1   **Characterizing the differential distribution and targets of Sumo paralogs in the**

2   **mouse brain**

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4   Terry R. Suk[1-4], Trina T. Nguyen[1-4], Zoe A. Fisk[1-4], Miso Mitkovski[5], Haley M. Geertsma[1-

5   4], Jean-Louis A. Parmasad[1-4], Meghan M. Heer[1-4], Steve M. Callaghan[1-4], Nils Brose[6],

6   Marilyn Tirard[6,*], and Maxime W.C. Rousseaux[1-4,*]

7

8   [1]University of Ottawa Brain and Mind Research Institute, Ottawa, Canada

9   [2]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada

10   [3]Eric Poulin Center for Neuromuscular Diseases, Ottawa, Canada

11   [4]Ottawa Institute of Systems Biology, Ottawa, Canada

12   [5]Imaging Facility, Max Planck Institute of Multidisciplinary Sciences, Göttingen, Germany

13   [6]Department of Molecular Neurobiology, Max Planck Institute of Multidisciplinary

14   Sciences, Göttingen, Germany.

15   *Co-corresponding Authors: max.rousseaux@uottawa.ca or tirard@mpinat.mpg.de

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Author Contributions

18   T.R.S., T.T.N., Z.A.F., M.M., H.M.G., J.L-A.P., M.M.H., S.M.C., M.T. investigation, formal

19   analysis; M.W.C.R., M.T. and N.B., resources; M.W.C.R., M.T. and N.B.,

20   conceptualization; T.R.S., M.W.C.R., M.T. and N.B., writing original draft and editing;

21   M.W.C.R., M.T. and N.B., supervision and funding acquisition. All authors reviewed and

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ABSTRACT

SUMOylation is an evolutionarily conserved and essential mechanism whereby Small Ubiquitin Like Modifiers, or SUMO proteins (Sumo in mice), are covalently bound to protein substrates in a highly dynamic and reversible manner. SUMOylation is involved in a variety of basic neurological processes including learning and memory, and central nervous system development, but is also linked with neurological disorders. However, studying SUMOylation *in vivo* remains challenging due to limited tools to study Sumo proteins and their targets in their native context. More complexity arises from the fact that Sumo1 and Sumo2 are ~50% homologous, whereas Sumo2 and Sumo3 are nearly identical and indistinguishable with antibodies. While Sumo paralogues can compensate for one another's loss, Sumo2 is highest expressed and only paralog essential for embryonic development making it critical to uncover roles specific to Sumo2 *in vivo*. To further examine the roles of Sumo2, and to begin to tease apart the redundancy and similarity between key Sumo paralogs, we generated (His$_6$-)HA epitope-tagged Sumo2 knock-in mouse alleles, expanding the current Sumo knock-in mouse tool-kit comprising of the previously generated His$_6$-HA-Sumo1 knock-in model. Using these HA-Sumo mouse lines, we performed whole brain imaging and mapping to the Allen Brain Atlas to analyze the relative distribution of the Sumo1 and Sumo2 paralogues in the adult mouse brain. We observed differential staining patterns between Sumo1 and Sumo2, including a partial localization of Sumo2 in nerve cell synapses of the hippocampus. Combining immunoprecipitation with mass spectrometry, we identified native substrates targeted by Sumo1 or Sumo2 in the mouse brain. We validated select hits using proximity ligation assays, further providing insight into the subcellular distribution of neuronal Sumo2-conjugates. These mouse models thus serve as valuable tools to study the cellular and biochemical roles of SUMOylation in the central nervous system.

INTRODUCTION

Post translational modifications (PTMs) provide a dynamic mode of regulation over protein functions altering the secondary, tertiary, or quaternary structures of proteins. Although the most frequently characterized PTMs typically involve the covalent conjugation of small molecules, such as phosphate, glycosyl, or acetyl groups, protein

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54   function can also be modulated by the covalent conjugation of large molecules such as

55   the protein Ubiquitin. Indeed, several Ubiquitin-like proteins have been identified to

56   function as PTMs in eukaryotes including NEDD8, Atg8, Atg12, and Small Ubiquitin-like

57   Modifiers (SUMOs) (Ilic et al., 2022; Mahajan et al., 1997; Matunis et al., 1996).

58   The covalent addition of SUMOs through SUMOylation is a dynamic and essential

59   process that is highly conserved throughout eukaryote evolution(Celen and Sahin, 2020;

60   Ilic et al., 2022). First, immature SUMO proteins are matured through Sentrin Specific

61   Protease (SENP) cleavage at a diglycine motif that is essential for covalent conjugation

62   (Xu and Au, 2005). Next, SUMOs are activated in an ATP dependent manner and

63   conjugated to the E1 ligase heterodimer complex consisting of SUMO Activating Enzyme

64   1 (SAE1) and Ubiquitin-Like Modifier Activating Enzyme 2 (UBA2)(Gong et al., 1999).

65   SUMOs are then transferred to the sole E2 ligase, SUMO conjugating enzyme UBC9

66   (UBC9, encoded by *UBE2I*), which is essential for SUMOylation to occur(Desterro et al.,

67   1997; Gong et al., 1997; Johnson and Blobel, 1997; Lee et al., 1998; Saitoh et al., 1998;

68   Seufert et al., 1995). SUMOylation can then occur at a lysine residue of a target substrate,

69   typically residing in a SUMO consensus motif (Ψ-K-x-D/E, Ψ=large hydrophobic amino

70   acid), directly from UBC9 or upon facilitation by E3 ligases (Rodriguez et al., 2001;

71   Sampson et al., 2001). Finally, SENPs can readily deSUMOylate the protein substrate,

72   and SUMO is recycled for subsequent rounds of SUMOylation, allowing for highly

73   dynamic regulation of substrate function (Nayak and Müller, 2014).

74   There are five known human SUMO paralogs (SUMO1 – SUMO5) with SUMO1 –

75   SUMO3 being the most extensively studied as they are ubiquitously expressed and found

76   in most vertebrates, whereas SUMO4 and SUMO5 are specific to humans and exhibit

77   tissue-specific expression (Bouchard et al., 2021; Citro and Chiocca, 2013). SUMO1

78   shares around 50% homology with SUMO2 and SUMO3, whereas mature SUMO2 and

79   SUMO3 share 97% homology leading to these proteins to be often referred together as

80   SUMO2/3 (Bohren et al., 2004). While SUMO paralogs often play redundant roles and

81   can exhibit some level of compensation, each paralog can also play unique roles in cells,

82   localizing to different subcellular regions and targeting different protein substrates (Citro

83   and Chiocca, 2013; Ilic et al., 2022). Additionally, *Sumo2*, but not *Sumo1* nor *Sumo3*, is

84   the only essential paralog whereby knock-out in mice results in major developmental

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85   defects leading to embryonic lethality by E10.5 (Wang et al., 2014b). The essentiality of

86   Sumo2 for proper development may be in part due to higher levels of Sumo2 in

87   comparison to its paralogs, particularly Sumo3 (Wang et al., 2014b; Yu et al., 2020).

88   Alternatively, it may also be the result of specific Sumo-conjugated proteomes between

89   Sumo2 and its paralogs conferring different roles in cells. Thus, characterizing the specific

90   roles of Sumo2 is crucial to not only help to uncover key mechanisms of cellular function

91   and advance our understanding of SUMOylation in basic biology, but to also provide

92   insight into disease processes.

93   Several challenges currently limit the study of protein SUMOylation: 1)

94   Differentiating between the Sumo paralogs using antibodies is limiting or not possible due

95   to the high degree of homology(Garvin et al., 2022); 2) The highly dynamic nature of

96   SUMOylation makes biological context such as tissue, cell type, and cell state, critical for

97   capturing and characterizing SUMOylation events; and 3) Tools to study SUMOylation

98   are limited and often rely on overexpression models, potentially confounding studies of

99   protein function. Consequently, protein SUMOylation is heavily studied in highly

100   proliferating cells where it is now well established that SUMOylation plays a key role in

101   regulating nuclear and DNA-related mechanisms, but the role of this PTM remains

102   unclear, and even debated, in more complex system, including post-mitotic cells such as

103   neurons (Daniel et al., 2018; Daniel et al., 2017).

104   To overcome these limitations, we expanded the current Sumo mouse knock-in

105   toolkit and generated and characterized in parallel two novel knock-in (KI) mouse models.

106   Specifically, sequences encoding affinity tags are knocked into the endogenous *Sumo2*

107   locus whereby an HA-Sumo2 or His_6-HA-Sumo2 fusion protein is generated, allowing for

108   the characterization of Sumo2 and its substrates *in vivo*. Analogous to our work with a

109   corresponding His_6-HA-Sumo1 KI allele (Daniel et al., 2018; Daniel et al., 2017; Tirard et

110   al., 2012), this approach allows us to directly compare the localization patterns between

111   Sumo1 and Sumo2 throughout the mouse central nervous system (CNS). We used whole

112   brain imaging to map the relative abundance of Sumo1 and Sumo2 throughout the adult

113   mouse brain and observed that, while Sumo levels are broadly distributed, they exhibit

114   regional differences throughout the CNS. We found that, unlike Sumo1 (Daniel et al.,

115   2018; Daniel et al., 2017; Tirard et al., 2012), Sumo2 is present in both nuclear and extra-

116   nuclear compartments, including neuronal synapses. Based on this distinct subcellular

117   distribution, we explored native Sumo1 and Sumo2 targets using immunoprecipitation

118   and mass spectrometry. We identified genuine Sumo2-specific targets in the adult mouse

119   brain, including a subset of non-nuclear proteins. Moreover, we observed dynamic

120   changes between Sumo1- vs. Sumo2-selective substrates. Finally, we validated several

121   hits using proximity ligations assays to detect protein-Sumo interactions within primary

122   cortical neurons. This approach did not only provide an additional mode of validating

123   native interactors of Sumo2 in wild-type neurons, but also yielded spatial information,

124   demonstrating both nuclear and cytoplasmic interactions between Sumo2 and its

125   substrates. Together, these new mouse lines and our data provide an important new

126   resource that lays the foundation of a "Sumo-code" which provides a new layer of

127   complexity to brain function.

128

129   **RESULTS**

130   ***Generation of (His$_6$-)HA-Sumo2 mouse lines***.

131   The use of epitope tags provides a versatile way to streamline the study of proteins by

132   taking advantage of the highly selective nature of antibodies raised against these tags. In

133   parallel institutions, we generated two independent alleles to facilitate the study of Sumo2

134   *in vivo*. We took advantage of a CRISPR/Cas9 approach to knock-in a 6xHis-

135   hemagglutinin (*His$_6$-HA*) tag into the amino-terminus of *Sumo2* (*His$_6$-HA-Sumo2*) in

136   C57BL/6J mice (Figure 1—figure supplement 1A) in one case, mirroring that of a

137   previously generated mouse line to study Sumo1 *in vivo* (Tirard et al., 2012). Moreover,

138   we also generated an HA-Sumo2 knock-in mouse that does not have the His$_6$ tag. We

139   observed that knock-in of either *His$_6$-HA* or *HA* to *Sumo2* resulted in hydrocephaly and

140   premature death in heterozygous mice, indicating a likely hypomorphic effect of the

141   epitope tag on a C57BL/6J background. Interestingly, backcrossing the mice to an FVB/N

142   background alleviated these effects, resulting in no overt differences between mutant and

143   wild-type FVB/N (WT) littermates in either line. Nevertheless, given the importance of

144   Sumo2 for life, and the robustness of the HA tag for visualization and biochemistry, we

145   performed the bulk of our analyses on heterozygous His$_6$-HA-Sumo2 (and heterozygous

146   His$_6$-HA-Sumo1; also crossed to an FVB/N background) knock-in mice.

147

148 ***His$_6$-HA-Sumo2 enables the comparison of endogenous Sumo2 levels in the CNS.***

149 To determine whether the addition of the HA epitope tag affects native Sumo levels, we

150 assessed the levels of Sumo2/3 conjugates by Western blot, comparing total levels of

151 unconjugated Sumo ("Free Sumo") and Sumo-conjugated proteins from mouse brain

152 lysates. We observed no change in the abundance of Sumo2/3 conjugates in the brains

153 of adult His$_6$-HA-Sumo2 mice compared to His$_6$-HA-Sumo1 or WT mice (Figure 1—figure

154 supplement 1B). As decreased levels of Sumo1 conjugates were previously observed in

155 homozygotes His$_6$-HA-Sumo1 mouse brain (Daniel et al., 2017), we thus measured

156 whether there were any changes in the abundance of native Sumo1 conjugates in

157 heterozygous His$_6$-HA-Sumo1 and His$_6$-HA-Sumo2 KI mice (Wang et al., 2014b). Again,

158 no changes were observed between either Sumo KI line and their WT counterparts,

159 indicating that the heterozygous His$_6$-HA-Sumo2 or His$_6$-HA-Sumo1 allele does not alter

160 overall levels of Sumo conjugates in mouse brain (Figure 1—figure supplement 1C).

161 Additionally, we did not observe a significant effect on the expression of *Sumo1* and

162 *Sumo2* transcript levels in either of the His$_6$-HA-Sumo KI mice (Figure 1—figure

163 supplement 1D). Thus, the novel His$_6$-HA-Sumo2 KI mice model enable the study of

164 Sumo2 at endogenous levels *in vivo* without changes to the Sumo equilibrium*.*

165 The uniformity of the HA-tag epitope between the His$_6$-HA-Sumo1 and His$_6$-HA-

166 Sumo2 mice allows for a more direct comparison of endogenous Sumo1 and Sumo2

167 levels *in vivo*. We assessed the relative levels of Sumo1 and Sumo2 in the central nervous

168 system, specifically the cortex (CTX), cerebellum (CBM), olfactory bulbs (OB), spinal cord

169 (SC) and remainder of the brain (termed "striatum-thalamus-brainstem for simplicity [S-

170 Th-B]") from adult mice (Figure 1). Western blot analysis from CNS regional lysates shows

171 that levels of unconjugated and conjugated Sumo2 are significantly higher than Sumo1

172 throughout the CNS (2-way ANOVA; Sumo effect: $P = 0.0046$ and $P = 0.0003$ for high

173 molecular weight Sumo and free Sumo, respectively). The CTX, CBM, and OB are

174 specifically enriched for Sumo2 whereas the S-Th-B and SC contains lower levels of

175 Sumo2. Taken together, Sumo1 and Sumo2 are broadly distributed throughout the CNS,

176 however regional differences in Sumo abundance and Sumo paralog predominance can

177 be observed, indicating possible unique regional roles throughout the CNS.

178

Regional Analysis of Sumo1 and Sumo2 Throughout the Mouse Brain

As differences in Sumo levels are detectable from regional crude brain lysates, we leveraged the specificity of the HA-tag and performed whole brain clearing and mapping of Sumo1 and Sumo2 signals in the adult mouse brain. His_6-HA-Sumo1 and His_6-HA-Sumo2 mouse brains were cleared and immunolabeled against the HA-tag and NeuN before imaging using light sheet microscopy (Figure 2A; Figure 2—figure supplement videos 1 & 2). Images were then analyzed and aligned to the Allen Brain Atlas to determine the relative levels of the His_6-HA-Sumo signals in defined brain regions (Table 1). It is worth noting that due to the different absolute abundance of Sumo1 (low) versus Sumo2 (high) in the brain, only qualitative Sumo1 vs Sumo2 comparisons were made. To generate relative maps of HA-Sumo distribution, the mean regional HA signal intensity was first normalized to the regional density of NeuN to account for changes in cell density before being averaged across hemibrains to account for tissue variability. Generally, Sumo1 and Sumo2 showed similar distributions throughout the mouse brain (Figure 2B-E) and we observed that both Sumo1 and Sumo2 were most abundant in cerebellar nuclei.

While Sumo levels were generally evenly distributed when characterizing gross anatomical regions, clear nuances in Sumo abundance were detectable at higher anatomical resolution. For example, in the hippocampus, Sumo levels were highest in the parasubiculum and presubiculum, and lowest in the *Fasciola cinerea* (Figure 2—figure supplement 1A & B). In the neocortex, we observed that Sumo levels were generally enriched in layers 2/3 (Figure 2B & D; Figure 2—figure supplement 1C), an area critical for integrative processing (Feldmeyer, 2012). Interestingly, while Sumo1 and Sumo2 often share similar distributions, we observed that Sumo2 is enriched in the anterior hypothalamus (Figure 2—figure supplement 1D), particularly in regions involved in the circadian rhythm, including the suprachiasmatic nucleus and subparaventricular zone (Drunen and Eckel-Mahan, 2021).

We investigated whether the brain-wide imaging findings could be validated by an orthogonal immunofluorescence assay (Figure 2—figure supplement 2). Looking at multiple brain regions, we found that Sumo1 and Sumo2 are broadly distributed through

209   the somatosensory cortex. Interestingly, Sumo1 maintains a strictly nuclear localization,

210   whereas Sumo2 is observed throughout the nucleus and in the cytoplasm and axons of

211   cortical neurons. In the cerebellum, we found that Sumo2 is specifically enriched in

212   granule cells, contrasting the strong Purkinje cell staining observed in His_6-HA-Sumo1

213   mice (Tirard *et al.* 2012). The hippocampus also displays characteristic nuclear staining

214   for His_6-HA-Sumo1 in both the CA1 and CA3, whereas Sumo2 is seen throughout the

215   nucleus and in the somata, and axons of hippocampal neurons. Thus, while Sumo1 and

216   Sumo2 may share regional expression in the brain, the distinct localizations of Sumo1

217   and Sumo2 *in vivo* indicate different targets of SUMOylation or different metabolism or

218   conjugation dynamics of free Sumo likely play unique roles in neuronal regulation.

