

1 A human stem cell resource to decipher the biochemical and cellular 2 basis of neurodevelopmental defects in Lowe Syndrome

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27 Abstract

28 Human brain development is a complex process where multiple cellular and developmental events are
29 co-ordinated to generate normal structure and function. Alteration in any of these events can impact
30 brain development, manifesting clinically as neurodevelopmental disorders. Human genetic disorders
31 of lipid metabolism often present with features of altered brain function. Lowe syndrome (LS), is a X-
32 linked recessive disease with features of altered brain function. LS results from mutations in *OCRL1*
33 that encodes a phosphoinositide 5-phosphatase enzyme. However, the cellular mechanisms by which
34 loss of *OCRL1* leads to brain defects remain unknown. Human brain development involves several
35 cellular and developmental features not conserved in other species and understanding such mechanisms
36 remains a challenge. Rodent models of LS have been generated, but failed to recapitulate features of
37 the human disease. Here we describe the generation of human stem cell lines from LS patients. Further,
38 we present biochemical characterization of lipid metabolism in patient cell lines and demonstrate their
39 use as a “*disease-in-a-dish*” model for understanding the mechanism by which loss of *OCRL1* leads to
40 altered cellular and physiological brain development.

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55 Introduction

56 Phosphoinositides are key regulators of the organization and function of eukaryotic cells (Schink et al.,
57 2016). Of the seven species of phosphoinositides, phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] is
58 the most abundant and is required to regulate multiple sub-cellular processes including membrane
59 turnover, cytoskeletal function and the organization of the plasma membrane (Kolay et al., 2016).
60 PI(4,5)P₂ exerts its control over cellular functions both through binding and allosteric regulation of
61 protein activity and also through its ability to serve as a substrate for phospholipase C (PLC) and Class
62 I PI3K signalling (Katan and Cockcroft, 2020). Therefore, the accurate regulation of PI(4,5)P₂ levels
63 at cellular membranes is critical for normal function. PI(4,5)P₂ levels are regulated through enzymes
64 that regulate its synthesis, the phosphatidylinositol 4-phosphate 5-kinases (PIP5K) (van den Bout and
65 Divecha, 2009) and also by enzymes that catalyze its metabolism. In addition to PLC and Class I PI3K
66 that utilise PI(4,5)P₂ to generate signalling molecules, lipid phosphatases that can dephosphorylate
67 PI(4,5)P₂ have also been described. These include 4-phosphatase enzymes that generate PI5P, but their
68 function *in vivo* remains unclear (Ungewickell et al., 2005). A large family of 5-phosphatases that can
69 dephosphorylate PI(4,5)P₂ at position 5 to generate phosphatidylinositol 4-phosphate (PI4P) have been
70 described (Ooms et al., 2009). This 5-phosphatase activity is encoded in all major eukaryotic genomes,
71 with mammalian genomes encoding upto ten genes for this family of proteins; many of these gene-
72 products have been linked to human diseases (Ramos et al., 2019). The significance of encoding a single
73 enzyme activity through such a large gene family remains to be understood.

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75 The Oculocerebrorenal syndrome of Lowe gene (*OCRL*) gene encodes a 901 amino acid inositol
76 polyphosphate 5-phosphatase enzyme, that is able to catalyse the removal of the 5' phosphate from
77 PI(4,5)P₂ to generate PI4P. *OCRL* was originally identified as the gene underlying the human inherited
78 disease Lowe Syndrome (LS) (Attree et al., 1992). In human patients with LS, sequencing studies have
79 revealed a large diversity of mutations in *OCRL*, including deleterious missense and nonsense mutations
80 in all of the major domains of the protein including the 5-phosphatase domain, PH, ASH and RhoGAP
81 domain (Staiano et al., 2015). *OCRL* is widely expressed across many human tissues or organs and at
82 all stages of life (Raghu et al., 2019). The function of *OCRL* has been studied by overexpression or
83 depletion in a number of common human cell lines and the protein has been reported to localize to and
84 affect the function of many cellular organelles (Mehta et al., 2014).

85

86 LS is a rare (~ 1/500,000 males), X-linked, recessive disorder characterized by the triad of congenital
87 cataracts, intellectual or neurodevelopmental impairment and proximal renal tubular dysfunction

