distinct modules of proteins that strongly covaried together

(Supplemental Table 3).

To evaluate modules that were changing between WT and MUT genotypes, we

extended the GLM framework to test for protein differential abundance. Modules were

summarized as the sum of their proteins and fit with a GLM, with fraction as a blocking

factor. In this statistical design, we were interested in the average effect of genotype on

all proteins in a module. For plotting, module abundance was adjusted for fraction

differences using the removeBatchEffect function (package: limma). We utilized the

Bonferroni method to adjust P-values for 255 module level comparisons and considered

modules with an adjusted P-value less than 0.05 were considered significant (n=37).

SUPPLEMENTARY FILES

- **Supplementary Table 1:** WASH iBioID raw and normalized data and the corresponding statistical results.

- **Supplementary Table 2:** SWIP^{P1019R} TMT raw and normalized proteomics data and the corresponding statistical results.

- **Supplementary Table 3:** Module-level data and statistical results from network analysis of SWIP^{P109R} proteomics.

- **Supplementary Table 4:** DNA oligonucleotides used in this study.

SUPPLEMENTAL FIGURE TITLES AND LEGENDS

Figure S1. Overexpression of SWIP^{P1019R} decreases WASH complex binding in cultured cells; related to Figure 2

(A) Schematic showing overexpression of WT or MUT SWIP^{P1019R} 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 B normalized to WT. Strumpellin (WT 100.0 ± 6.8%, MUT 54.8 ± 8.0%, $t_{5.9}$=4.290, p=0.0054), WASH1 (WT 100.0 ± 7.3%, MUT 41.4 ± 4.4%, $t_{4.9}$=6.902, p=0.0011), HA (WT 100.0 ± 4.1%, MUT 107.8 ± 4.1%, $t_{6.0}$=1.344, p=0.2275). Data reported as mean ± SEM, error bars are SEM. **p<0.01, two-tailed t-tests.

Figure S2. SWIP^{P1019R} MUT brain displays significant alterations in protein abundance compared to WT; related to Figures 2 and 3

(A) Interactome of altered proteins. Nodes reflect protein 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 database (López et al., 2015). Color reflects cellular function seen in B. Nodes with red borders delineate WASH complex proteins.

(B) 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:

1522   (C) Proteins with increased (red) or decreased (blue) abundance in MUT brains compared

1523   to WT.

1524   (D) Known protein components of the WASH complex and their previously reported

1525   interactors.

1526   (E) Proteins with lysosomal function.

1527   (F) Proteins identified in the WASH1-BioID2 proteome (Figure 1).

1528   (G) Proteins with links to intellectual disability (I.D.) or neurodegeneration (Degen.).

1529

1530   **Figure S3. Multiple protein networks display significant alterations in MUT brain**

1531   **compared to WT; related to Figures 2 and 3**

1532   (A) PCA of all proteins identified by TMT proteomics. Each protein is color-coded

1533   according to its module assignment. PC = principal component, PVE = percent variance

1534   explained.

1535   Network graphs of:

1536   (B) Module 14 (M14) containing endosomal proteins.

1537   (C) Module 83 (M83) containing endoplasmic reticulum (ER) stress response proteins.

1538   (D-E) Modules 143 and 35 (M143, M35, respectively) containing synaptic proteins.

1539   Network attributes graphs in B-E: Node size denotes its weighted degree centrality

1540   (~importance in module), colored node indicates altered abundance in MUT brain relative

1541   to WT, black node border denotes proteins identified in the WASH1-BioID proteome

1542   (Figure 1), black edges indicate known protein-protein interactions, and grey-red edges

1543   denote the relative strength of protein covariation within a module (gray = weak, red =

1544   strong).

1545 (F) Summary box plots of module abundance for all seven brain fractions analyzed

1546 presented as $\log_2$(adjusted module intensities). All modules display significant differences

1547 between WT and MUT groups (M14: WT 13.94 ± 0.002 MUT 13.82 ± 0.002, p=0.0207;

1548 M83: WT 14.46 ± 0.004, MUT 14.70 ± 0.003, p= 0.0088; M143: WT 14.03 ± 0.002, MUT

1549 13.92 ± 0.002, p= 0.0148; M35: WT 14.19 ± 0.001, MUT 14.10 ± 0.001, p= 0.0030).