219

220   ***HA-Sumo2 localizes to synapses in vivo.***

221   Consistent with our previous work, we observed that Sumo1 strictly localizes to neuronal

222   nuclei (Figure 2–figure supplement 2) (Tirard et al., 2012). However, we noticed that

223   Sumo2 signal can also be found outside the nuclear compartment (Figure 2–figure

224   supplement 2). The role of Sumo in extra-nuclear compartments, specifically at synapses,

225   remains a topic of debate, particularly in the context of Sumo1(Daniel et al., 2018; Daniel

226   et al., 2017). Thus, we further characterized extra-nuclear Sumo2 signals using

227   homozygous HA-Sumo2 mice. As in the His_6-HA-Sumo2 KI model, levels of Sumo2

228   conjugates were unaltered in total brain lysates from homozygous HA-Sumo2 mice as

229   compared to WT littermates, ruling out an effect of the HA tag on overall Sumo2

230   expression and conjugation levels (Figure 3–figure supplement 1A) (Tirard et al., 2012).

231   Western blot analysis of brain subcellular fractions using an anti-HA antibody highlighted

232   not only the difference in global levels of Sumo1 and Sumo2 conjugates, but also revealed

233   that Sumo2 conjugates are abundant in non-nuclear fractions, including synaptic cytosol

234   fractions S2 and S3 (Figure 3–figure supplement 1B-C). A weak but specific signal was

235   also observed in crude synaptosomes and other synaptic membrane fractions (Figure 3–

236   figure supplement 1B-D).

237   Next, we used homozygous HA-Sumo2 mice to further characterize extra-nuclear

238   Sumo2 by performing anti-HA immunolabeling of the hippocampal CA3 area, a region

239   enriched in synapses (Figure 2–figure supplement 2). Brain sections from WT and HA-

240　Sumo2 mice were immunolabeled for HA and neuronal markers for dendrites and

241　synaptic compartments, and anti-HA immunosignals were quantified in HA-Sumo2 mice

242　as compared to WTs (Figure 3). Using confocal microscopy, we confirmed the presence

243　of Sumo2 in extra-nuclear compartments, specifically along dendrites and in synapses

244　(Figure 3A). Quantification of HA-Sumo2 immunosignals in DAPI positive area confirmed

245　the strong enrichment of Sumo2 conjugates in neuronal nuclei, in agreement with the

246　well-known role of Sumos as nuclear regulators (Figure 3A and B) (Hendriks and

247　Vertegaal 2016, Hendriks and Vertegaal 2015). In addition, HA-Sumo2 immunosignal

248　was also observed in MAP2-positive regions and, strikingly, in synapses positives for

249　Synapsin1, Shank2, Gephyrin, and/or vGlut1 (Figure 3C and D). Higher magnification

250　images confirmed the presence of Sumo2 along MAP2 dendrites and at synapses (Figure

251　4; Figure 4–figure supplement 1). Quantification of the 3D object-based co-localization

252　between the anti-HA immunosignal with the various synaptic markers confirmed the

253　presence of Sumo2 at synapses, with HA-Sumo2 signal being significantly co-localized

254　with Shank2, Synapsin 1, vGlut1 and vGAT (Figure 4C and Figure 4–figure supplement

255　1, which shows a typical 3D reconstruction generated in Imaris). Co-localization of Sumo2

256　with Gephyrin was not obvious, most likely due to difficulties in immunolabeling inhibitory

257　synapses with the method optimized for HA visualization. Altogether, our data

258　demonstrate the presence of Sumo2 at synapses.

259

260　***HA-Sumo mice reveal convergent and contrasting neuronal Sumo1 and Sumo2***

261　***substrates.***

262　The observation that there are regional and subcellular differences between Sumo1 and

263　Sumo2 indicate divergent roles for the Sumo paralogs in the mouse brain. To uncover

264　such molecular differences between Sumo paralogs, we performed HA-tag

265　immunoprecipitation, under denaturing conditions to break apart standard protein-protein

266　interactions, from whole brain lysate of WT, His_6-HA-Sumo1, and His_6-HA-Sumo2 mice,

267　followed by mass spectrometric analysis to identify candidate targets of Sumo1 and

268　Sumo2 *in vivo* (Figure 5A; Table 2). To rank proteins identified from the mass

269　spectrometry dataset, we summated the total peptides from 4 replicates per genotype

270　and then filtered proteins with >2-fold enrichment over proteins identified in the WT

271 condition. Next, to determine high-stringency candidates, proteins were further filtered

272 based on peptide counts being identified in at least 2/4 replicates in the HA-Sumo

273 conditions, and no more than 1/4 replicate in the respective WT condition. Using these

274 criteria, we identified 131 proteins enriched in the HA-Sumo1 and 75 proteins enriched in

275 the HA-Sumo2 immunoprecipitations (Table 2). Gene ontology and Reactome terms

276 related to protein SUMOylation process (Figure 5B) were significantly enriched in both

277 datasets (Figure 5C; Figure 5—figure supplement 1C; Table 3). Indeed, we identified

278 critical components of the SUMOylation pathway including E1 SUMO ligases Sae1 and

279 Uba2, the E2 SUMO ligase Ube2i, and E3 ligases Ranbp2(Pichler et al., 2002),

280 Trim28(Liang et al., 2011), and Pml(Chu and Yang, 2011), validating the use of our mouse

281 model to identify molecular substrates involved with SUMOylation.

282   To determine whether we could identify specific interactors of Sumo2, we

283 compared the relative fold change of peptides identified in the His_6-HA-Sumo2

284 immunoprecipitation to that of the His_6-HA-Sumo1 immunoprecipitation and found that

285 there are shared, specific, and preferential interactors of Sumo2 vs. Sumo1 (Figure 5D;

286 Figure 5—figure supplement 1D; Table 2). We selected several hits from our mass

287 spectrometry screen for validation via Western blot (Figure 5D). Ranbp2 and Rangap1

288 are consistently identified amongst the most abundantly SUMOylated proteins (Gareau

289 et al., 2012; Geiss-Friedlander and Melchior, 2007; Saitoh et al., 1998) and were indeed

290 validated to be shared targets of Sumo1 and Sumo2 in the mouse brain. Additionally,

291 both proteins displayed a preference for Sumo1 modification in the mouse brain as

292 previously described (Gareau et al., 2012). Conversely, the E3 ligase and transcriptional

293 repressor Trim28 displayed a preference for Sumo2 modification in the adult mouse brain.

294 Interestingly, the transcription factor Prox1, involved in neurogenesis and a marker of

295 granule cells in the dentate gyrus and cerebellum (Lavado et al., 2010), was one of the

296 most abundantly SUMOylated proteins targeted specifically by Sumo2 in our screen. We

297 further tested the sensitivity of the model to detect more modestly SUMOylated proteins

298 targeted by Sumo2 in the mouse brain, as measured by relative abundance in our

299 immunoprecipitation coupled with mass spectrometry experiment. We confirmed that

300 both Histone Deacetylase 2 (Hdac2) and General Transcription Factor 2i (Gtf2i, also

301 known as Tf2-i) were Sumo2-modified (Figure 5D). Because of the dynamic nature of

302 SUMOylation and the fact that Sumo only targets a fraction of the total pool of most

303 substrates, biologically meaningful interactions may be buried under strong, constitutively

304 SUMOylated targets in our screen. As a proof of concept, we picked a hit with low

305 enrichment in the His$_6$-HA-Sumo2 immunoprecipitation by expanding the filtering criteria

306 to a >1.5 fold enrichment over wild-type: Matrin3 (Matr3). We found that Matr3 was

307 selectively SUMOylated in the His$_6$-HA-Sumo2 pulldown (Figure 5D), indicating that even

308 at milder cut-off thresholds, SUMOylated substrates identified by mass spectrometry can

309 be validated in an orthogonal assay. Taken together, these results show that the His$_6$-

310 HA-Sumo2 mouse model is effective to identify SUMOylated proteins, including proteins

311 SUMOylated at relatively low levels *in vivo*, and can be used for targeted studies of protein

312 substrates.

313

314 ### *Subcellular Localization of Sumo Interaction in Neurons*

315 Sumo1 and Sumo2 predominantly reside in the nucleus, and unsurprisingly, many of the

316 interactors identified here and in other studies of protein SUMOylation are nuclear

317 proteins. However, previous studies hinted at roles for SUMOylation outside of the

318 nucleus(Hasegawa et al., 2014; Ilic et al., 2022; Wang et al., 2014a; Watanabe et al.,

319 2008; Yang and Paschen, 2015). During the validation of whole brain imaging, we

320 observed that His$_6$-HA-Sumo2, while extensively localized throughout the nucleus, could

321 also be observed within the somata and synapses of neurons *in vivo*, contrasting the

322 predominantly nuclear membrane staining of His$_6$-HA-Sumo1 (Figure 2—figure

323 supplement 2; Figure 3). The cytoplasmic localization of His$_6$-HA-Sumo2 in neurons

324 indicates unique extranuclear roles *in vivo.* Indeed, we identified several predominantly

325 extranuclear proteins via mass spectrometry in the anti-HA affinity purification from the

326 His$_6$-HA-Sumo2 knock-in mice. To better visualize the subcellular localization of Sumo,

327 we cultured primary cortical neurons from heterozygous His$_6$-HA-Sumo1, His$_6$-HA-

328 Sumo2, and WT animals and co-stained them for HA and Map2 as a cytoplasmic marker.

329 As previously observed, Sumo2 is predominantly distributed uniformly throughout the

330 nucleus except for heterochromatic foci, but is also observed at low levels in the

331 cytoplasm (Figure 6—figure supplement 1A), contrasting with the anti-HA immunosignal

332 observed for Sumo1, which predominantly localizes at the nuclear membrane. This

333 indicates that Sumo2 specifically may play a larger role in regulating proteins outside of
334 the nucleus.

335   To better assess the subcellular localization of neuronal SUMOylated proteins
336 targeted by Sumo2, and to provide an additional level of validation in an orthogonal
337 system, we used proximity ligations assays (PLA) against endogenous Sumo2/3 and
338 targets of Sumo2 identified from our mass spectrometry screen to visualize interactions
339 within wild-type primary cortical neurons (Figure 6A) (Ristic et al., 2016; Sahin et al.,
340 2016). In this assay, proteins colocalizing within 40 nm will produce a PLA signal
341 suggesting a potential interaction. We selected proteins expected to interact with
342 Sumo2/3 in the nucleus, Gtf2i and Matr3, as well as expected cytoplasmic interactors
343 Rangap1, Ctsd, Alix, and Kars1 for additional validation (Figure 6B & C). Gtf2i is
344 distributed almost exclusively throughout the nucleus and interactions between Sumo2/3
345 and Gtf2i, inferred by the presence of PLA foci, are seen throughout the nucleoplasm.
346 Interestingly, PLA foci were often observed to cluster around constitutive heterochromatin
347 (Figure 6C, inset), indicating a potential role for Sumo2 in regulating Gtf2i at the periphery
348 of heterochromatin in neurons. Matr3 is also localized throughout the nucleus and to a
349 lesser extent in the cytoplasm, consistent with its identification in cytoplasmic processing
350 bodies (Rajgor et al., 2016). Interestingly, PLA signals for Matr3 and Sumo2/3 are robust,
351 indicating extensive interactions between the two proteins. Immunofluorescence staining
352 of Rangap1, Ctsd, Alix, and Kars1 demonstrate these proteins predominantly reside
353 outside of the nucleus (Figure 6C). PLA between these hits and Sumo2/3 display an
354 increase in the proportion of cytoplasmic foci relative to nuclear foci (Figure 6B), in
355 addition to a substantial decrease in total nuclear PLA foci compared to nuclear localized
356 proteins Gtf2i and Matr3 (Figure 6—figure supplement 1B). Importantly, for all PLA
357 assays conducted, negative controls did not elicit a PLA signal (Figure 6—figure
358 supplement 1C). Taken together, our PLA analyses of wild-type neurons provide an
359 additional layer of validation of Sumo2-substrate interactions. With the added benefit of
360 providing spatial resolution to the interactions, PLA data support a role for neuronal
361 Sumo2 outside the nucleus.

DISCUSSION

Protein SUMOylation plays a variety of essential and conserved roles (Ilic et al., 2022). Increasing evidence indicates that SUMOylation plays roles in the brain, e.g. in neuronal development or learning and memory(Ripamonti et al., 2020). Furthermore, SUMOylation has been linked with various brain disorders, including Autism Spectrum Disorder, Epilepsy, Schizophrenia, and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (Bernstock et al., 2018; Krumova and Weishaupt, 2013; Osmanovic et al., 2022; Rousseaux et al., 2016). However, despite a clear connection with neuronal function and dysfunction, SUMOylation has remained enigmatic due to technical challenges and limitations for studying this modification (Daniel et al., 2017). We generated His_6-HA-Sumo2 and HA-Sumo2 KI mouse alleles to overcome these technical limitations and to provide insight into the role of native SUMOylation *in vivo.* These mice complement the previously generated His_6-HA-Sumo1 mouse line (Tirard et al., 2012), which together enable direct comparisons of Sumo1 and Sumo2 *in vivo* to further understand the specific roles conferred by Sumo2 and native roles of SUMOylation.

Using whole brain imaging against the HA epitope in His_6-HA-Sumo1 and His_6-HA-Sumo2 mice, we found that Sumo1 and Sumo2 are broadly distributed throughout the mouse brain. Deeper anatomical analysis revealed clear patterns of Sumo differences across brain structures as well as some divergence of Sumo1 and Sumo2 levels in the brain. Indeed, we observed that levels of His_6-HA-Sumo1 was generally evenly distributed amongst cortical layers, whereas His_6-HA-Sumo2 levels were higher in layers 2/3 (Figure 2—figure supplement 1D). In the hypothalamus, we found that Sumo levels vary across anatomical regions (Figure 2—figure supplement 1E). Interestingly, regions involved in circadian rhythm including the subparaventricular zone (SBPV) and suprachiasmatic nucleus (SCH) show high levels of Sumo2. These findings are consistent with previous reports that have linked both Sumo1 and Sumo2 differentially affecting circadian clock related proteins such as PER2 and CLOCK, ultimately supporting a role for SUMOylation in regulating circadian rhythm processes (Chen et al., 2021; Drunen and Eckel-Mahan, 2021; Lee et al., 2015).

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392   We observed differential subcellular distribution patterns between Sumo1 and

393   Sumo2. Western blot analysis of brain subcellular fractions showed that Sumo2 is more

394   abundantly expressed than Sumo1 (Figure 1), and that Sumo2 conjugates are present

395   outside of the nuclear compartment, including synaptic fractions (Figure 3–figure

396   supplement 1B & C). This is of importance, as the role of Sumo proteins at synapses has

397   been debated, particularly in the context of Sumo1(Daniel et al., 2018; Daniel et al., 2017).

398   We previously showed that Sumo1 conjugates are not present in synaptic compartments,

399   in contrast to several other reports. Here, we used anti-HA immunolabeling of brain

400   material from HA-Sumo2 mouse brains, and used WT littermates as negative controls to

401   assess non-specific anti-HA immunolabeling (Figures 3, 4 and Figure 4–figure

402   Supplement 1). Strikingly, the quantification of anti-HA immunosignal intensity confirmed

403   a co-localization of Sumo2 with pre- and post-synaptic markers, at both excitatory and

404   inhibitory synapses (Figures 3A-B, 4A-C). This comparative and quantitative approach of

405   investigating the HA-Sumo mouse models allows for the specific assessment of Sumo2

406   immunosignals within various neuronal sub-compartments, and we provide here the first

407   robust dataset identifying the presence of a Sumo paralog, Sumo2, at synapses in the

408   mammalian brain. These data suggest that Sumo1 and Sumo2 do not only have divergent

409   patterns of expression in mouse brain, but also show differential distributions in the

410   various subcellular compartments, with only Sumo2 present in synapses.

411   Sumo2 is the only essential Sumo paralog, and has key roles in

412   neurodevelopment, nerve cell function, and neurological diseases(Krumova and

413   Weishaupt, 2013; Ripamonti et al., 2020; Stankova et al., 2018; Wang et al., 2014b). To

414   uncover some of the unique clientele targeted by Sumo2, we performed HA tag

415   immunoprecipitation paired with mass spectrometry. As SUMOylation results in the

416   covalent interaction between Sumo and the target substrate, mouse brains were lysed

417   under denaturing conditions to promote the dissociation of protein complexes prior to

418   immunoprecipitation to help identify bona fide SUMOylation substrates. Using stringent

419   criteria, 75 proteins were identified to potentially interact with Sumo2, ~1/2 of which were

420   selective to Sumo2. Conversely, using the same criteria, we identified 131 proteins that

421   interact with Sumo1, 98 of which were selective to Sumo1. Pathway analysis of the

422   Sumo2 dataset revealed that gene ontology terms related to SUMOylation were

423   significantly enriched in the dataset, validating this method to identify potentially

424   SUMOylated substrates. Moreover, pathway analysis of the Sumo1 dataset revealed

425   enrichment of proteins involved in protein transport consistent with roles for Sumo1 in

426   nucleocytoplasmic shuttling (Pichler et al., 2002; Salinas et al., 2004; Westman et al.,

427   2010). Prox1 was validated via Western blot as one of the strongest interactors specific

428   to Sumo2 in the mouse brain. Interestingly, previous studies demonstrated that

429   SUMOylation of Prox1 by Sumo1 occurs *in vitro* and in cancer cell lines (Pan et al., 2009;

430   Shan et al., 2008). During development, Prox1 was found to be SUMOylated by Sumo1,

431   and this interaction is critical for neurogenesis in the neural tube development. However,

432   the role of Prox1 in the mature brain remains unclear, although some data indicate an

433   involvement in adult neurogenesis (Correa-Vázquez et al., 2021). Due to the extent of

434   the Prox1 and Sumo2 interaction observed in this study, further analysis may uncover

435   key roles of SUMOylated Prox1 in the adult mouse brain. We further found that the RNA

436   binding protein Matr3 interacts with Sumo2 *in vivo*, indicating that even a mild enrichment

437   over wild-type in our dataset may identify *bona fide* biological interactors. Interestingly,

438   many of the enriched computationally-predicted SUMOylation sites in Matr3 (K588 and

439   K843) reside within a structurally disordered region of the protein that also happens to be

440   enriched for mutations causing Amyotrophic Lateral Sclerosis (ALS) (Johnson et al.,

441   2014). Structurally disordered regions are thought to be critically involved in the formation

442   of protein aggregates in neurodegenerative diseases, and given the key role of SUMO in

443   controlling protein solubility and the recent links between SUMOylation and ALS,