88 (<https://omim.org/entry/300535>) (Bökenkamp and Ludwig, 2016; De Matteis et al., 2017). The brain
89 phenotypes in LS include delayed and impaired cognitive milestones, hypotonia, febrile seizures and
90 hyperechoic changes in the periventricular zone of the cerebral cortex. There are two enigmatic and
91 unresolved observations in relation to the clinical presentation of LS: (i) Although LS is a monogenic
92 disorder, there is substantial variability in the clinical presentation between individual patients, even in
93 those individuals with mutations in *OCRL* with equivalent molecular consequences (e.g truncating
94 nonsense mutations prior to the start of the phosphatase domain). For e.g, while some patients present
95 with severe neurodevelopmental phenotypes, others show relatively mild deficits in brain function.
96 These disparities suggest that in addition to the mutation in *OCRL*, other changes in the genetic
97 background of the individual may impact the clinical outcome of loss of *OCRL*. (ii) Although *OCRL*
98 is widely expressed in human tissues, it remains a mystery as to why only three organs are affected in
99 LS, namely the eye, brain and kidney. One possibility is that the phenotypic changes seen in human
100 patients may arise due to the requirement of *OCRL* only in specific cell types of the affected organs.
101 Therefore, the relevant cellular changes may only be seen when studying eye, brain or renal tissue during
102 development. Although rodent models of *OCRL* were generated, they failed to show phenotypes that
103 recapitulate the human disease (Jänne et al., 1998). A zebrafish model of *OCRL* depletion has been
104 generated that recapitulates some aspects of the human phenotype but there remains a lack of models
105 that allow the brain phenotype to be studied (Ramirez et al., 2012). A limited number of studies have
106 been done on LS fibroblasts and renal biopsies, but there is presently no understanding of how loss of
107 *OCRL* leads to neurodevelopmental phenotypes. Thus, there is a requirement for a model system in
108 which the cellular and physiological changes in the brain during development can be studied.

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110 One possible route to obtaining a suitable model system arises from the ability to use modern stem cell
111 technology to generate human induced pluripotent stem cells (hiPSC) (Shi et al., 2017) from the
112 somatic tissues of patients with LS. These hiPSC can then be differentiated into specific adult tissues
113 and the trajectory of development along with the cellular and molecular changes in any particular patient
114 derived line can be analysed (Russo et al., 2015; Zeng et al., 2014). In the context of LS, a limited
115 number of studies have reported individual hiPSC lines derived from patients (Barnes et al., 2018;
116 Hsieh et al., 2018; Liu et al., 2021; Qian et al., 2021). In this study, we present the generation of hiPSC
117 lines and neural derivatives from a family with a unique genetic structure and clinical features that should
118 allow an understanding of the cellular basis of the neurodevelopmental phenotype in LS. We also
119 present a biochemical analysis of phosphoinositide levels and an insight into the biochemical
120 compensation for the loss of the 5-phosphatase activity of *OCRL* in LS cells.

121

122 Results

123 Generation of hiPSCs from a family with Lowe syndrome

124 For this study, we selected a family whose genetic structure (Fig. 1A i) is uniquely suited for the analysis
125 of neurodevelopmental phenotypes in LS (Ahmed P et al., 2021). Briefly, in this family, the patients
126 with LS are children of two female siblings, both of whom carry the identical mutation in *OCRL*. While
127 all three children show the triad of eye, renal and brain phenotypes characteristic of LS, the brain
128 phenotype of LSPH004 is much more severe than that of the identical twins LSPH002 and LSPH003.
129 To understand the cellular and developmental mechanisms that underlie this neurodevelopmental
130 defect, we generated hiPSC from each of these patients. Peripheral blood mononuclear cells (PBMC)
131 were isolated from each patient, immortalized into lymphoblastoid cells lines (LCLs) which were then
132 reprogrammed to generate hiPSC (Iyer et al., 2018) (Fig. 1A ii). The hiPSC lines so generated did not
133 express the OCRL protein as determined by immunocytochemistry with an antibody to OCRL (Fig.
134 1B). The hiPSC lines showed expression of pluripotency markers SOX2 and SSEA4 as determined by
135 immunocytochemistry (Fig. 1C) and SSEA4 and OCT4 as determined by single cell quantitative
136 fluorescence activated cell sorting (FACS) analysis (Supplementary Fig. 1A-C). We differentiated these
137 hiPSC into embryoid bodies and established their ability to differentiate into each of the three germ
138 layers by detecting the expression of transcripts characteristic of each layer (SOX1, Nestin-ectoderm,
139 Nodal-mesoderm and GATA4-endoderm) (Fig. 1D). All hiPSC lines were determined to be of a
140 normal karyotype (Fig. 1E, Supplementary Fig. 2D, E) and short tandem repeat analysis was used to
141 determine and track the identity of each cell line (Supplementary Table 1). These hiPSC lines offer a
142 unique resource from which tissue specific differentiation, for example into brain tissue can be carried
143 out; a comparison of the cellular and molecular differences between control and patient derived hiPSC
144 lines during brain development can provide important insights into how loss of OCRL results in altered
145 neurodevelopment.