1550 (G) Normalized protein abundance for all proteins in each module across the seven

1551 subcellular fractions analyzed. Plots correspond to modules seen in F, and data colors

1552 reflect genotypes in F. Number of proteins in each module are indicated at the top of each

1553 graph. Data reported as mean ± SEM, error bars are SEM. *p<0.05, **p<0.01, ***p<0.001,

1554 GLM (model: 0 ~ Fraction + Module) and empirical Bayes quasi-likelihood F-test with

1555 Bonferroni correction (G).

1556

1557 **Figure S4. There is no significant difference in striatal or cerebellar cell death**

1558 **between WT and MUT mice; related to Figure 5**

1559 (A) Representative image of adolescent (P42) WT striatum stained with cleaved

1560 caspase-3 (CC3, green).

1561 (B) Representative image of adolescent (P42) MUT striatum stained with cleaved

1562 caspase-3 (CC3, green).

1563 (C and D) DAPI co-stained images of A and B, respectively.

1564 (E) Anatomical representation of mouse brain with striatum highlighted in red, adapted

1565 from Allen Brain Atlas (Oh et al., 2014).

1566 (F) Representative image of adult (10 mo) WT striatum stained with CC3 (green).

1567 (G) Representative image of adult (10 mo) MUT striatum stained with CC3 (green).

1568 (H and I) DAPI co-stained images of F and G, respectively. Scale bars for A-I are 15 µm.

1569 (J) Graph depicting the % of DAPI+ nuclei that are positive for CC3 per image. No

1570 difference is seen between genotypes at either age (P42 WT 3.70 ± 0.99%, P42 MUT

1571 1.95 ± 0.49%, 10mo WT 16.77 ± 2.09%, 10mo MUT 24.86 ± 2.17%, H=61.87, p<0.0001).

1572 (K) Representative image of adolescent (P42) WT cerebellum stained with cleaved

1573 caspase-3 (CC3, green).

1574 (L) Representative image of adolescent (P42) MUT cerebellum stained with cleaved

1575 caspase-3 (CC3, green).

1576 (M and N) DAPI and Calbindin co-stained images of K and L, respectively.

1577 (O) Anatomical representation of mouse cerebellum, adapted from Allen Brain Atlas (Oh

1578 et al., 2014). Red region highlights area used for imaging.

1579 (P) Representative image of adult (10 mo) WT cerebellum stained with CC3 (green).

1580 (Q) Representative image of adult (10 mo) MUT cerebellum stained with CC3 (green). No

1581 significant CC3 staining is observed at either age.

1582 (R and S) DAPI and Calbindin co-stained images of P and Q, respectively. Scale bars for

1583 K-S are 50 µm.

1584 (T) Graph depicting the number of Calbindin+ somas per image, a marker for Purkinje

1585 cells. No difference is seen between genotypes at either age (P42 WT 20.50 ± 0.53, P42

1586 MUT 20.67 ± 0.59, 10mo WT 21.42 ± 0.85, 10mo MUT 22.63 ± 0.74, H=4.891, p=0.1799).

1587 Data obtained from four animals per condition, and reported as mean ± standard error of

1588 the mean (SEM), with error bars as SEM. Kruskal-Wallis test (J and T).

1589

Figure S5. SWIP^P1019R mutant mice do not display deficits in spatial working memory or novel object recognition; related to Figure 6

(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) Graph depicting the percent alternations achieved for each mouse at adolescence (WT 51.48 ± 2.47%, MUT 50.81 ± 2.19%, t_{24}=0.2036, p=0.8404).

(C) Graph depicting the percent alternations achieved for each mouse at adulthood (WT 56.12 ±1.53%, MUT 57.93 ± 2.56%, t_{18}=0.6074, p=0.5511).

(D) Graphs of the number of direct revisits mice made to the arm they just explored reveal no difference between genotypes at adolescence (WT 2.64 ± 0.50, MUT 2.42 ± 0.42, t_{24}=0.3482, p=0.7308).

(E) Graphs of the number of direct revisits mice made at adulthood (WT 1.36 ± 0.33, MUT 1.42 ± 0.36, t_{24}=0.1231, p=0.9031).

(F) Similar to D-E, there were no differences in the number of indirect revisits (ex: arm A→ arm B→arm A) between genotypes at adolescence (WT 10.21 ± 1.03, MUT 12.00 ± 1.48, U=69, p=0.4515)

(G) Indirect revisits at adulthood (WT 12.86 ± 1.26, MUT 15.17 ± 1.43, t_{23}=1.211, p=0.2380).

(H) There were no significant differences in total distance travelled at adolescence, suggesting that motor function did not affect Y-maze performance (WT 2401 ± 98.9 cm, MUT 2406 ± 121.0 cm, t_{22}=0.03281, p=0.9741).