444   SUMOylation of Matr3 may provide key insights into Matr3 biology related to this disease.

445   SUMOylation occurs in a context-specific manner and can regulate protein

446   localization within cells. One of the best characterized examples concerns the regulation

447   of nucleocytoplasmic shuttling across nuclear pore complexes (Pichler et al., 2002;

448   Salinas et al., 2004; Westman et al., 2010). However, SUMOylation can also regulate

449   sub-compartmental localizations, such as protein targeting to the nucleolus or to

450   heterochromatin (Andreev et al., 2022; Mo et al., 2002; Rawat et al., 2021). As Sumo2

451   was observed throughout the nucleus and outside the nucleus *in vivo* and in cultured

452   neurons, we sought to determine the subcellular localization of Sumo2 interactions with

453   hits identified via IP-MS using a PLA-based approach (Figure 6). Gtf2i was SUMOylated

454 almost exclusively in the nucleus but interestingly appeared to cluster around
455 heterochromatin. Gtf2i can function as both a transcriptional activator and repressor in
456 response to various signals. For example, the specific splice isoforms β and ∆ undergo
457 changes in subcellular localization to differentially regulate the immediate early gene *c-*
458 *Fos* in response to growth factor signaling(Hakre et al., 2006). However, roles for Gtf2i in
459 regulating heterochromatin remain elusive. SUMOylation may provide a dynamic
460 mechanism in response to neuronal activity to regulate both targeted expression at the
461 gene level and global transcriptional changes(Niskanen et al., 2015). Finally, interactions
462 between Matr3 and Sumo2/3 occurred broadly throughout the nucleus. However,
463 interactions between Matr3 and Sumo2 in mouse brain occurred at relatively low levels
464 based on immunoprecipitation assays. This discrepancy observed in the nucleus may be
465 explained via non-covalent interactions with Sumo2 through larger protein complexes as
466 Matr3 typically functions in complex with other DNA and RNA binding proteins (Banani et
467 al., 2016; Keiten-Schmitz et al., 2021, Keiten-Schmitz et al., 2020). Extranuclear proteins
468 Rangap1, Ctsd, Alix, and Kars1, despite having a certain level of PLA signal within the
469 nucleus, displayed an increase in the proportion of cytoplasmic PLA foci relative to
470 nuclear PLA foci implying increased interactions with Sumo2/3 outside the nucleus.
471 Indeed, Rangap1 plays roles in nuclear import, but can also be found to some extent
472 within the nucleus (Cha et al., 2015). PLA foci between Rangap1 and Sumo2/3 were often
473 found near the nuclear membrane, likely at nuclear pore complexes (Figure 6C, inset).
474 Ctsd is a lysosomal protease predominantly localizing to lysosomes, thus interactions
475 between Sumo2/3 and Ctsd may suggest that its SUMOylation may play a role in
476 lysosomal metabolism(Nakanishi, 2003). Previous reports have linked Sumo with the
477 apoptosis pathway(Basu-Shrivastava et al., 2022; Besnault-Mascard et al., 2005; Mojsa
478 et al., 2015); here, we observe potential interactions between Sumo and the apoptotic
479 protein Alix further inferring a role for Sumo in the apoptotic process. Finally extranuclear
480 PLA foci could be observed for the lysyl-tRNA Synthetase Kars1, consistent with reports
481 of Sumo interacting with tRNA-related proteins(Chymkowitch et al., 2017; Chymkowitch
482 and Enserink, 2018; Rohira et al., 2013). Together these results suggest that Sumo2 may
483 play a variety of roles outside the nucleus in various subcellular compartments. While
484 PLA assays provide spatial information of putative protein interactions (i.e. interactions

485   occurring within 40 nm), this does not discount indirect interactions (e.g. protein

486   complexes) (Ristic et al., 2016; Sahin et al., 2016). Thus, differentiating between covalent

487   SUMOylation, interactions with SUMO interacting motifs (SIMs), and protein complexes

488   containing Sumo are not possible using this approach. Extensive localization and

489   biochemical analyses, including arginine to lysine mutagenesis to block covalent

490   SUMOylation, should be performed to properly assess how Sumo confers regulation on

491   a specific target throughout the cell.

492   In sum, the novel (His$_6$-)HA-Sumo2 knock-in mouse lines described here represent

493   powerful tools to facilitate the study of protein SUMOylation via Sumo2 *in vivo*. The HA

494   epitope tag allows for specific yet versatile modes to detect and capture Sumo2 in its

495   native context, without altering its function. Furthermore, these mouse models add to the

496   previously generated His$_6$-HA-Sumo1 allele, and thus expand the Sumo knock-in mouse

497   toolkit to begin exploring differential SUMOylation in parallel systems. The versatility of

498   these models extends to all other tissue systems, as Sumo proteins are broadly

499   distributed beyond the brain (Hendriks et al., 2018; Uhlén et al., 2015). Beyond basic

500   biology, these alleles can be used to explore disease processes, e.g. by crossing these

501   mouse lines into disease models, thus enabling the study of the Sumo-linked disease

502   proteome (Stankova et al., 2018). Ultimately, these tools will advance our understanding

503   of the essential biological processes and potential disease targets regulated by

504   SUMOylation.

505

506   **MATERIALS AND METHODS**

507   ***Mouse Husbandry***

508   His$_6$-HA-Sumo1 and His$_6$-HA-Sumo2 mice, kept on an FVB/N background, were housed

509   with up to 5 mice per cage on a 12 h light–dark cycle. Mice were fed *ad libitum* and all

510   husbandry was performed by the uOttawa Animal Care and Veterinary Services staff. All

511   animal work for the His$_6$-HA-Sumo1 and His$_6$-HA-Sumo2 mouse lines were done under

512   the breeding (CMMb-3009 and CMMb-3904) protocols approved under the uOttawa

513   Animal Care Committee.

514   All experiments regarding the HA-Sumo2 KI mice were performed in accordance with the

515   guidelines for the welfare of experimental animals issued by the State Government of

516   Lower Saxony, Germany (LAVES). Animals were hosted in a pathogen free facility at the

517   Max Planck Institute of experimental Medicine and were maintained in groups in

518   accordance with European Union Directive 63/2010/EU and ETS 123 (individually

519   ventilated cages, specific pathogen-free conditions, 21 ± 1°C, 55% relative humidity, 12

520   h/12 h light/dark cycle). Mice received food and tap water *ad libitum* and were provided

521   with bedding and nesting material. Cages were changed once a week. Animal health was

522   controlled daily by caretakers and by a veterinarian. Health monitoring (serological

523   analyses; microbiological, parasitological, and pathological examinations) was done

524   quarterly according to FELASA recommendations with either NMRI sentinel mice or

525   animals from the colony. The mouse colony used for experiments did not show signs of

526   pathogens. All experiments were performed during a light cycle.

527

528   ***Mouse Genotyping***

529   Genomic DNA (gDNA)was isolated from small tail samples collected from mouse

530   embryos (primary cortical neuron experiments), pups (pre-weaning), or adult mice

531   (endpoint). His_6-HA-Sumo2 mice were genotyped using parallel reactions with primers

532   targeting the *His_6-HA* knock-in tag and corresponding genomic loci upstream (Forward:

533   5′- AGGAAGAGAGCGAGAGAGGAA-3′, Reverse: 5′-CACCACCACTACCCATACGA-3′,

534   224bp product) and downstream (Forward: 5′-TCGTATGGGTAGTGGTGGTG-3′,

535   Reverse: 5′-AGGAGGAGGGGTGGTTATGT-3′, 276bp product) of the knock-in. 25µL

536   reactions were prepared using <10ng gDNA, primers (400nM final), 1.25µL DMSO (5%

537   final), and 2X GoTaq (Promega). Thermocycler parameters: 95°C for 2 min, (95°C for

538   30 s, 57.6°C for 30 s, 72°C for 40 s) repeated for 35 cycles, final denature at 72°C for

539   5 min. HA-Sumo2 mice were genotyped with primers flanking the *HA* knock-in tag

540   (Forward: 5′-GCCCTCTGCCTCGTCCAC-3′, Reverse: 5′-CCGCCGCGAGCTCACCTTG

541   -3′, WT Allele: 160bp product, KI Allele: 187bp product). 20µL reactions were prepared

542   using 1µL clean gDNA, primers (4 pmol final), and 5X Biozym Hot-Start Taq DNA

543   Polymerase plus extra Mg^{2+} (Biozym 331620XL). Thermocycler parameters: 96°C for

544   3 min, (94°C for 30 s, 62°C for 60 s, 72°C for 60 s) repeated for 32 cycles, final denature

545   at 72°C for 7 min. His_6-HA-Sumo1 mice were genotyped as previously described (Tirard

546   et al., 2012).

547

Biochemical Analysis of Adult Mouse Brain and Spinal Cord

549 Mice were anesthetized using isoflurane inhalation and sacrificed via decapitation. Brains
550 were quickly isolated, dissected for regional protein analysis, and flash frozen on dry ice.
551 Analysis of total Sumo1 and Sumo2/3 levels were performed on whole brain lysates.
552 Spinal cords were removed using hydraulic extrusion(Richner et al., 2017). Samples were
553 thawed and immediately lysed using a dounce homogenizer in RIPA buffer (9.1 mM
554 dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM sodium
555 chloride, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS) containing 50 mM freshly
556 prepared N-Ethylmaleimide (Sigma), 0.25 % β-mercaptoethanol, and Xpert Protease
557 (GenDEPOT) and Xpert Phosphatase (GenDEPOT) inhibitor cocktails. Whole brain
558 lysates were lysed in 11 mL of RIPA buffer described above and centrifuged at 125,000
559 x g for 2 hours at 4 °C. Regional lysates were centrifuged at 21,000 x *g* for 20 minutes at
560 4 °C and the supernatant was removed and suspended in 4X laemmli buffer (BioRad)
561 with 2-mercaptaethanol (BioRad) and boiled at 95 °C for 5 minutes. Samples were run on
562 8 % polyacrylamide gels and transferred onto 0.45μm nitrocellulose membranes at 340
563 mA for 90 minutes and stained with ponceau to normalize protein levels between lysates.
564 Normalized lysates were run on a 4-15 % GTx Mini-PROTEAN gel (BioRad) and
565 transferred onto a 0.45 μm nitrocellulose membrane at 340 mA for 2 hours for western
566 blot analysis. Densitometry was performed using BioRad Image Lab software and
567 FiJi/ImageJ by normalizing the HA intensity to ponceau for High Molecular Weight (>70
568 kDa) and free Sumo levels.

569

Real Time Quantitative PCR (RT-qPCR) analysis

571 RNA was extracted from mouse brain homogenate using Trizol-Chloroform extraction
572 (Invitrogen™ User Guide: TRIzol Reagent version B.0). Briefly, mouse brains were
573 homogenized in 3 ml of PEPI Buffer [5 mM EDTA, 1X protease inhibitor (GenDEPOT cat#
574 P3100-020), in 1X PBS] using a dounce homogenizer. 3 % of homogenate was added to
575 1 ml of TRIzol Reagent (Fisher Scientific cat# 15-596-026) and RNA was isolated as per
576 the user guide referenced above. cDNA was synthesized using 5X All-in-One RT Master
577 Mix (Bio Basic cat# HRT025-10) following manufacturer's instructions. RT-qPCR was

578 performed using Green-2-Go qPCR Master Mix (Bio Basic cat# QPCR004-S) with 25 ng

579 cDNA per reaction and primers targeting mouse *Sumo1* (NM_009460.2) (Forward: 5′-

580 GCTGATAATCATACTCCGAAAGAAC-3′, Reverse: 5′-CCCCGTTTGTTCCTGATAAA-

581 3′), *Sumo2*(NM_133354.2) (Forward: 5′-GGACAGGATGGTTCTGTGGTGC-3′, Reverse:

582 5′-CCCATCAAACCGGAATCTGATCTGC-3′), and *Hprt1*(NM_013556.2) (Forward: 5′-

583 TGATAGATCCATTCCTATGACTGTAGA-3′, Reverse: 5′-

584 AAGACATTCTTTCCAGTTAAAGTTGAG-3′). Reactions were run on BioRad CFX96

585 thermocycler (protocol: 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 60 s, then

586 melting curve). *Sumo1* and *Sumo2* Ct values were standardized to the Ct values of *Hprt1*.

587

588 ### Brain preparation for whole brain clearing and HA immunolabeling.

589 Mice were sedated via intraperitoneal injection of 16.25 mg sodium pentobarbital

590 (Euthanyl, DIN 00141704). Once sedated, mice were perfused using 10 mL 1X PBS + 10

591 U/mL heparin (Millipore Sigma, H3393-50KU) followed by 10 mL of freshly prepared 4 %

592 paraformaldehyde. Brains were carefully isolated and stored in 13 mL of 4 %

593 paraformaldehyde at 4 °C overnight with gentle shaking. Brains were rinsed with 1X PBS

594 then shipped to LifeCanvas Technologies (MA) in 1X PBS + 0.02 % sodium azide.

595

596 ### Whole mouse brain processing, staining, and imaging

597 Whole mouse brains were processed using the SHIELD protocol (LifeCanvas

598 Technologies (Park et al., 2018)). Samples were cleared for 1 day at 42 °C then actively

599 immunolabeled using SmartBatch+ (LifeCanvas Technologies) based on eFLASH

600 technology integrating stochastic electrotransport (Kim et al., 2015) and SWITCH (Murray

601 et al., 2015). Each sample was labeled with 60 µg anti-NeuN (Encor, MCA-1B7) and 36

602 µg rabbit anti-HA-tag (Cell Signalling Technology #3724) followed by fluorescently

603 conjugated secondary antibodies in a 3:2 primary:secondary molar ratio (Jackson

604 ImmunoResearch). Samples were incubated in EasyIndex (LifeCanvas Technologies) for

605 refractive index of RI = 1.52 and imaged at 3.6X using a SmartSPIM axially-swept light

606 sheet microscope (LifeCanvas Technologies). Images were tile corrected, de-striped, and

607 registered to the Allen Brain Atlas (Allen Institute: https://portal.brain-map.org/). NeuN

608 channels for each brain were registered to 8-20 atlas-aligned reference samples using

609 successive rigid, affine, and b-spline warping (SimpleElastix:

610 https://simpleelastix.github.io/). Average alignment to the atlas was generated across all

611 intermediate reference sample alignments to serve as the final atlas alignment value per

612 sample. Fluorescent measurements from the acquired images were projected onto the

613 Allen Brain Atlas to quantify the total fluorescence intensity per region defined by the Allen

614 Brain Atlas. These values were then divided by the volume of the corresponding regional

615 volume to calculate the intensity per voxel measurements.

616

HA Immunoprecipitation from mouse brain lysates

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618 Immunoprecipitation was performed based on the previously established protocol(Tirard

619 et al., 2012; Tirard and Brose, 2016). Briefly, mice aged 9-16 weeks old were anesthetized

620 and sacrificed via decapitation and the brain was quickly removed and homogenized

621 using a dounce homogenizer in RIPA buffer (9.1 mM dibasic sodium phosphate, 1.7 mM

622 monobasic sodium phosphate, 150 mM sodium chloride, 1 % NP-40, 0.5 % sodium

623 deoxycholate, 0.1 % SDS) containing 50 mM freshly prepared N-Ethylmalamide (Sigma),

624 0.25 % β-mercaptoethanol, and Xpert Protease (GenDEPOT) and Xpert Phosphatase

625 (GenDEPOT) inhibitor cocktails. Samples were lysed on ice for 20 minutes with vigorous

626 vortexing every 5 minutes before ultracentrifugation at an average of 100,000 x g for 30

627 minutes at 4 °C. Supernatants were removed and spiked with 50 mM N-ethylmalamide

628 before being added to 50 μL of magnetic protein G Dynabeads (Invitrogen) pre-

629 conjugated with 10 μg mouse HA antibody (in house) per sample. Samples were then

630 placed on rotator at 4 °C for 1 hour. Beads were washed three times in 10 mL of RIPA

631 buffer containing 20 mM N-Ethylmalamide, protease, and phosphatase inhibitors. Wash

632 buffer was thoroughly removed, and beads were eluted using 75 μg synthetic HA peptide

633 (Sino Biological Inc.).

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635

Sample Preparation for Mass Spectrometry

Two thirds of eluted protein sample from HA Immunoprecipitation were run on a 4-15% Mini-PROTEAN (Bio-Rad) gel to separate proteins and remove synthetic HA peptide. Samples were stained using Silver Stain for Mass Spectrometry Kit (Thermo Scientific, 24600). Lanes were cut into 6-7 gel slices per sample and stored in 1% acetic acid until analysis via LC-MS/MS.

Protein Identification by LC-MS/MS

Proteomics analysis was performed at the Ottawa Hospital Research Institute Proteomics Core Facility (Ottawa, Canada). Proteins were digested in-gel using trypsin (Promega) according to the method of Shevchenko (Shevchenko et al., 2006), but without the use of iodoacetamide for cysteine alkylation due to the treatment of 50 mM N-ethylmaleimide (Sigma) during the sample immunoprecipitation. Peptide extracts were concentrated by Vacufuge (Eppendorf). LC-MS/MS was performed using a Dionex Ultimate 3000 RLSC nano HPLC (Thermo Scientific) and Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific). MASCOT software version 2.7.0 (Matrix Science, UK) was used to infer peptide and protein identities from the mass spectra. The observed spectra were matched against *Mus musculus* sequences from SwissProt (version 2021-02) and against an in-house database of common contaminants. The results were exported to Scaffold (Proteome Software, USA) for further validation and viewing and will be uploaded to the ProteomXchange Consortium.

Gene Ontology analysis

Top His_6-HA-Sumo1 and His_6-HA-Sumo2 interactors were analyzed using gProfiler2 web tool (https://biit.cs.ut.ee/gprofiler/gost)(Peterson et al., 2020). Lists were arranged in descending order based on relative peptide abundance and analyzed as an ordered query for *Mus musculus* proteins using Benjamini-Hochberg FDR significance threshold with an alpha of 0.05.

Immunohistochemistry of Mouse Brain

Mice were anesthetized with 30 µl of 120 mg/kg Euthanyl (DIN00141704) and then perfused with 10 ml 1x phosphate buffered saline (PBS) and 10 ml 4 % paraformaldehyde (PFA). Brain and spinal cord tissue were collected and stored for 48 hours in 4 % PFA. Brain and spinal cord tissue were then dehydrated in 10 %, 20 % and 30 % sucrose solutions for 48 hours each before being flash frozen in -40 °C isopentane for 1 minute. Tissues were then sectioned at 20 µm and -21 °C on the Thermo Scientific HM 525 NX cryostat at the Louise Pelletier Histology Core at the University of Ottawa and stored free floating in 1x PBS + 0.02 % NaN_3 at 4 °C until use in staining. Brains tissue were incubated for 24 hours in blocking buffer (1.5 % Triton X-100, 5 % cosmic calf serum in 1X PBS), 24 hours in primary antibody (1:500 HA-Tag C29F4 rabbit monoclonal antibody (Cat: 3724S, Cell Signaling Technology), and 1 hour in secondary antibody (1:500 Alexa Fluor 568 donkey anti mouse antibody, Cat: A10037, Lot: 1917938)) with DAPI (1:1,000 Millipore Sigma, D9542-1MG). Tissue was washed 5 times for 5 minutes each in 1x PBS between each treatment and mounted on Fisherbrand Superfrost Plus slides. Slides were left to dry for 24 hours and covered with DAKO mounting medium (Cat: S3023, Lot: 11347938) and #1.5 coverslips.

For the analysis of the synaptic localization of Sumo2, HA-Sumo2 KI brains were used (Figure 1—figure supplement 1B). Mice were anaesthetized (250 mg/Kg Avertin i.p.) and transcardially perfused with PBS and then with 4 % (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 at 4 °C for 10 min. Brains were removed and post-fixed for 1 h at 4 °C. The tissue was cryoprotected in 30 % (w/v) sucrose in phosphate-buffered saline (PBS). Brains were frozen in the cryostat and sagittal 35 µm sections were prepared with a cryostat and collected free-floating in PBS. For immunohistochemistry, sections were pre-incubated for 24 h in PB containing 3 % horse serum (HS), 3 % fish skin gelatin (FSG), and 0.3 % Triton X-100, and were then incubated for 3 days at 4 °C in primary antibodies diluted in PBS containing 3 % HS, 3 % FSG and 0.3 % Triton X-100. After washing repeatedly in PBS and overnight, sections were incubated overnight in dye coupled secondary antibodies, repeatedly washed, and mounted on slides with Aquapolymount (Polysciences). The antibodies used are listed in Table 4.

Primary Cortical Neuron Cultures

Pregnant mice were euthanized between at gestation E14.5-15.5 with 48 mg Pentobarbital Sodium (Bimeda-MTC, 8015E) delivered via intraperitoneal injection. Embryos were removed, washed in chilled PBS (Wisent Bioproducts, 311-010-CL), and cortices were isolated in chilled HBSS (Sigma Aldrich, H9394). Cortices were dissociated for 20 minutes with trypsin (Thermo Scientific, 90305) at 37 °C before adding trypsin inhibitor and DNase solution to quench reaction. Cells were pelleted at 2,500 x g for 5 minutes at 4 °C and washed with trypsin inhibitor with DNase solution. Cortical neurons were pelleted at 2,500 x g for 5 minutes at 4 °C and resuspended in 1 mL Neurobasal outgrowth media (Thermo Scientific, 21103049), supplemented with B-27 (Thermo Scientific, 17504044), N-2 (Thermo Scientific, 21103049), 500 µM L-Glutamine (Wisent Bioproducts, 609-065-EL), and 0.5 % penicillin/streptomycin (GE Healthcare Life Sciences, SV30010) before plating. Cultures were maintained for 7 days in vitro with a half media change after 3-4 days.

Immunofluorescence in Primary Cortical Neurons

Micro Coverglass #1.5 coverslips (Electron Microscopy Sciences) coverslips were pre-coated with poly-D-lysine (50 µg/ml) overnight at 37 °C, then washed with distilled water three times and air-dried at room temperature for at least two hours. Primary mouse cortical neurons were seeded at 75,000 cells per coverslip were seeded and cultured as described in Primary Cortical Neuron Cultures. On day 7, neurons were fixed using 10 % phosphate buffered formalin for (Fisher Chemical, SF100-4) for 10 minutes followed by 3 x 5-minute washes in 1 mL of 1X PBS. Neurons were blocked in 500 µL of blocking buffer (1.5 % Triton X-100, 10 % cosmic calf serum in 1X PBS) for 1 hour, then incubated in 300 µL of primary antibody diluted in blocking buffer overnight at 4 °C. The following day, the neurons were washed for 4 x 5-minute washes in 1 mL of 1X PBS then incubated in 300 µL of secondary antibody diluted in blocking buffer for 2 hours at room temperature. Next, the neurons were washed for 4 x 5-minute washes in 1 mL of 1X PBS, dried, and then placed on slides with Vectashield Antifade Mounting Medium with DAPI (MJS Biolynx Inc., H-1200). Z-Stack images were obtained on a Zeiss AxioObserverZ1 LSM800

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727 Confocal Microscope at 63X magnification through a Z distance of 10 μm per image using

728 optimal 0.27 μm spacing per slice. The dimensions were set to 1,024 x 1,024 pixels.

729

Proximity Ligation Assay in Primary Cortical Neurons

731 Primary mouse cortical neurons were cultured and fixed as described for

732 immunofluorescence experiments. Individual coverslips for Proximity Ligation Assay

733 experiments (PLA) were transferred to 12-well plates and outlined with a hydrophobic

734 pen. Neurons were blocked using 40 μL of Duolink blocking buffer (Sigma Aldrich,

735 DUO82007) at 37 °C for 1 hour and were then washed for 3 x 5 minutes washes in 1 mL

736 1X PBS. Next, the neurons were incubated in 300 μL of primary antibody diluted in

737 blocking buffer (1.5 % Triton X-100, 10 % cosmic calf serum in 1X PBS) overnight at 4

738 °C. The following day, the neurons were washed for 3 x 5 minutes washes in 1 mL of

739 Duolink Wash Buffer A (0.01 M Tris-Base, 0.15 M NaCl, 0.05 % Tween-20, pH 7.4)

740 followed by incubation in 40 μL of Duolink PLA MINUS (Sigma Aldrich, DUO82004) and

741 PLUS probes (Sigma Aldrich, DUO82002) at 37 °C for 1 hour. Duolink PLA probes were

742 diluted in antibody diluent (Sigma Aldrich, DUO82008) at a 1:5 dilution. Neurons were

743 washed for 3 x 5 minutes washes in Duolink Wash Buffer A and then incubated in 40 μL

744 of ligase (Sigma Aldrich, DUO82027) at 37 °C for 30 minutes. Ligase was diluted in 1X

745 ligation buffer (Sigma Aldrich, DUO82009) at a 1:40 dilution. Neurons were washed for 3

746 x 5-minute washes in Duolink Wash Buffer A and then incubated in 40 μL of polymerase

747 (Sigma Aldrich, DUO82028) at 37 °C for 90 minutes. Polymerase was diluted in 1X

748 amplification buffer (Sigma Aldrich, DUO82011) at a 1:80 dilution. Then, neurons were

749 washed 2 x 10 minutes in Duolink Wash Buffer B (0.2 M Tris-Base, 0.1 M NaCl, pH 7.5)

750 and then again in 1 mL of Duolink Wash Buffer B diluted at 1:100 for 1 minute. Coverslips

751 were briefly air dried and then mounted on slides using Vectashield Antifade Mounting

752 Medium with DAPI. Z-Stack images were obtained on a Zeiss AxioObserverZ1 LSM800

753 Confocal Microscope at 63X magnification with a 5X digital zoom through a Z distance of

754 10 μm per image using optimal 0.27 μm spacing per slice with dimensions set to 512 x

755 512 pixels. Images were analyzed and quantified using the Spots function on the Imaris

756 (ver. 9.9.1 Bitplane, Switzerland) software. Localization of the foci (nuclear versus

757 cytoplasmic) was determined using the Orthogonal Views function for colocalization with

758 DAPI signal.

759

Tissue Lysis

761 Dissected mouse organs were lysed in 1X RIPA buffer (9.1 mM Na_2HPO_4, 1.7 mM

762 NaH_2PO_4, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 1X

763 protease inhibitor (GenDEPOT, P3100), 1X phosphatase inhibitor (GenDEPOT, P3200)

764 and 50 mM N-ethylmaleimide (Sigma-Aldrich, E3876-5G) using a Dounce homogenizer.

765 Tissue lysates were centrifuged at ~21,000 x g for 15 minutes at 4°C and the resulting

766 supernatant was transferred into a fresh microcentrifuge tube.

Subcellular fractionation

768 Western blot analysis of brain subcellular fraction was performed as described

769 previously(Jones and Matus, 1974; Tirard et al., 2012).

770

Western blot analysis

772 SDS-PAGE was performed with standard discontinuous gels or with commercially

773 available 4%-12% Bis-Tris gradient gels (Invitrogen). Western blots were probed using

774 primary and secondary antibodies as indicated in Table 4. Blots were routinely developed

775 using enhanced chemiluminescence (GE Healthcare) and imaged using an INTAS ECL

776 Chemostar PLUS Imager HR 6.0. For quantitative Western blotting, transferred proteins

777 were visualized using the total protein stain MemCode (Invitrogen). Western blot signals

778 were visualized using an INTAS ECL Chemostar PLUS Imager HR 6.0 apparatus and

779 quantified using ImageJ, and a ratio of the antibody signal relative to the total protein stain

780 as revealed by the MemCode was performed.

Confocal Imaging and Image analysis

782 MAP2, HA-Sumo2, DAPI and the respective synaptic marker fluorescence intensities

783 were acquired from the hippocampal CA3 pyramidal layer and the *stratum radiatum*. In

784 particular, multi-channel z-stacks of 354 x 354 μm-sized fields of view were acquired with

785 a 40x oil immersion objective (1.4 NA) of a Nikon Ti, Yokogawa W1 spinning disk

786 microscope with Andor iXon Ultra 888 EMCCD camera. A custom macro written within

787   the FIJI software package (Schindelin et al., 2012) automatically identified the z-plane of

788   highest HA-Sumo2 intensity, out of which all channels were extracted. The DAPI, MAP2

789   and synaptic marker channel of this z-plane were segmented with the Trainable WEKA

790   Segmentation FIJI plugin (Arganda-Carreras et al., 2017), resulting in binary masks

791   (Figure 3A, bottom row) that were subsequently used to extract the respective HA-Sumo2

792   mean intensity.

793   To more closely investigate HA-Sumo2 colocalization with the respective synaptic

794   markers, high-resolution, multi-channel fields of view (x, y: 30, 30 μm) of the respective

795   stains within the Mossy fiber region were acquired using 63x oil-immersed objective

796   (NA=1.4) on a Leica SP8 confocal laser scanning microscope. Oversampled (x, y, z: 30,

797   30, 100 nm), 1.2 μm-sized z-stacks within the mossy fiber region were acquired for each

798   fluorescent channel. Signal to noise ratio and resolution were subsequently enhanced

799   and sample geometry was corrected with the SVI Huygens deconvolution software

800   package (ver. 22.04, Hilversum, Netherlands). To obtain object-based colocalization

801   information, an Imaris (ver. 9.9.1 Bitplane, Switzerland) batch process was created to

802   automatically process all three-dimensional fields-of-view identically. All HA-Sumo2 and

803   the respective synaptic marker objects were created, yielding the total object number for

804   the respective stain. Synaptic marker objects were designated as collocating with the HA-

805   Sumo2 objects only if they overlapped with HA-Sumo2 objects and if they contained a

806   minimum HA-Sumo2 average intensity. Data resulting from both analysis workflows was

807   assembled and quantified with the KNIME software package (ver. 4.5.2, Zurich,

808   Switzerland).

809

810   **Statistical Analysis**

811   Statistical tests were performed using PRISM 9.4. Test type was picked based on the

812   number of comparisons made. Levels of statistical significance are indicated in figure

813   legends.

814

815

Figure Legends

Figure 1: HA-epitope knock-in enables direct comparisons of differential levels of Sumo1 and Sumo2 and their conjugates in the murine central nervous system.

Western blot analysis of various regions of His_6-HA-Sumo1 and His_6-HA-Sumo2 mouse central nervous system. Brain regions highlighted in the schematic (Top Left) were dissected for protein extraction and analyzed by Western blot using anti-HA antibody. HA signal corresponding to SUMOylated protein (bracket on the left side of the top left panel, HMW: High Molecular Weight) was normalized to ponceau (right top panel) and quantified via densitometry (bottom panels), N=3.

Figure 1—figure supplement 1: Heterozygous His_6-HA-Sumo2 and His_6-HA-Sumo1 mice exhibit normal Sumo levels.

(A). Schematic of His_6-HA-Sumo2 and HA-Sumo2 knock in mice. (B). Anti-Sumo2/3 Western blot analysis of total brain homogenates from heterozygous His_6-HA-Sumo1, His_6-HA-Sumo2 mice and WT controls. Anti-Sumo2/3 signal (bracket on the right side of the top left panel) was analyzed by densitometry. Signal from either heterozygous His_6-HA-Sumo1 or His_6-HA-Sumo2 mice was normalized to the Gapdh signal and is relative to WT controls (right panel), N=4. (C). Anti-Sumo1 Western blot analysis of total brain homogenates from heterozygous His_6-HA-Sumo1, His_6-HA-Sumo2 mice and WT controls. Anti-Sumo1 signal (bracket on the right side of the top left panel) was analyzed by densitometry. Signal from heterozygous His_6-HA-Sumo1 and His_6-HA-Sumo2 mice was normalized to the Gapdh signal (bottom left panel) and is relative to WT controls (right panel), N=4. (D). RT-qPCR analysis of *Sumo1* and *Sumo2* transcript levels in whole brain from heterozygous His_6-HA-Sumo1, His_6-HA-Sumo2 mice and WT controls. *Sumo1* and *Sumo2* transcript levels were normalized to *Hprt1* as a housekeeping control. All statistical tests were analyzed using an ordinary One Way ANOVA with Tukeys multiple comparisons tests.

Figure 2: Whole brain imaging reveals the topographical distribution of Sumo paralogs.

(A). Schematic of brain clearing and light sheet microscopy for whole brain imaging with a 3D Imaris render of a His_6-HA-Sumo2 brain. (B). Heatmaps of anti-HA immunosignal

847   intensity normalized to regional density quantified by NeuN staining and alignment to the

848   Allen Brain Atlas across the His$_6$-HA-Sumo2 KI depicted brain region, averaged between

849   hemibrains and biological replicates (N=2, two hemispheres). (C). Bar plot depicting the

850   relative levels of anti-HA immunosignal per voxel averaged across across His$_6$-HA-

851   Sumo2 brain regions defined by the Allen Brain Atlas. (D). Heatmaps of anti-HA

852   immunosignal intensity normalized to regional density quantified by NeuN staining and

853   alignment to the Allen Brain Atlas across the His$_6$-HA-Sumo1 KI depicted brain region,

854   averaged between hemibrains and biological replicates (N=2, two hemispheres). (E). Bar

855   plot depicting the relative levels of anti-HA immunosignal intensity normalized to regional

856   density across His$_6$-HA-Sumo1 brain regions defined by the Allen Brain Atlas. Each

857   datapoint in (C) and (D) is from the mean intensity from a single hemisphere (N=2, two

858   hemispheres).

859

860   Figure 2—figure supplement 1: Detailed examination of regional anatomical Sumo

861   paralog distribution.

862   (A). Bar plot depicting the relative levels of anti-HA immunosignal in the regions of His$_6$-

863   HA-Sumo2 hippocampus. Each datapoint is the mean intensity from a single hemisphere

864   (N=2, two hemispheres). Middle and Left panels depict a representative image and zoom

865   of the hippocampal formation overlayed with a mask depicting anatomical regions defined

866   by the Allen Brain Atlas. Scale Bar: 1000 μm. (B). Bar plot depicting the relative levels of

867   anti-HA immunosignal across His$_6$-HA-Sumo1brain regions. Middle and Left panels

868   depict a representative image and zoom of the hippocampal formation overlayed with a

869   mask depicting anatomical regions defined by the Allen Brain Atlas. Scale Bar: 1000 μm.

870   (C). Bar plot depicting relative anti-HA immunosignal levels in the primary somatosensory

871   area–mouth (SSp-m) layers of His$_6$-HA-Sumo1 (Top) and His$_6$-HA-Sumo2 (Bottom) brain

872   (D). Bar plot depicting relative anti-HA immunosignal levels in regions of the

873   hypothalamus of His$_6$-HA-Sumo1 (Top) and His$_6$-HA-Sumo2 (Bottom). Each datapoint

874   from the brain atlases depict the mean intensity from a single hemisphere (N=2, two

875   hemispheres). For each abbreviated brain region, refer to Table 1 for legend.

876

877   Figure 2—figure supplement 2: Sumo1 and Sumo2 share similar and distinct anatomical

878   locations and subcellular compartments.

879   Confocal microscopy analysis of anti-HA (red) and DAPI (blue) nuclear labeling of wild-

880   type (untagged, top lane), His$_6$-HA-Sumo1 (middle lane) and His$_6$-HA-Sumo2 KI (bottom

881   lane) cortex layer 2/3, cerebellum, and hippocampal CA1 and CA3 regions. Z-projection

882   images are representative of three independent replicates. Scale bar: 50 µm

883

884   Figure 2—figure supplement video 1: Imaris 3D render of anti-HA immunosignal in whole

885   His$_6$-HA-Sumo1 brain. Scale bar: 3000 µm

886

887   Figure 2—figure supplement video 2: Imaris 3D render of anti-HA immunosignal in whole

888   His$_6$-HA-Sumo2 brain. Scale bar: 3000 µm

889

890   Figure 3: Extra-nuclear localization of Sumo2 in neurons of the hippocampal CA3 region.

891   (A). Anti-HA (purple), DAPI (blue), MAP2 (red), and anti-Shank2 (green) immunostaining

892   of wild-type (left) and HA-Sumo2 KI (right) hippocampal CA3 region. Scale bar: 50 µm.