(I) No difference in distanced travelled at adulthood (WT 2761 ± 111.6 cm, MUT 3124 ± 191.1 cm, t_{18}=1.638, p=0.1189).

1613 (J) Novel object task. Mice first performed a 5-minute trial in which they were placed in

1614 an arena with two identical objects and allowed to explore freely, while their behavior was

1615 tracked with video software. Mice were returned to their home cage, and then re-

1616 introduced to the arena a half an hour later, where one of the objects had been replaced

1617 with a novel object, and their behavior was again tracked. Twenty-four hours later the

1618 same test was performed, but the novel object was replaced with another new object.

1619 (K) Graph depicting adolescent animals' preference for the novel object during the three

1620 phases of the task, training (Train), short-term memory (STM), and long-term memory

1621 (LTM). No significant difference in object preference is seen between genotypes for any

1622 phase (Train WT -0.092 ± 0.065, Train MUT -0.123 ± 0.072, STM WT 0.488 ± 0.076, STM

1623 MUT 0.315 ± 0.094, LTM WT 0.479 ± 0.046, LTM MUT 0.373 ± 0.076, $F_{1,22}$=1.840,

1624 p=0.1887).

1625 (L) Graph depicting adult animals' preference for the novel object. (Train WT -0.066 ±

1626 0.053, Train MUT -0.130 ± 0.089, STM WT 0.416 ± 0.060, STM MUT 0.274 ± 0.096, LTM

1627 WT 0.316 ± 0.059, LTM MUT 0.306 ± 0.050, $F_{1,22}$=0.9735, p=0.3345).

1628 (M) Graph depicting the total amount of time (in seconds) adolescent animals spent

1629 exploring both objects in each phase of the task. No significant difference in exploration

1630 time was observed across genotypes, suggesting that genotype does not hinder object

1631 exploration (Train P44 WT 38.10 ± 2.45 s, Train P44 MUT 50.04 ± 5.44 s, STM P44 WT

1632 55.02 ± 4.31 s, STM P44 MUT 63.60 ± 4.50 s, LTM P45 WT 46.75 ± 3.34 s, LTM P45

1633 MUT 68.08 ± 7.54 s, $F_{1,22}$=7.373, p=0.0126).

1634 (N) Graph depicting the total time adult animals spent exploring both objects in each the

1635 task (Train WT 39.10 ± 3.62 s, Train MUT 51.15 ± 4.91 s, STM WT 44.75 ± 4.87 s, STM

1636 MUT 49.56 ± 6.16 s, LTM WT 39.02 ± 5.29 s, LTM MUT 55.15 ± 7.34 s, $F_{1,22}$=2.936,

1637 p=0.1007). For all Y-maze measures, WT n=14, MUT n=12. For all novel object

1638 measures, WT n=13, MUT n=11. Data reported as mean ± SEM, error bars are SEM.

1639 Two-tailed t-tests or Mann-Whitney U tests (B-I), two-way ANOVAs (K-N).

1640

1641 **Figure S6. SWIP^{P1019R} mutant mice do not have significant deficits in contextual**

1642 **fear memory recall, auditory perception, or tactile sensation; related to Figure 6**

1643 (A) Experimental fear conditioning scheme. After acclimation, mice received a mild

1644 aversive 0.4mA footshock paired with a 2900Hz tone in a conditioning chamber. 24 hours

1645 later, the mice were placed back in the same chamber to assess freezing behavior

1646 (without footshock or tone).

1647 (B) Experimental startle response setup used to assess hearing and somatosensation.

1648 Mice were placed in a plexiglass tube atop a load cell that measured startle movements

1649 in response to stimuli.

1650 (C) Line graphs of WT and MUT freezing response during the contextual memory recall

1651 task. Data represented as average freezing per genotype in 30 second time bins. The

1652 task was performed with two different cohorts for the different ages, P42 (top) and 6.5mo

1653 (bottom). Top: no significant difference in freezing at P42 (Two-way repeated measure

1654 ANOVA, Genotype effect, $F_{1,18}$=0.088, p=0.7698. Sidak's post-hoc analysis, 30 s

1655 p=0.8388, 60 s p=0.9990, 90 s p=0.9964, 120 s p=0.3281), Bottom: no significant

1656 difference in freezing at 6.5mo (Two-way repeated measure ANOVA, Genotype effect,

1657 $F_{1,22}$= 3.723, p=0.0667. Sidak's post-hoc analysis, 30 s p=0.8977, 60 s p=0.1636, 90 s

1658 p=0.9979, 120 s p=0.0037).

1659   (D) Graphs showing the average total freezing time per animal during context exposure.