893   (B) Anti-HA signal in masked regions for each marker (exemplified for Shank2 in the

894   bottom panel A) was quantified in both wild-type (WT) and HA-Sumo2 KI, and average

895   intensity was calculated. N=3, *p<0.05, **p<0.005, ***p<0,0005, ****p<0,0001.

896

897   Figure 3–figure supplement 1: Differential levels and sub-cellular distribution of Sumo1

898   and Sumo2 conjugates in whole mouse brain fractions.

899   (A). Total Protein stain (left panel) and anti-HA Western blot (right panel) analysis of whole

900   brain lysates from homozygous HA-Sumo2 and corresponding WT littermates. The black

901   bar on the right indicates Sumo2 signal used for the quantification shown by the dot plot

902   (right panel).

903   (B and C). Total protein stain (right panel) and anti-HA (left panel) Western blot analysis

904   of subcellular fractions from homozygous His$_6$-HA-Sumo1 (B) and HA-Sumo2 KI (C).

905   Western blot analysis using anti-Synaptophysin and PSD95 (bottom panels) validates the

906   subcellular fractionation procedure. The black line between the HA panel indicates that

907   different exposure times are depicted, as the HA signal in the P1 fraction saturates faster

908 than in the other fractions. The black line on the right of the HA panels indicate the Sumo

909 signal used for quantification in E. H, homogenate; P1, nuclear pellet S1; supernatant

910 after P1 sedimentation; P2, crude synaptosomal pellet; S2, supernatant after P2

911 sedimentantion; P3, pellet after ultracentrifugation of the S2, cytosolic pellet; S3,

912 supernatant after P3 sedimentation; LP1, lysed synaptosomal membranes; LS1,

913 supernatant after LP1 sedimentation; LP2, pellet after sedimentation of the LS1; LS2,

914 supernatant after LP2 sedimentation; SPM, synaptic plasma membranes. (D). Bar plot

915 depicting the quantification of anti-HA signal as indicated by the black line on the right

916 side of each anti-HA Western blot in C and D, relative to the total protein stain.

917

918 Figure 4: Sumo2 localizes at synapses *in vivo*.

919 (A). High magnification images of wild-type (left) and HA-Sumo2 KI (right) hippocampal

920 CA3 region labelled with anti-HA (purple), DAPI (blue), MAP2 (red), and synaptic markers

921 (green) Shank2, Gephyrin, Synapsin1, vGlut1 and vGAT1. Scale bar, 10 μm. (B). High

922 magnification images with equally scaled anti-HA intensity corresponding to the insets

923 (white boxes) in A showing anti-HA (purple) immunostaining of wild-type (left) and HA-

924 Sumo2 KI (right) hippocampal CA3 region immunolabelled with the synaptic markers

925 (green) Shank2, Gephyrin, Synapsin1, vGLUT1 and vGAT1. Scale bar: 5 μm. N=3. (C).

926 The percentage of co-localization between each synaptic marker and the anti-HA signal

927 was quantified in both wild-type (WT) and HA-Sumo2 KI. N=3, *p<0.05, **p<0.005,

928 ****p<0,0001.

929

930 Figure 4–figure supplement 1: Imaris-based representation of the synaptic Sumo2 co-

931 localization with Shank2 and Synapsin1.

932 Blended representation generated in Imaris using images from Figure 4B and depicting

933 the colocalization between HA-Sumo2 and Shank2 (top panels) and Synapsin 1 (bottom

934 panels). The right panels include the MAP2 (grey) immunolabeling. Scale bar: 5 μm.

935

936 Figure 5: Neuronal Sumo2 has shared and distinct substrates compared to Sumo1 *in*

937 *vivo.*

31

938 (A). Schematic of approach to identify His$_6$-HA-Sumo interactors from the adult mouse
939 brain via anti-HA affinity immunoprecipitation and mass spectrometry (N=4). (B). Heat
940 map depicting the relative peptide abundance of His$_6$-HA-Sumo2 interactors relative to
941 levels in His$_6$-HA-Sumo1 immunoprecipitation. (C). gProfiler2 Gene Ontology analysis for
942 His$_6$-HA-Sumo2 interactors. (D). Anti-HA affinity Immunoprecipitation followed by
943 Western Blot analysis of His$_6$-HA-Sumo1 and His$_6$-HA-Sumo2 interactors as listed on the
944 left side (N=3-6). Black arrowheads indicate protein SUMOylation inferred by shift in
945 molecular weight.

946

947 Figure 5—figure supplement 1: Neuronal Sumo1 has shared and distinct substrates
948 compared to Sumo2 *in vivo.*
949 (A). Anti-HA Western blot analysis of eluates from anti-HA affinity immunoprecipitation
950 (IP: HA) from WT, His$_6$-HA-Sumo1, and His$_6$-HA-Sumo2 mouse brain lysates
951 demonstrating HA signal patterns of Sumo1 and Sumo2 and their corresponding
952 conjugates (B). Heat map depicting the relative peptide abundance of His$_6$-HA-Sumo1
953 interactors relative to levels in His$_6$-HA-Sumo2 immunoprecipitation. (C). gProfiler2 Gene
954 Ontology analysis for His$_6$-HA-Sumo1 interactors. (D). Venn diagram depicting unique
955 and common His$_6$-HA-Sumo1 and His$_6$-HA-Sumo2 interactors.

956

957 Figure 6: Established and newly identified Sumo2 substrates are present both in nuclear
958 and extranuclear compartments in neurons.
959 (A). Schematic of the proximity ligation assay (PLA) strategy for native Sumo2/3 and
960 target proteins in WT primary cortical neurons. (B). The relative proportion of PLA foci
961 between the selected target proteins and Sumo2/3 was quantified and normalized within
962 the nucleus (grey) versus outside of the nucleus (red) (N=3). (C) Representative Z-
963 projected immunofluorescent images and PLA assays for selected target proteins
964 identified from the mass spectrometry screen (N=3). White arrowheads indicate
965 cytoplasmic PLA foci. Scale bar: 10 µm.

966

967 Figure 6—figure supplement 1: Localization of Sumo2 to extranuclear compartments and
968 proof of PLA assay specificity.

969   (A). Confocal microscopy analysis of anti-Map2 (purple) and anti-HA (grey)

970   immunolabeling of WT (top lanes), His_6-HA-Sumo1 (middle lanes), and His_6-HA-Sumo2

971   primary cortical neurons (bottom lanes). Scale bar: 10 μm. N=3 (B). Bar plot depicting the

972   total number of nuclear (left plot) and cytoplasmic (right plot) PLA foci for the indicated

973   antibody (N=3). Data are presented as a mean ± S.E.M. (C). Representative Z-projected

974   images of proximity ligation assays performed with only a single antibody (indicated on

975   top, top lanes) merged with DAPI (bottom panels). Scale bar: 10 μm.

976

997

COMPETING INTERESTS

999   The authors have no competing interests to declare.

1000

REFERENCES

Andreev VI, Yu C, Wang J, Schnabl J, Tirian L, Gehre M, Handler D, Duchek P, Novatchkova M, Baumgartner L, Meixner K, Sienski G, Patel DJ, Brennecke J. 2022. Panoramix SUMOylation on chromatin connects the piRNA pathway to the cellular heterochromatin machinery. *Nat Struct Mol Biol* **29**:130–142. doi:10.1038/S41594-022-00721-X

Banani SF, Rice AM, Peeples WB, Lin Y, Jain S, Parker R, Rosen MK. 2016. Compositional Control of Phase-Separated Cellular Bodies. *Cell* **166**:651–663. doi:10.1016/J.CELL.2016.06.010

Basu-Shrivastava M, Mojsa B, Mora S, Robbins I, Bossis G, Lassot I, Desagher S. 2022. Trim39 regulates neuronal apoptosis by acting as a SUMO-targeted E3 ubiquitin-ligase for the transcription factor NFATc3. *Cell Death Differ*. doi:10.1038/S41418-022-01002-2

Bernstock JD, Yang W, Ye DG, Shen Y, Pluchino S, Lee YJ, Hallenbeck JM, Paschen W. 2018. SUMOylation in brain ischemia: Patterns, targets, and translational implications. *J Cereb Blood Flow Metab* **38**:5–16. doi:10.1177/0271678X17742260

Besnault-Mascard L, Leprince C, Auffredou MT, Meunier B, Bourgeade MF, Camonis J, Lorenzo HK, Vazquez A. 2005. Caspase-8 sumoylation is associated with nuclear localization. *Oncogene* **24**:3268–3273. doi:10.1038/SJ.ONC.1208448

Bohren KM, Nadkarni V, Song JH, Gabbay KH, Owerbach D. 2004. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *Journal of Biological Chemistry* **279**:27233–27238. doi:10.1074/jbc.M402273200

Bouchard D, Wang W, Yang WC, He S, Garcia A, Matunis MJ. 2021. SUMO paralogue–specific functions revealed through systematic analysis of human knockout cell lines and gene expression data. *Mol Biol Cell* **32**:1849. doi:10.1091/MBC.E21-01-0031

Celen AB, Sahin U. 2020. Sumoylation on its 25th anniversary: mechanisms, pathology, and emerging concepts. *FEBS Journal*. doi:10.1111/febs.15319

Cha K, Sen P, Raghunayakula S, Zhang XD. 2015. The Cellular Distribution of RanGAP1 Is Regulated by CRM1-Mediated Nuclear Export in Mammalian Cells. *PLoS One* **10**. doi:10.1371/JOURNAL.PONE.0141309

Chen LC, Hsieh YL, Tan GYT, Kuo TY, Chou YC, Hsu PH, Hwang-Verslues WW. 2021. Differential effects of SUMO1 and SUMO2 on circadian protein PER2 stability and function. *Sci Rep* **11**. doi:10.1038/S41598-021-93933-Y

Chu Y, Yang X. 2011. SUMO E3 ligase activity of TRIM proteins. *Oncogene* **30**:1108–1116. doi:10.1038/onc.2010.462

Chymkowitch P, Enserink JM. 2018. Regulation of tRNA synthesis by posttranslational modifications of RNA polymerase III subunits. *Biochim Biophys Acta Gene Regul Mech* **1861**:310–319. doi:10.1016/J.BBAGRM.2017.11.001

Chymkowitch P, Nguéa P A, Aanes H, Robertson J, Klungland A, Enserink JM. 2017. TORC1-dependent sumoylation of Rpc82 promotes RNA polymerase III assembly and activity. *Proc Natl Acad Sci U S A* **114**:1039–1044. doi:10.1073/PNAS.1615093114

1045   Citro S, Chiocca S. 2013. Sumo paralogs: Redundancy and divergencies. *Frontiers in*
1046   *Bioscience - Scholar* **5 S**:544–553. doi:10.2741/S388/PDF

1047   Correa-Vázquez JF, Juárez-Vicente F, García-Gutiérrez P, Barysch S v., Melchior F,
1048   García-Domínguez M. 2021. The Sumo proteome of proliferating and neuronal-
1049   differentiating cells reveals Utf1 among key Sumo targets involved in neurogenesis.
1050   *Cell Death & Disease 2021 12:4* **12**:1–14. doi:10.1038/s41419-021-03590-2

1051   Daniel JA, Cooper BH, Palvimo JJ, Zhang FP, Brose N, Tirard M. 2018. Response:
1052   Commentary: Analysis of SUMO1-conjugation at synapses. *Front Cell Neurosci*
1053   **12**:117. doi:10.3389/FNCEL.2018.00117/BIBTEX

1054   Daniel James A., Cooper BH, Palvimo JJ, Zhang FP, Brose N, Tirard M. 2017. Analysis
1055   of SUMO1-conjugation at synapses. *Elife* **6**. doi:10.7554/ELIFE.26338

1056   Daniel James A, Cooper BH, Palvimo JJ, Zhang F-P, Brose N, Tirard M. 2017. Analysis
1057   of SUMO1-conjugation at synapses. *Elife* **6**. doi:10.7554/eLife.26338

1058   Desterro JMP, Thomson J, Hay RT. 1997. Ubch9 conjugates SUMO but not ubiquitin.
1059   *FEBS Lett* **417**:297–300. doi:10.1016/S0014-5793(97)01305-7

1060   Drunen R van, Eckel-Mahan K. 2021. Circadian Rhythms of the Hypothalamus: From
1061   Function to Physiology. doi:10.3390/clockssleep3010012

1062   Feldmeyer D. 2012. Excitatory neuronal connectivity in the barrel cortex. *Front*
1063   *Neuroanat* **0**:24. doi:10.3389/FNANA.2012.00024/BIBTEX

1064   Gareau JR, Reverter D, Lima CD. 2012. Determinants of small ubiquitin-like modifier 1
1065   (SUMO1) protein specificity, E3 ligase, and SUMO-RanGAP1 binding activities of
1066   nucleoporin RanBP2. *J Biol Chem* **287**:4740–4751. doi:10.1074/JBC.M111.321141

1067   Garvin AJ, Lanz AJ, Morris JR. 2022. SUMO monoclonal antibodies vary in sensitivity,
1068   specificity, and ability to detect SUMO conjugate types. *bioRxiv*.
1069   doi:10.1101/2022.03.19.484974

1070   Geiss-Friedlander R, Melchior F. 2007. Concepts in sumoylation: a decade on. *Nat Rev*
1071   *Mol Cell Biol* **8**:947–956. doi:10.1038/NRM2293

1072   Gong L, Kamitani T, Fujise K, Caskey LS, Yeh ETH. 1997. Preferential interaction of
1073   sentrin with a ubiquitin-conjugating enzyme, Ubc9. *Journal of Biological Chemistry*
1074   **272**:28198–28201. doi:10.1074/jbc.272.45.28198

1075   Gong L, Li B, Millas S, Yeh ETH. 1999. Molecular cloning and characterization of
1076   human AOS1 and UBA2, components of the sentrin-activating enzyme complex.
1077   *FEBS Lett* **448**:185–189. doi:10.1016/S0014-5793(99)00367-1

1078   Hakre S, Tussie-Luna MI, Ashworth T, Novina CD, Settleman J, Sharp PA, Roy AL.
1079   2006. Opposing functions of TFII-I spliced isoforms in growth factor-induced gene
1080   expression. *Mol Cell* **24**:301–308. doi:10.1016/J.MOLCEL.2006.09.005

1081   Hasegawa Y, Yoshida D, Nakamura Y, Sakakibara SI. 2014. Spatiotemporal distribution
1082   of SUMOylation components during mouse brain development. *Journal of*
1083   *Comparative Neurology* **522**:3020–3036. doi:10.1002/CNE.23563

1084   Hendriks IA, Lyon D, Su D, Skotte NH, Daniel JA, Jensen LJ, Nielsen ML. 2018. Site-
1085   specific characterization of endogenous SUMOylation across species and organs.
1086   *Nature Communications 2018 9:1* **9**:1–17. doi:10.1038/s41467-018-04957-4

1087   Hendriks IA, Vertegaal ACO. 2016. A comprehensive compilation of SUMO proteomics.
1088   *Nat Rev Mol Cell Biol* **17**:581–595. doi:10.1038/nrm.2016.81

1089   Hendriks IA, Vertegaal ACO. 2015. SUMO in the DNA damage response. *Oncotarget*
1090   **6**:15734–15735. doi:10.18632/ONCOTARGET.4605

1091 Ilic D, Magnussen HM, Tirard M. 2022. Stress - Regulation of SUMO conjugation and of
1092   other Ubiquitin-Like Modifiers. *Semin Cell Dev Biol*.
1093   doi:10.1016/j.semcdb.2021.12.010
1094 Johnson ES, Blobel G. 1997. Ubc9p is the conjugating enzyme for the ubiquitin-like
1095   protein Smt3p. *Journal of Biological Chemistry* **272**:26799–26802.
1096   doi:10.1074/jbc.272.43.26799
1097 Johnson JO, Pioro EP, Boehringer A, Chia R, Feit H, Renton AE, Pliner HA, Abramzon
1098   Y, Marangi G, Winborn BJ, Gibbs JR, Nalls MA, Morgan S, Shoai M, Hardy J,
1099   Pittman A, Orrell RW, Malaspina A, Sidle KC, Fratta P, Harms MB, Baloh RH,
1100   Pestronk A, Weihl CC, Rogaeva E, Zinman L, Drory VE, Borghero G, Mora G,
1101   Calvo A, Rothstein JD, Drepper C, Sendtner M, Singleton AB, Taylor JP, Cookson
1102   MR, Restagno G, Sabatelli M, Bowser R, Chio` A, Traynor BJ, Moglia C,
1103   Cammarosano S, Canosa A, Gallo S, Brunetti M, Ossola I, Marinou K, Papetti L,
1104   Pisano F, Pinter GL, Conte A, Luigetti M, Zollino M, Lattante S, Marangi G, la Bella
1105   V, Spataro R, Colletti T, Battistini S, Ricci C, Caponnetto C, Mancardi G, Mandich
1106   P, Salvi F, Bartolomei I, Mandrioli J, Sola P, Lunetta C, Penco S, Monsurrò MR,
1107   Conforti FL, Tedeschi G, Gambardella A, Quattrone A, Volanti P, Floris G, Cannas
1108   A, Piras V, Marrosu F, Marrosu MG, Murru MR, Pugliatti M, Parish LD, Sotgiu A,
1109   Solinas G, Ulgheri L, Ticca A, Simone I, Logroscino G, Pirisi A. 2014. Mutations in
1110   the Matrin 3 gene cause familial amyotrophic lateral sclerosis. *Nat Neurosci*
1111   **17**:664–666. doi:10.1038/NN.3688
1112 Jones DH, Matus AI. 1974. Isolation of synaptic plasma membrane from brain by
1113   combined flotation-sedimentation density gradient centrifugation. *Biochim Biophys*
1114   *Acta* **356**:276–87. doi:10.1016/0005-2736(74)90268-5
1115 Keiten-Schmitz J, Röder L, Hornstein E, Müller-McNicoll M, Müller S. 2021. SUMO:
1116   Glue or Solvent for Phase-Separated Ribonucleoprotein Complexes and Molecular
1117   Condensates? *Front Mol Biosci* **8**. doi:10.3389/FMOLB.2021.673038
1118 Keiten-Schmitz J, Wagner K, Piller T, Kaulich M, Alberti S, Müller S. 2020. The Nuclear
1119   SUMO-Targeted Ubiquitin Quality Control Network Regulates the Dynamics of
1120   Cytoplasmic Stress Granules. *Mol Cell* **79**:54-67.e7.
1121   doi:10.1016/J.MOLCEL.2020.05.017
1122 Kim SY, Cho JH, Murray E, Bakh N, Choi H, Ohn K, Ruelas L, Hubbert A, McCue M,
1123   Vassallo SL, Keller PJ, Chung K. 2015. Stochastic electrotransport selectively
1124   enhances the transport of highly electromobile molecules. *Proc Natl Acad Sci U S*
1125   *A* **112**:E6274–E6283.
1126   doi:10.1073/PNAS.1510133112/SUPPL_FILE/PNAS.1510133112.SM05.AVI
1127 Krumova P, Weishaupt JH. 2013. Sumoylation in neurodegenerative diseases. *Cell Mol*
1128   *Life Sci* **70**:2123–2138. doi:10.1007/S00018-012-1158-3
1129 Lavado A, Lagutin O v., Chow LML, Baker SJ, Oliver G. 2010. Prox1 is required for
1130   granule cell maturation and intermediate progenitor maintenance during brain
1131   neurogenesis. *PLoS Biol* **8**:43–44. doi:10.1371/JOURNAL.PBIO.1000460
1132 Lee GW, Melchior F, Matunis MJ, Mahajan R, Tian Q, Anderson P. 1998. Modification
1133   of Ran GTPpase-activating protein by the small ubiquitin- related modifier SUMO-1
1134   requires Ubc9, an E2-type ubiquitin-conjugating enzyme homologue. *Journal of*
1135   *Biological Chemistry* **273**:6503–6507. doi:10.1074/jbc.273.11.6503

1136 Lee Y, Chun SK, Kim K. 2015. Sumoylation controls CLOCK-BMAL1-mediated clock
1137   resetting via CBP recruitment in nuclear transcriptional foci. *Biochimica et*
1138   *Biophysica Acta (BBA) - Molecular Cell Research* **1853**:2697–2708.
1139   doi:10.1016/J.BBAMCR.2015.07.005
1140 Liang Q, Deng H, Li X, Wu X, Tang Q, Chang T-H, Peng H, Rauscher FJ, Ozato K, Zhu
1141   F. 2011. Tripartite Motif-Containing Protein 28 Is a Small Ubiquitin-Related Modifier
1142   E3 Ligase and Negative Regulator of IFN Regulatory Factor 7. *The Journal of*
1143   *Immunology* **187**:4754–4763. doi:10.4049/jimmunol.1101704
1144 Mahajan R, Delphin C, Guan T, Gerace L, Melchior F. 1997. A small ubiquitin-related
1145   polypeptide involved in targeting RanGAP1 to nuclear pore complex protein
1146   RanBP2. *Cell* **88**:97–107. doi:10.1016/S0092-8674(00)81862-0
1147 Matunis MJ, Coutavas E, Blobel G. 1996. A novel ubiquitin-like modification modulates
1148   the partitioning of the Ran-GTPase-activating protein RanGAP1 between the
1149   cytosol and the nuclear pore complex. *Journal of Cell Biology* **135**:1457–1470.
1150   doi:10.1083/JCB.135.6.1457
1151 Mo YY, Yu Y, Shen Z, Beck WT. 2002. Nucleolar delocalization of human
1152   topoisomerase I in response to topotecan correlates with sumoylation of the
1153   protein. *J Biol Chem* **277**:2958–2964. doi:10.1074/JBC.M108263200
1154 Mojsa B, Mora S, Bossowski JP, Lassot I, Desagher S. 2015. Control of neuronal
1155   apoptosis by reciprocal regulation of NFATc3 and Trim17. *Cell Death Differ*
1156   **22**:274–286. doi:10.1038/CDD.2014.141
1157 Murray E, Cho JH, Goodwin D, Ku T, Swaney J, Kim S-Y, Choi H, Park Y-G, Park J-Y,
1158   Hubbert A, McCue M, Vassallo S, Bakh N, Frosch MP, Wedeen VJ, Seung HS,
1159   Chung K. 2015. Simple, Scalable Proteomic Imaging for High-Dimensional Profiling
1160   of Intact Systems. *Cell* **163**:1500–1514. doi:10.1016/j.cell.2015.11.025
1161 Nakanishi H. 2003. Neuronal and microglial cathepsins in aging and age-related
1162   diseases. *Ageing Res Rev* **2**:367–381. doi:10.1016/S1568-1637(03)00027-8
1163 Nayak A, Müller S. 2014. SUMO-specific proteases/isopeptidases: SENPs and beyond
1164   1–7. doi:10.1186/s13059-014-0422-2
1165 Niskanen EA, Malinen M, Sutinen P, Toropainen S, Paakinaho V, Vihervaara A,
1166   Joutsen J, Kaikkonen MU, Sistonen L, Palvimo JJ. 2015. Global SUMOylation on
1167   active chromatin is an acute heat stress response restricting transcription. *Genome*
1168   *Biol* **16**:1–19. doi:10.1186/S13059-015-0717-Y/FIGURES/8
1169 Osmanovic A, Förster A, Widjaja M, Auber B, Anibh ·, Das M, Christians A, Brand ·
1170   Frank, Petri S, Weber RG. 2022. A SUMO4 initiator codon variant in amyotrophic
1171   lateral sclerosis reduces SUMO4 expression and alters stress granule dynamics.
1172   *Journal of Neurology 2022* **1**:1–9. doi:10.1007/S00415-022-11126-7
1173 Pan MR, Chang TM, Chang HC, Su JL, Wang HW, Hung WC. 2009. Sumoylation of
1174   Prox1 controls its ability to induce VEGFR3 expression and lymphatic phenotypes
1175   in endothelial cells. *J Cell Sci* **122**:3358–3364. doi:10.1242/JCS.050005
1176 Park YG, Sohn CH, Chen R, McCue M, Yun DH, Drummond GT, Ku T, Evans NB, Oak
1177   HC, Trieu W, Choi H, Jin X, Lilascharoen V, Wang J, Truttmann MC, Qi HW,
1178   Ploegh HL, Golub TR, Chen SC, Frosch MP, Kulik HJ, Lim BK, Chung K. 2018.
1179   Protection of tissue physicochemical properties using polyfunctional crosslinkers.
1180   *Nature Biotechnology 2018 37:1* **37**:73–83. doi:10.1038/nbt.4281

Peterson H, Kolberg L, Raudvere U, Kuzmin I, Vilo J. 2020. gprofiler2 -- an R package for gene list functional enrichment analysis and namespace conversion toolset g: Profiler. *F1000Res* **9**. doi:10.12688/F1000RESEARCH.24956.2/DOI

Pichler A, Gast A, Seeler JS, Dejean A, Melchior F. 2002. The nucleoporin RanBP2 has SUMO1 E3 ligase activity. *Cell* **108**:109–120. doi:10.1016/S0092-8674(01)00633-X

Rajgor D, Hanley JG, Shanahan CM. 2016. Identification of novel nesprin-1 binding partners and cytoplasmic matrin-3 in processing bodies. *Mol Biol Cell* **27**:3894–3902. doi:10.1091/MBC.E16-06-0346

Rawat P, Boehning M, Hummel B, Aprile-Garcia F, Pandit AS, Eisenhardt N, Khavaran A, Niskanen E, Vos SM, Palvimo JJ, Pichler A, Cramer P, Sawarkar R. 2021. Stress-induced nuclear condensation of NELF drives transcriptional downregulation. *Mol Cell* **81**:1013-1026.e11. doi:10.1016/J.MOLCEL.2021.01.016

Richner M, Jager SB, Siupka P, Vaegter CB. 2017. Hydraulic Extrusion of the Spinal Cord and Isolation of Dorsal Root Ganglia in Rodents. *J Vis Exp* **2017**. doi:10.3791/55226

Ripamonti S, Shomroni O, Rhee JS, Chowdhury K, Jahn O, Hellmann KP, Bonn S, Brose N, Tirard M. 2020. SUMOylation controls the neurodevelopmental function of the transcription factor Zbtb20. *J Neurochem* **154**:647–661. doi:10.1111/JNC.15008

Ristic M, Brockly F, Piechaczyk M, Bossis G. 2016. Detection of protein–protein interactions and posttranslational modifications using the proximity ligation assay: Application to the study of the SUMO pathwayMethods in Molecular Biology. Humana Press Inc. pp. 279–290. doi:10.1007/978-1-4939-3756-1_17

Rodriguez MS, Dargemont C, Hay RT. 2001. SUMO-1 Conjugation in Vivo Requires Both a Consensus Modification Motif and Nuclear Targeting. *Journal of Biological Chemistry* **276**:12654–12659. doi:10.1074/jbc.M009476200

Rohira AD, Chen CY, Allen JR, Johnson DL. 2013. Covalent small ubiquitin-like modifier (SUMO) modification of Maf1 protein controls RNA polymerase III-dependent transcription repression. *J Biol Chem* **288**:19288–19295. doi:10.1074/JBC.M113.473744

Rousseaux MWC, de Haro M, Lasagna-Reeves CA, de Maio A, Park J, Jafar-Nejad P, Al-Ramahi I, Sharma A, See L, Lu N, Vilanova-Velez L, Klisch TJ, Westbrook TF, Troncoso JC, Botas J, Zoghbi HY. 2016. TRIM28 regulates the nuclear accumulation and toxicity of both alpha-synuclein and tau. *Elife* **5**:e19809. doi:10.7554/eLife.19809

Sahin U, Jollivet F, Berthier C, de Thé H, Lallemand-Breitenbach V. 2016. Detection of protein SUMOylation in situ by proximity ligation assays. *Methods in Molecular Biology* **1475**:139–150. doi:10.1007/978-1-4939-6358-4_10/FIGURES/3

Saitoh H, Sparrow DB, Shiomi T, Pu RT, Nishimoto T, Mohun TJ, Dasso M. 1998. Ubc9p and the conjugation of SUMO-1 to RanGAP1 and RanBP2. *Current Biology* **8**:121–124. doi:10.1016/S0960-9822(98)70044-2

Salinas S, Briançon-Marjollet A, Bossis G, Lopez MA, Piechaczyk M, Jariel-Encontre I, Debant A, Hipskind RA. 2004. SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1. *J Cell Biol* **165**:767–773. doi:10.1083/JCB.200310136

Sampson DA, Wang M, Matunis MJ. 2001. The Small Ubiquitin-like Modifier-1 (SUMO-1) Consensus Sequence Mediates Ubc9 Binding and is Essential for SUMO-1

Modification. *Journal of Biological Chemistry* **276**:21664–21669. doi:10.1074/jbc.M100006200

Seufert W, Futcher B, Jentsch S. 1995. Role of a ubiquitin-conjugating enzyme in degradation of S- and M-phase cyclins. *Nature 1995 373:6509* **373**:78–81. doi:10.1038/373078a0

Shan S fang, Wang L fang, Zhai J wei, Qin Y, Ouyang H fang, Kong Y ying, Liu J, Wang Y, Xie Y hua. 2008. Modulation of transcriptional corepressor activity of prospero-related homeobox protein (Prox1) by SUMO modification. *FEBS Lett* **582**:3723–3728. doi:10.1016/J.FEBSLET.2008.09.057

Shevchenko A, Tomas H, Havliš J, Olsen J v., Mann M. 2006. In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat Protoc* **1**:2856–2860. doi:10.1038/NPROT.2006.468

Stankova T, Piepkorn L, Bayer TA, Jahn O, Tirard M. 2018. SUMO1-conjugation is altered during normal aging but not by increased amyloid burden. *Aging Cell* **17**. doi:10.1111/ACEL.12760

Tirard M, Brose N. 2016. Systematic Localization and Identification of SUMOylation Substrates in Knock-In Mice Expressing Affinity-Tagged SUMO1. *Methods Mol Biol* **1475**:291–301. doi:10.1007/978-1-4939-6358-4_20

Tirard M, Hsiao H-H, Nikolov M, Urlaub H, Melchior F, Brose N. 2012. In vivo localization and identification of SUMOylated proteins in the brain of His6-HA-SUMO1 knock-in mice. *Proc Natl Acad Sci U S A* **109**:21122–7. doi:10.1073/pnas.1215366110

Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson Å, Kampf C, Sjöstedt E, Asplund A, Olsson IM, Edlund K, Lundberg E, Navani S, Szigyarto CAK, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, Pontén F. 2015. Proteomics. Tissue-based map of the human proteome. *Science* **347**. doi:10.1126/SCIENCE.1260419

Wang L, Rodriguiz RM, Wetsel WC, Sheng H, Zhao S, Liu X, Paschen W, Yang W. 2014a. Neuron-specific Sumo1-3 knockdown in mice impairs episodic and fear memories. *J Psychiatry Neurosci* **39**:259–266. doi:10.1503/JPN.130148

Wang L, Wansleeben C, Zhao S, Miao P, Paschen W, Yang W. 2014b. SUMO 2 is essential while SUMO 3 is dispensable for mouse embryonic development . *EMBO Rep* **15**:878–885. doi:10.15252/embr.201438534

Watanabe M, Takahashi K, Tomizawa K, Mizusawa H, Takahashi H. 2008. Developmental regulation of Ubc9 in the rat nervous system.
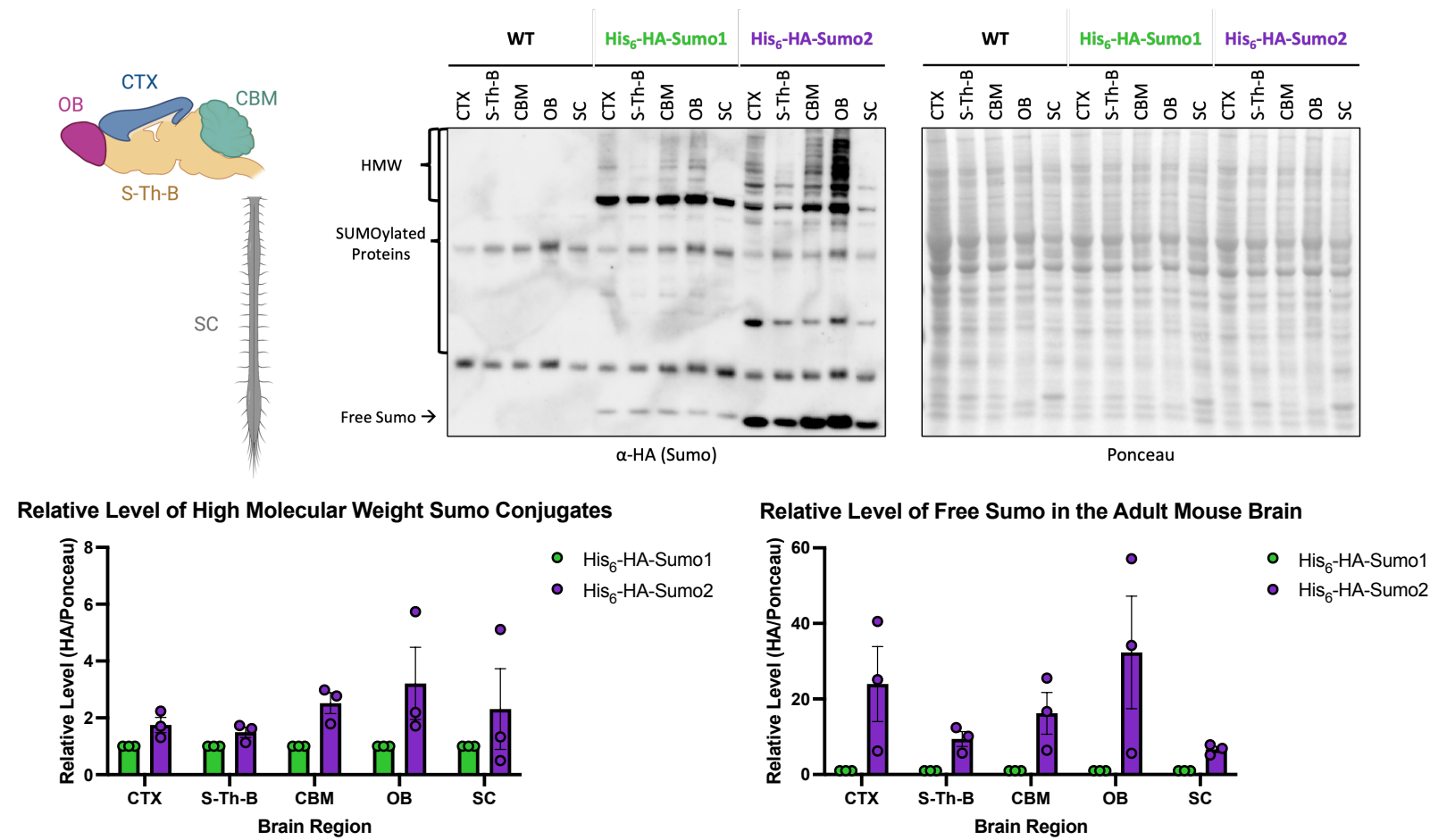
Westman BJ, Verheggen C, Hutten S, Lam YW, Bertrand E, Lamond AI. 2010. A proteomic screen for nucleolar SUMO targets shows SUMOylation modulates the function of Nop5/Nop58. *Mol Cell* **39**:618–631. doi:10.1016/J.MOLCEL.2010.07.025

Xu Z, Au SWN. 2005. Mapping residues of SUMO precursors essential in differential maturation by SUMO-specific protease, SENP1. *Biochem J* **386**:325–330.