1660   Top: no significant difference is seen between WT and MUT mice at P42 (WT 34.01 ±

1661   6.32%, MUT 36.99 ± 7.81%, t_{17}=2.985, p=0.7699). Bottom: no significant different is seen

1662   between genotypes at 6.5mo (WT 43.94 ± 6.00%, MUT 28.73 ± 4.80%, $t_{21.6}$=1.980,

1663   p=0.0606).

1664   (E) Graphs of individual animals' startle response to a 2900Hz tone played at 80dB. MUT

1665   mice were not significantly more reactive to the tone than WT at P50 (WT 25.96 ± 4.95,

1666   MUT 40.68 ± 5.05, U=35, p=0.2799), or at 6.5mo (WT 14.07 ± 3.27, MUT 14.85 ± 1.49,

1667   U=47, p=0.2768).

1668   (F) Graphs of individual animals' startle response to a 0.4mA footshock. No significant

1669   difference observed between genotypes at either age (P55 WT 1527 ± 215.7, P55 MUT

1670   1996 ± 51.0, U=28.50, p=0.0542; 6.5mo WT 1545 ± 179.5, 6.5mo MUT 1817 ± 119.1,

1671   U=47, p=0.2360). Startle response reported in arbitrary units (A.U.). For all adolescent

1672   measures: WT n=10, MUT n=10. For adult freezing measures: WT n=13, MUT n=11. For

1673   adult startle responses: WT n=13, MUT n=10. Data reported as mean ± SEM, error bars

1674   are SEM.*p<0.05, **p<0.01, two-way repeated measure ANOVAs (C), two-tailed t-tests

1675   (D), and Mann-Whitney U tests (E-F).

1676

1677   **Figure S7. Progressive gait changes in SWIP^P1019R mutant mice are not restricted**

1678   **to rear limbs; related to Figure 7**

1679   (A) Graph of average swing time per stride for front limbs. At P45 (top), there is no

1680   significant difference in front swing time (WT 177.5 ± 10.9 ms, MUT 175.3 ± 8.7 ms,

1681 t_{24}=0.1569, p=0.8766). At 5.5 mo (bottom), MUT mice take significantly longer to swing

1682   their forelimbs (WT 178.6 ± 6.2 ms, MUT 206.3 ± 7.2 ms, $t_{21.4}$=2.927, p=0.0079).

1683   (B) Graph of average stride length for front limbs. At P45 (top), there is no difference in

1684   WT and MUT stride length (WT 57.0 ± 0.9 mm, MUT 59.2 ± 1.4 mm, U=68, p=0.2800).

1685   At 5.5mo (bottom), MUT mice take significantly longer strides with their forelimbs (WT

1686   60.0 ± 0.6 mm, MUT 63.7 ± 0.9 mm, t_{17}=3.545, p=0.0024).

1687   (C) Graph of average homolateral coupling, the fraction of a reference foot's stride when

1688   its ipsilateral foot starts its stride. At P45, there is no significant difference in homolateral

1689   coupling (WT 0.48 ± 0.005, MUT 0.48 ± 0.004, U=90, p=0.9713), but at 5.5mo, MUT mice

1690   display decreased homolateral coupling (WT 0.48 ± 0.002, MUT 0.45 ± 0.005, $t_{13.5}$=3.469,

1691   p=0.0039).

1692   (D) Graph of average track width between front limbs. At P45 (top), there is a significantly

1693   narrower front track width in MUT compared to WT (WT 16.99 ± 0.15 mm, MUT 15.12 ±

1694   0.33 mm, t17=5.192, p<0.0001). This difference persists into adulthood at 5.5mo (WT

1695   19.36 ± 0.23 mm, MUT 16.74 ± 0.46 mm, t_{15}=5.055, p=0.0001).

1696   (E) Graph of average track width between rear limbs. At P45 (top), there is no difference

1697   in WT and MUT rear track widths (WT 29.58 ± 0.51 mm, MUT 28.77 ± 0.36 mm, t_{23}=1.292,

1698   p=0.2091). At 5.5mo (bottom), mutants display significantly narrower rear track widths

1699   (WT 32.59 ± 0.34 mm, MUT 30.01 ± 0.46 mm, $t_{19.4}$=4.502, p=0.0002). For P45 measures:

1700   WT n=14, MUT n=13; for 5.5mo measures: WT n=14, MUT n=11. Data reported as mean

1701   ± SEM, error bars are SEM. **p<0.01, ***p<0.001, ****p<0.0001, two-tailed t-tests or

1702   Mann-Whitney U tests.

1703

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