Yang W, Paschen W. 2015. SUMO proteomics to decipher the SUMO-modified proteome regulated by various diseases. *Proteomics* **15**:1181–1191. doi:10.1002/PMIC.201400298
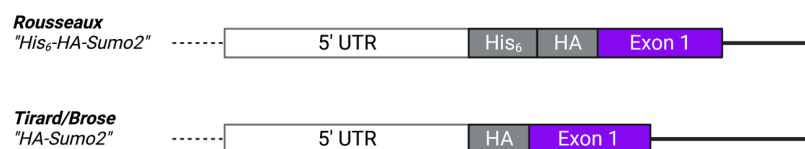
1272 Yu S, Galeffi F, Rodriguiz RM, Wang Z, Shen Y, Lyu J, Li R, Bernstock JD, Johnson
1273   KR, Liu S, Sheng H, Turner DA, Wetsel WC, Paschen W, Yang W. 2020. Small
1274   ubiquitin-like modifier 2 (SUMO2) is critical for memory processes in mice. *FASEB*
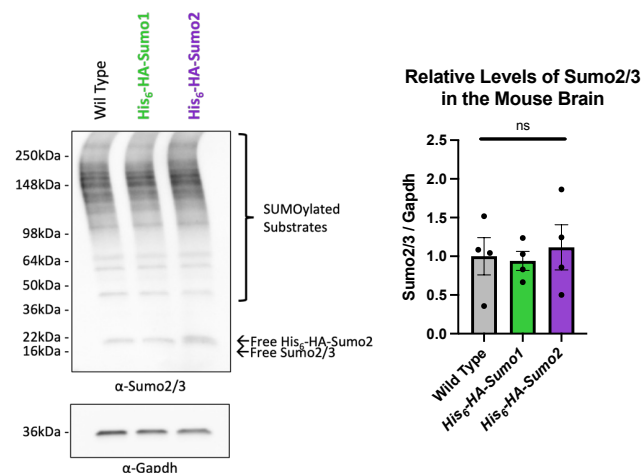1275   *J* **34**:14750–14767. doi:10.1096/FJ.202000850RR
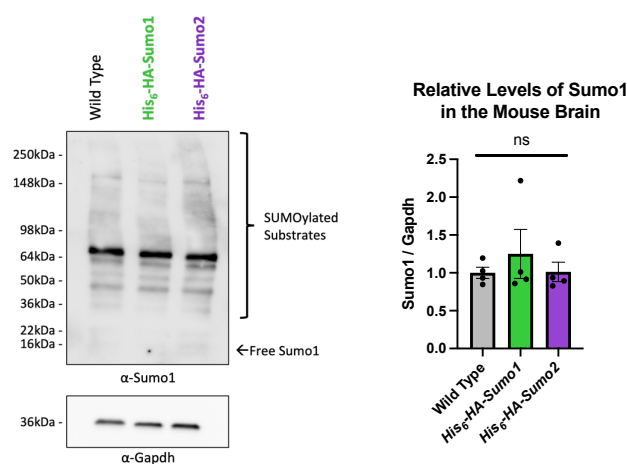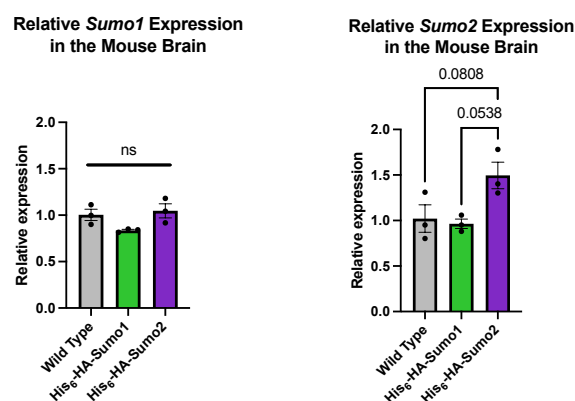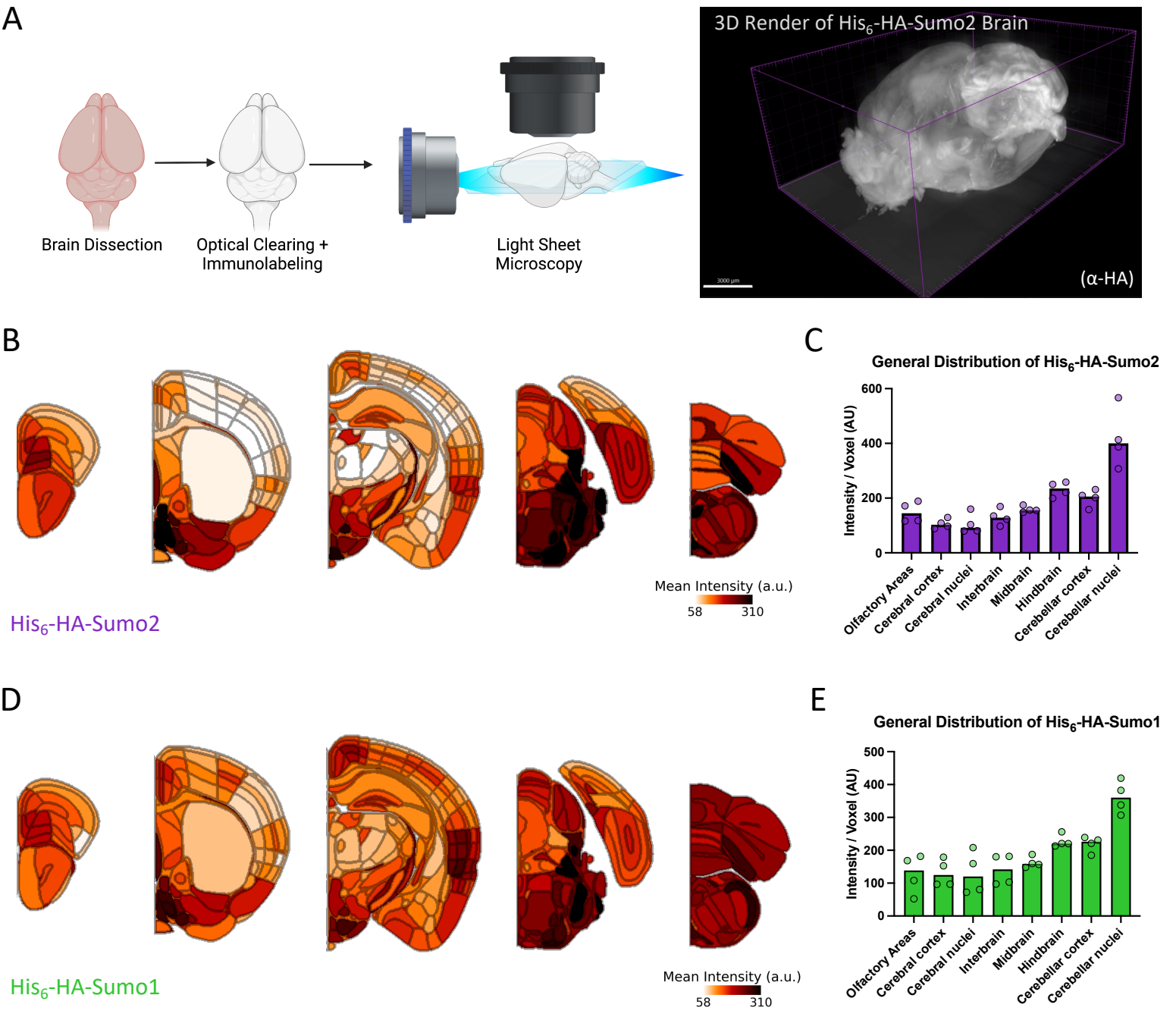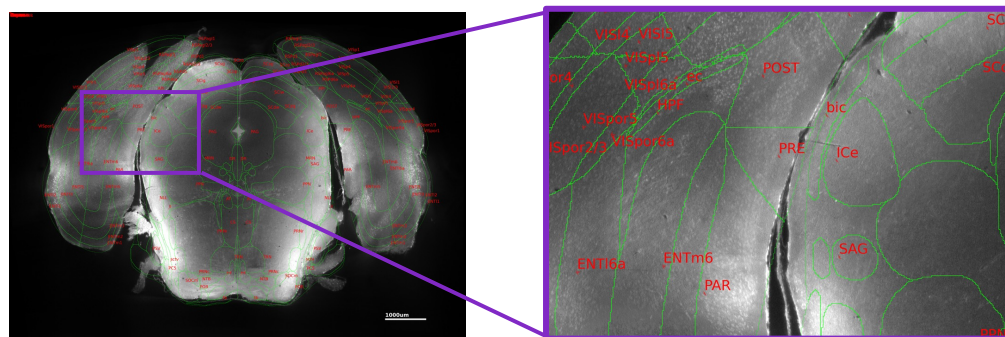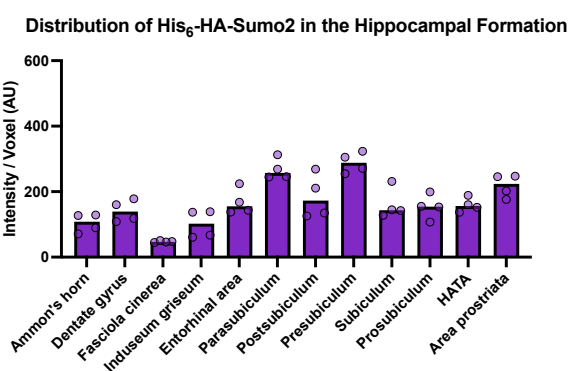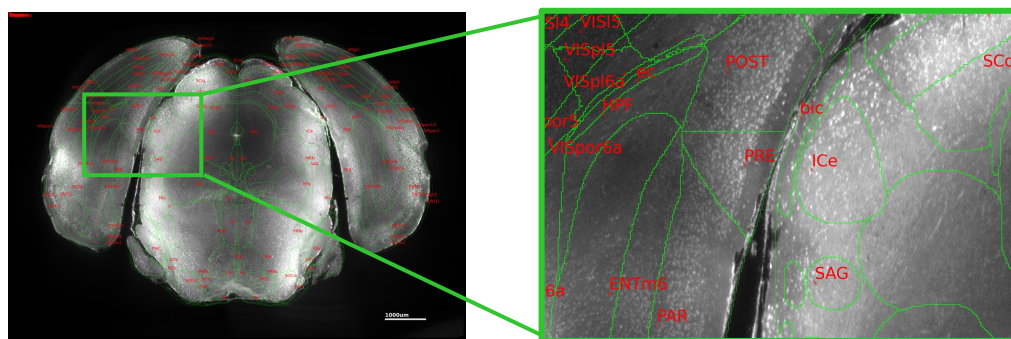1276

Figure 1

Figure 1–figure supplement 1

Figure 2

A

Brain Dissection

Optical Clearing + Immunolabeling

Light Sheet Microscopy

3D Render of His$_6$-HA-Sumo2 Brain

3000 µm

(α-HA)

B

His$_6$-HA-Sumo2

Mean Intensity (a.u.)

58   310

C

General Distribution of His$_6$-HA-Sumo2

Intensity / Voxel (AU)

Olfactory Areas, Cerebral cortex, Cerebral nuclei, Interbrain, Midbrain, Hindbrain, Cerebellar cortex, Cerebellar nuclei

D

His$_6$-HA-Sumo1

Mean Intensity (a.u.)

58   310

E

General Distribution of His$_6$-HA-Sumo1

Intensity / Voxel (AU)

Olfactory Areas, Cerebral cortex, Cerebral nuclei, Interbrain, Midbrain, Hindbrain, Cerebellar cortex, Cerebellar nuclei

Figure 2–figure supplement 1

Figure 2—figure supplement 2

Figure 3

Figure 3 – figure supplement 1

A

B

His6-HA-Sumo1

WB: HA

WB: Synaptophysin

WB: PSD95

Total Protein

C
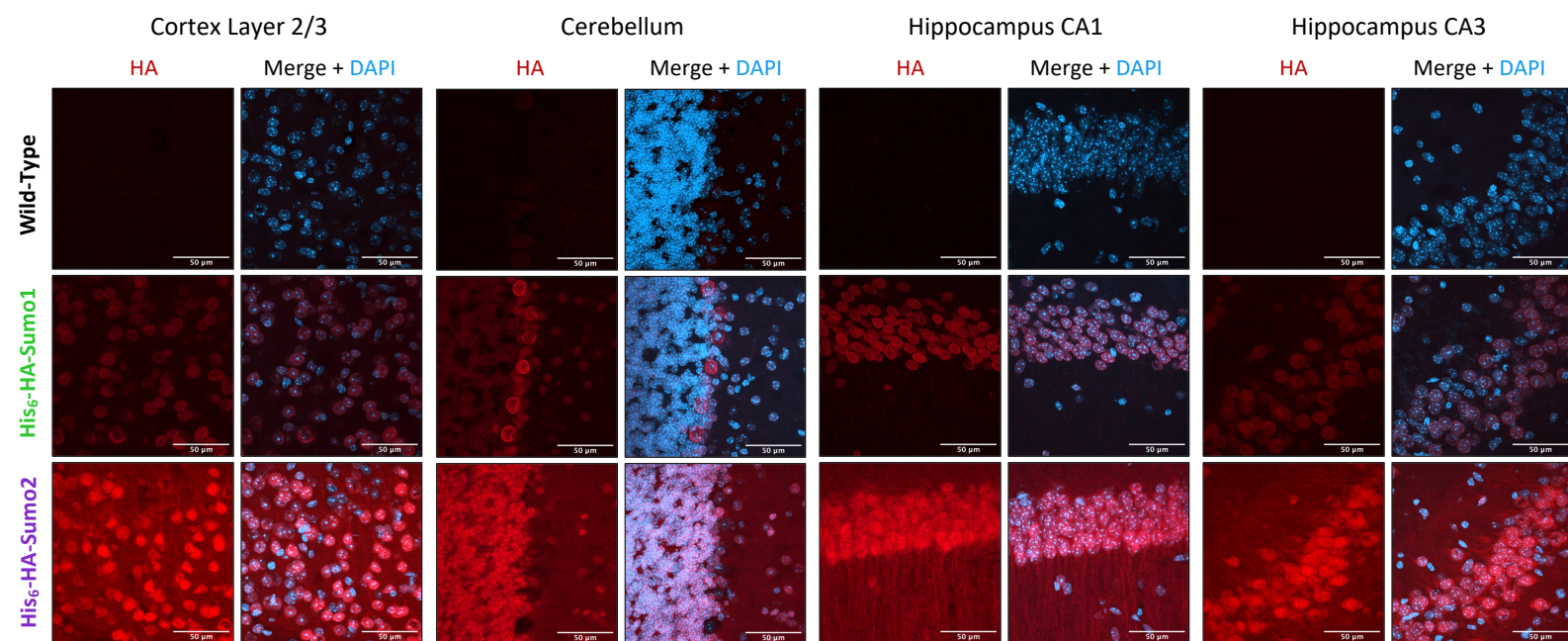
HA-Sumo2

WB: HA

WB: Synaptophysin

WB: PSD95

Total Protein

D

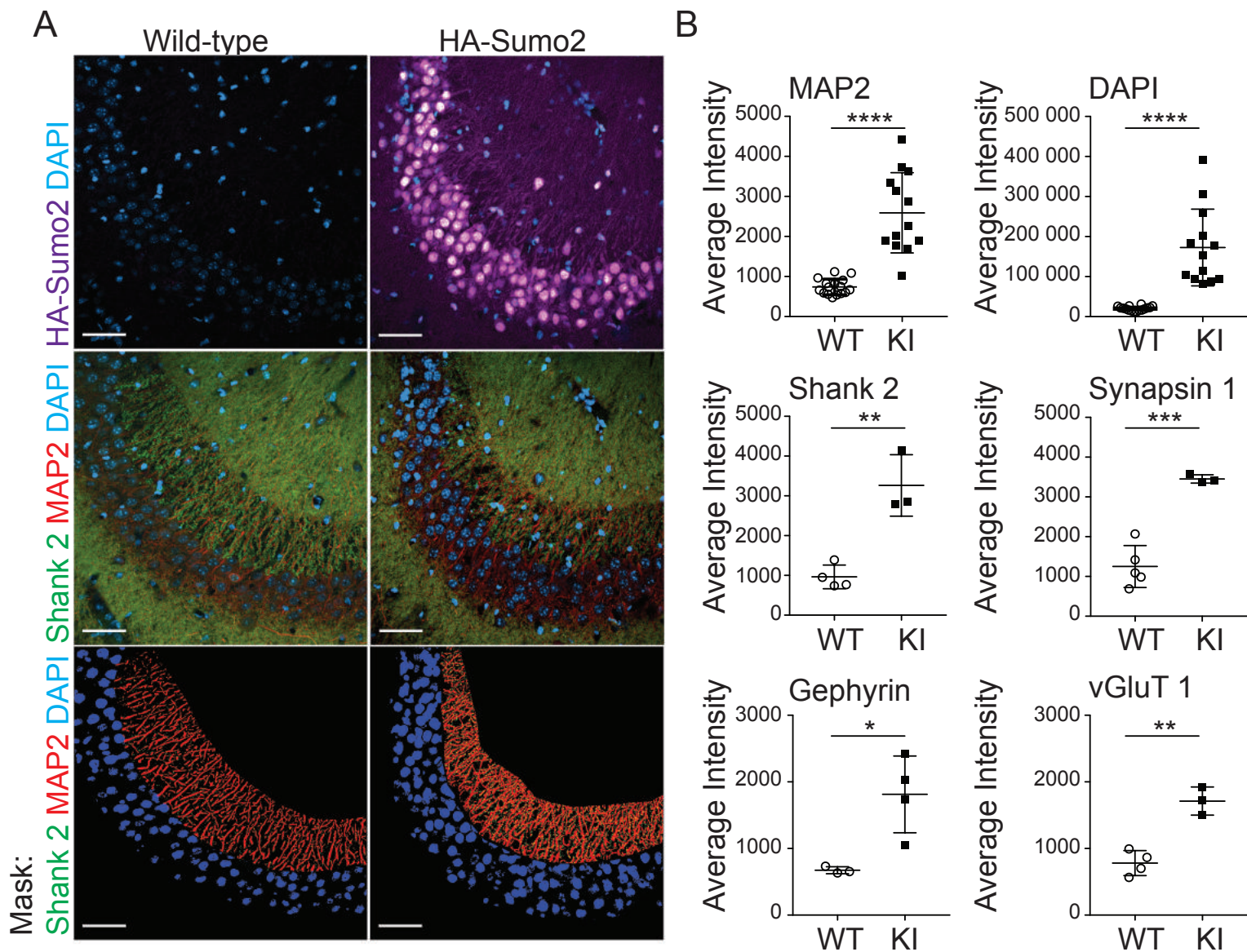# Figure 4

Figure 4—figure supplement 1

Figure 5

A — Whole Mouse Brain Dissection → Denaturing Lysis → HA Immunoprecipitation → Mass Spectrometry

B — Enrichment of His$_6$-HA-Sumo2 Interactors Relative to His$_6$-HA-Sumo1

C

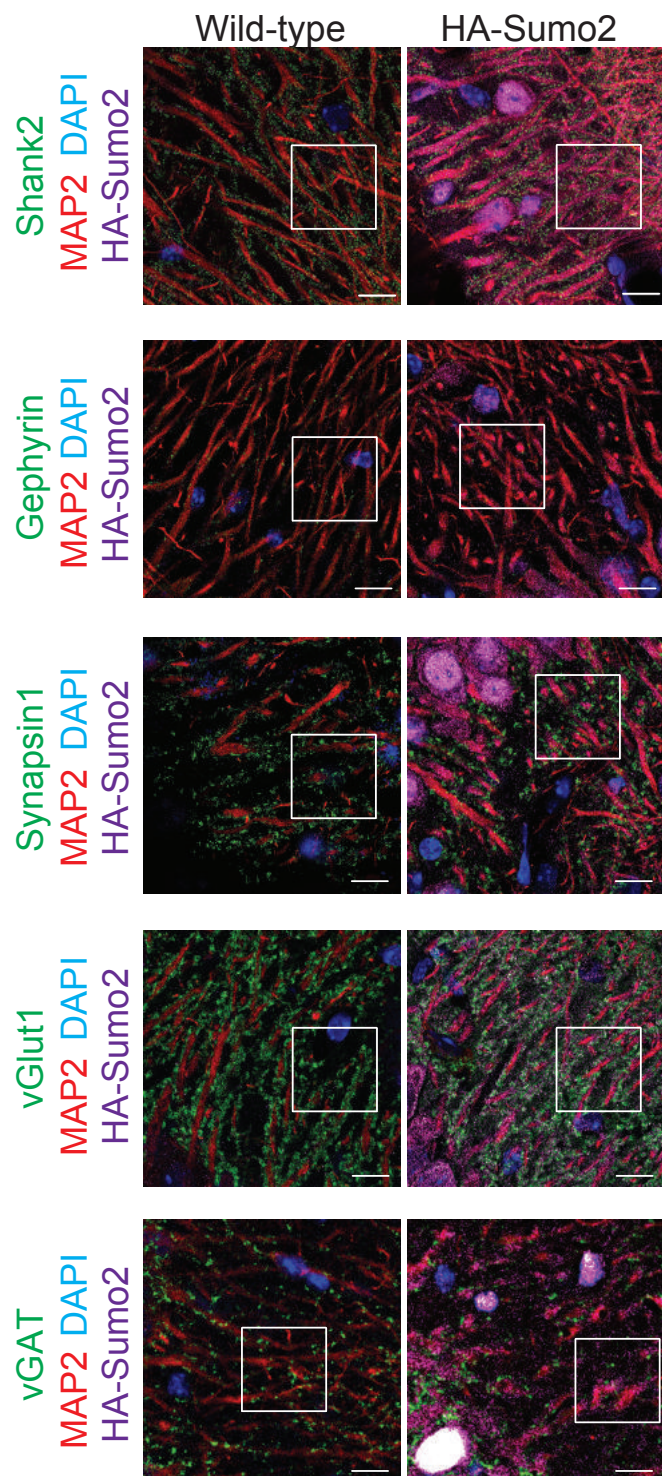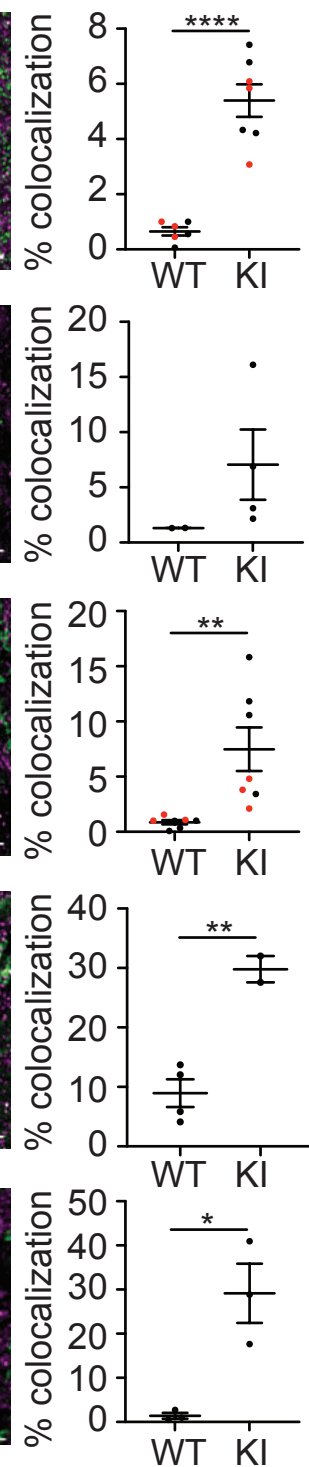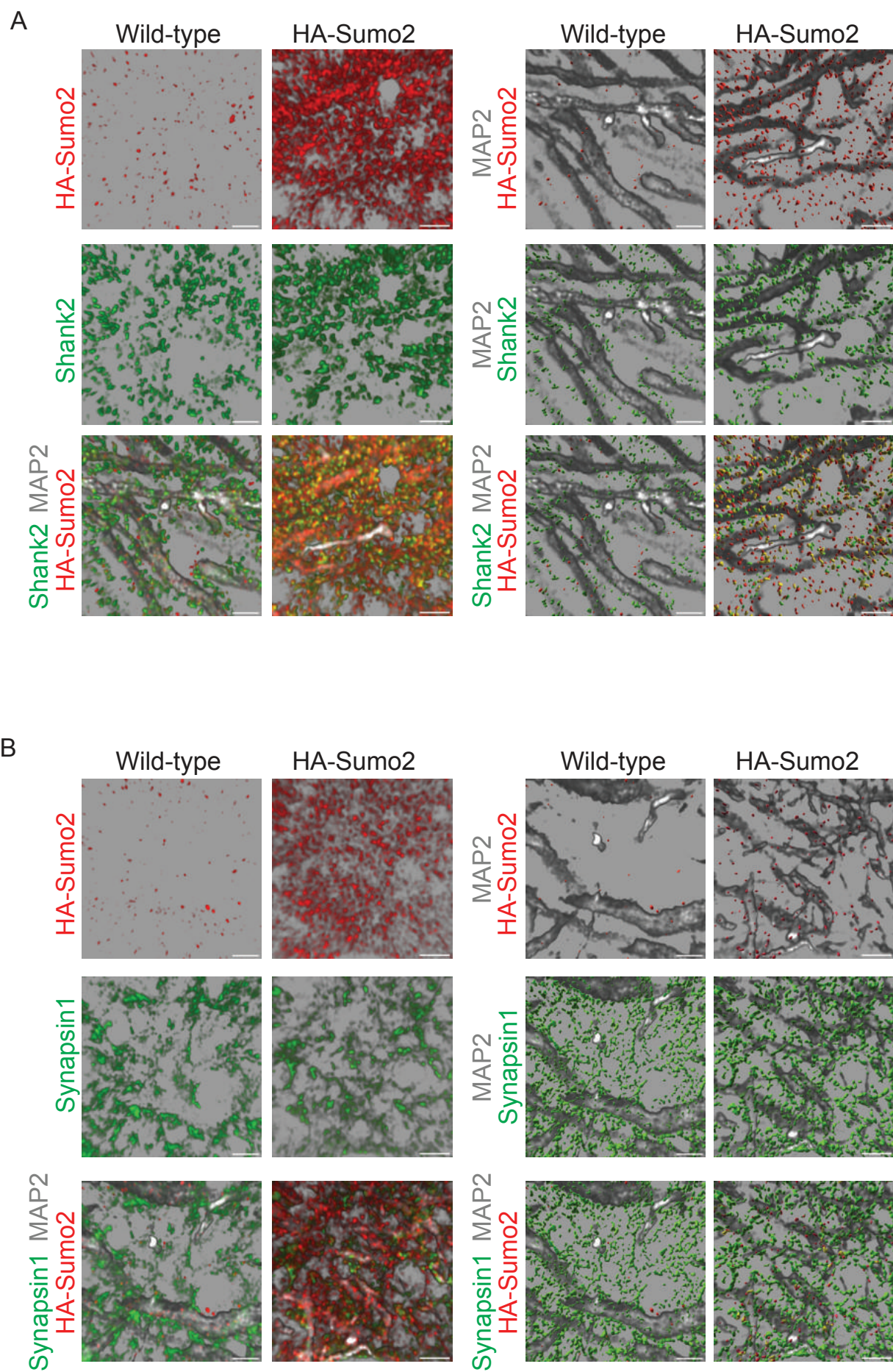| ID	Source	Term ID	Term Name	p$_{adj}$ (query_1)
1	GO:BP	GO:0016925	protein sumoylation	4.392×10^{-13}
2	GO:MF	GO:0019899	enzyme binding	1.977×10^{-10}
3	GO:MF	GO:0019789	SUMO transferase activity	1.550×10^{-9}
4	GO:MF	GO:0044388	small protein activating enzyme binding	9.720×10^{-9}
5	GO:BP	GO:0018205	peptidyl-lysine modification	8.689×10^{-8}
6	GO:BP	GO:0070647	protein modification by small protein conjugati...	7.630×10^{-7}
7	REAC	REAC:R-MMU-2990846	SUMOylation	1.279×10^{-7}

D

Figure 5—figure supplement 1

A

B

Enrichment of His$_6$-HA-Sumo1 Interactors Relative to His$_6$-HA-Sumo2

C

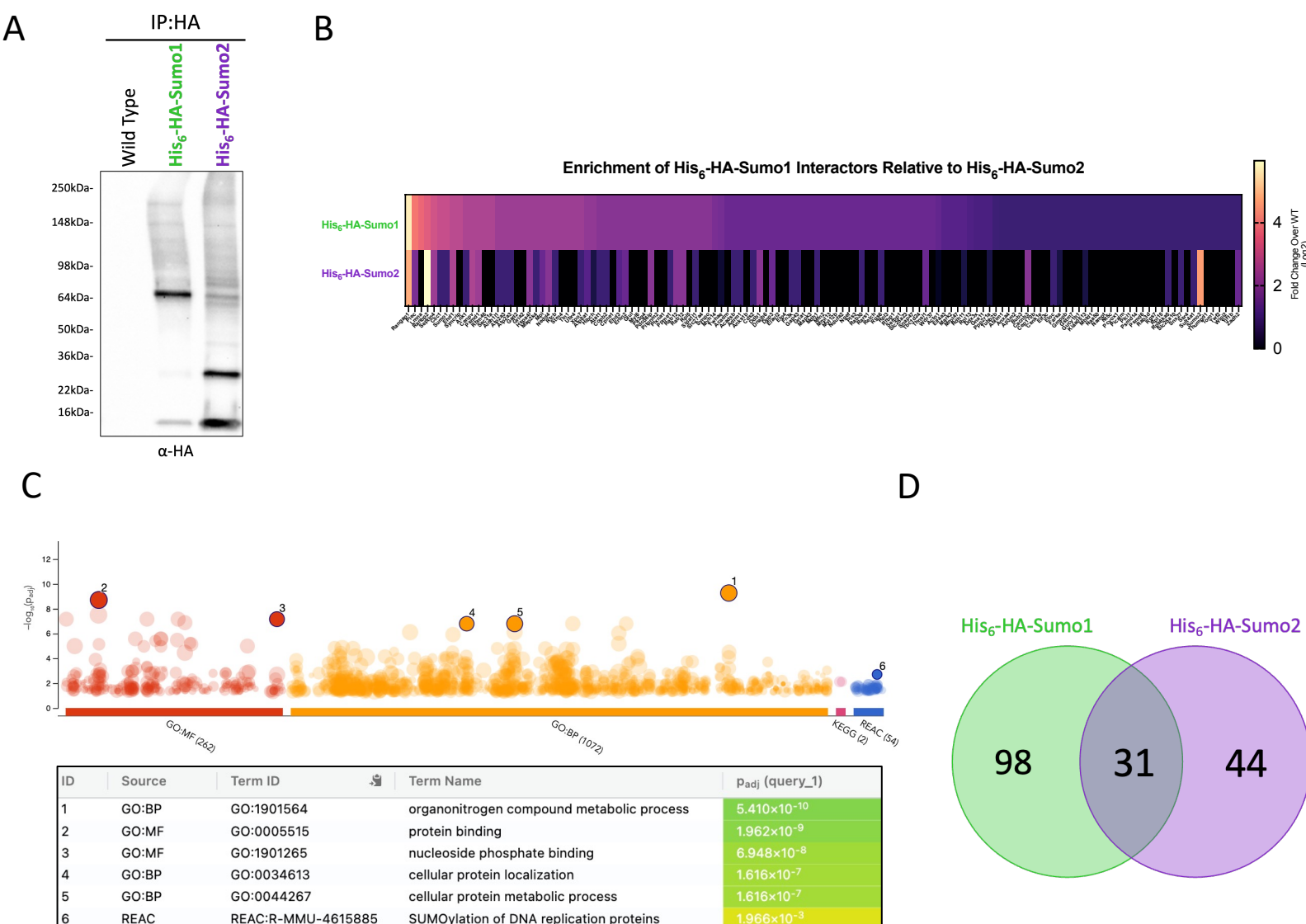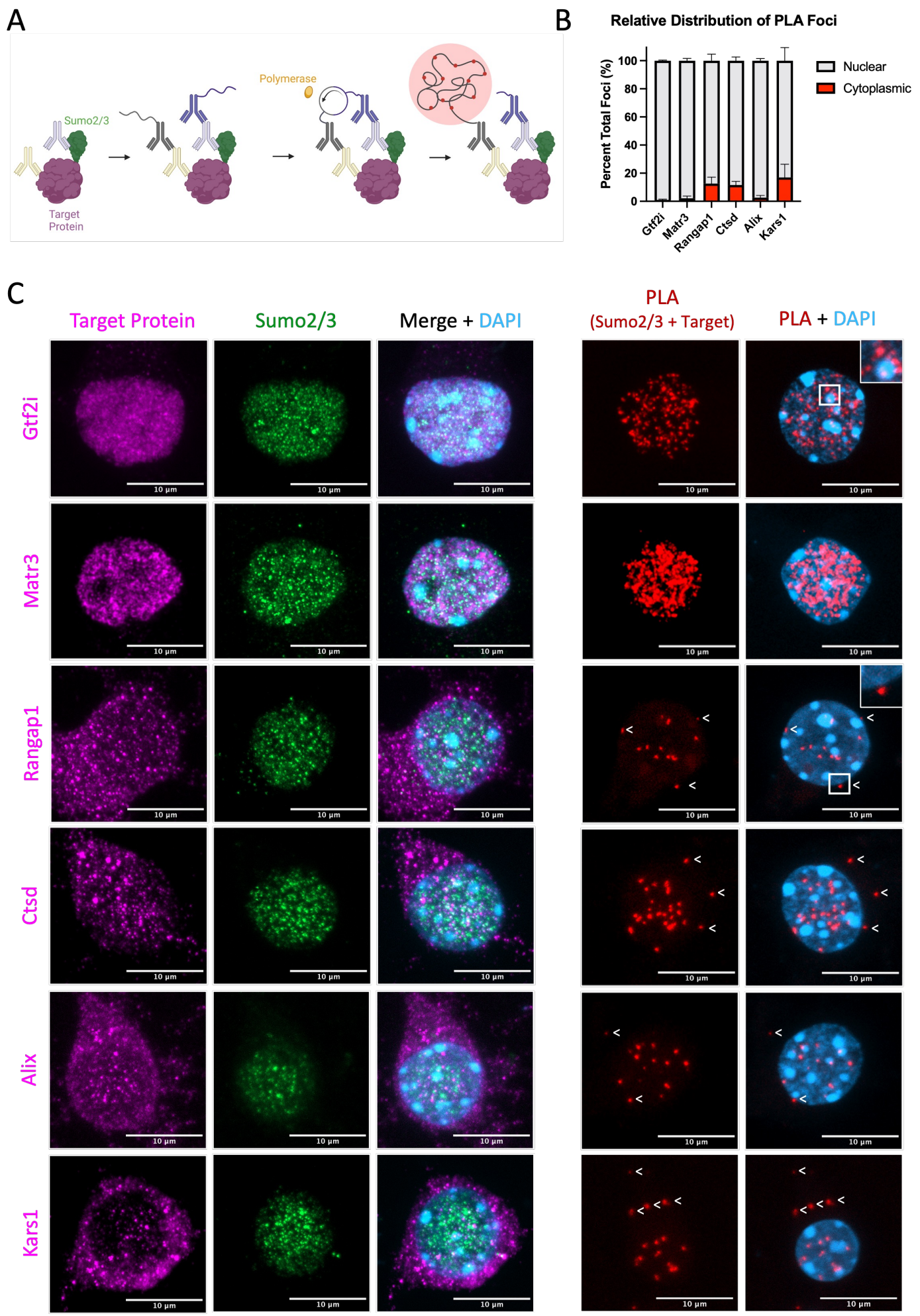| ID	Source	Term ID	Term Name	p$_{adj}$ (query_1)
1	GO:BP	GO:1901564	organonitrogen compound metabolic process	5.410×10^{-10}
2	GO:MF	GO:0005515	protein binding	1.962×10^{-9}
3	GO:MF	GO:1901265	nucleoside phosphate binding	6.948×10^{-8}
4	GO:BP	GO:0034613	cellular protein localization	1.616×10^{-7}
5	GO:BP	GO:0044267	cellular protein metabolic process	1.616×10^{-7}
6	REAC	REAC:R-MMU-4615885	SUMOylation of DNA replication proteins	1.966×10^{-3}

D

Figure 6

Figure 6 – figure supplement 1