146

147 Generation of Neural Stem Cells (NSC) from hiPSC

148 During the development of the brain, a key step in the conversion of pluripotent, early embryonic stem
149 cells into brain cells is the formation of neural stem cells (NSC) which then both divide and differentiate
150 to generate the different cell types of the brain. Thus, with the exception of microglia, NSC can be
151 differentiated into all cell types in the brain. Since the neurodevelopmental phenotype was strongest in
152 LSPH004, we generated NSC to understand the brain development phenotype of LS. As a control, we
153 generated NSC from D149 hiPSC (Iyer et al., 2018) that was originally derived from an unaffected
154 individual of similar population background (Fig 1A ii). The NSC so generated were characterized by

155 confirming expression of established NSC protein markers such as Nestin, SOX1, SOX2, PAX6 and
156 Musashi-1 along with the proliferation marker Ki-67 (Fig. 2A). They were also confirmed to be
157 karyotypically normal (Supplementary Fig. 3A-B). Lastly, these NSC were differentiated into cultures
158 of forebrain cortical neurons. The neurons so generated showed the characteristic morphology and
159 molecular markers of neuronal development including MAP2, DCX and Synapsin-1 (Fig. 2B) as
160 previously reported (Sharma et al., 2020). Western blot analysis of protein extracts from these lysates
161 revealed the band corresponding to OCRL in wild type neurons and absent in LSPH004 (Fig. 2C). To
162 test the physiological status of these neurons, we monitored them for the presence of intracellular
163 calcium transients $[Ca^{2+}]_i$, a characteristic feature associated with neuronal development (Rosenberg
164 and Spitzer, 2011). We found that in 30 days *in vitro* (DIV) cultures, robust $[Ca^{2+}]_i$ transients were
165 observed in neurons derived from both D149 (Fig. 2D i) and LSPH004 (Fig. 2D ii). In addition, we
166 also monitored the development of electrical activity in the differentiating neurons as a function of age
167 *in vitro* using whole cell, patch clamp electrophysiology in both D149 and LSPH004. For example, in
168 10 DIV cultures of D149, abortive action potentials were noted (Fig. 2E i); by 20 DIV single action
169 potentials were seen (Fig. 2E ii), by 30 DIV multiple action potentials were noted (Fig. 2E iii) and by
170 40 DIV repetitive firing was observed (Fig. 2E iv).

171

172 **Tools for controlled expression of proteins in NSC**

173 In order to understand the cellular basis of the neurodevelopmental defect in LS, it is essential to
174 monitor in real time, ongoing cellular and molecular processes using protein based reporters such as
175 those used to monitor phosphoinositide turnover at specific cellular membranes (Hammond and Balla,
176 2015). Likewise, in order to understand the function of specific domains of OCRL including the 5-
177 phosphatase domain, it will be necessary to achieve carefully controlled reconstitution of OCRL variants
178 in LS cells during development. Current methods of protein expression in neural cells, such as lentiviral
179 mediated transduction of transgenes, result in variable levels of expression between cell lines and
180 experiments. One strategy for the controlled expression of proteins in stem cells is integration of genetic
181 constructs at specific locations in the genome that have been found to be suitable for expression of a
182 transgene/biosensor without any major adverse consequences due to insertion (Pei et al., 2015). Briefly,
183 transcription activator-like effector nucleases (TALENs) are used to insert a Lox cassette at adeno-
184 associated virus integration site 1 (AAVS1) [referred to as Genomic safe harbour sites (GSH)] locus on
185 chromosome 19 thus generating a master cell line for subsequent insertion of transgenes at this location.
186 Transgenes of interest, under suitable promoters, can be inserted into this safe harbour site by Lox-
187 P/Cre-recombinase mediated cassette exchange (RMCE) (Fig. 3A). The advantage of this approach is
188 that there is no rapid loss of transgene expression after transfection and no variation in copy number of

189 transgenes between experiments or cell lines as might occur with transient transfection or lentiviral
190 transduction of transgenes.

191

192 Using this approach, we inserted GSHs into D149 and LSPH004 NSCs. Insertion of GSHs was
193 monitored by observing GFP expression and using a junction PCR (Fig. 3B) and the NSC line so
194 generated continued to express characteristic protein markers (Fig. 3C). RMCE was used to insert a
195 Lox cassette into GSHs of D149 (control) and LSPH004 (patient) NSCs; this cassette included the
196 cDNA for a protein probe for PI(4,5)P₂, the PH domain of PLCδ fused to mCherry (mCherry::PH-
197 PLCδ) (Várnai and Balla, 1998). In this cassette, the mCherry::PH-PLCδ is expressed under a CAG
198 promoter that is active in NSC. NSC in which the mCherry::PH-PLCδ transgene is recombined into
199 the GSH show mCherry fluorescence, NSC in which only one of two copies of the GSH have
200 undergone RMCE show both mCherry and GFP fluorescence and cells in which no recombination has
201 taken place show only GFP fluorescence (Fig. 3D). We purified NSC with mCherry fluorescence only
202 using FACS (Fig. 3E). Western blot analysis on these purified cells showed that they express a protein
203 with a M_r corresponding to that of mCherry::PH-PLCδ (Fig. 3F).

204

205 **Impact of OCRL depletion on total cellular PI(4,5)P₂**

206 Since OCRL is a PI(4,5)P₂ 5-phosphatase, it is expected that cellular PI(4,5)P₂ levels might be elevated
207 and PI4P levels reduced in LS patient cells. To test this, we extracted total lipids from hiPSC and NSC
208 of both control and patient derived LSPH004 cells. PIP₂ levels were measured using liquid
209 chromatography coupled with mass spectrometry (LCMS) from whole cell lysates (Sharma et al., 2019);
210 although this method cannot distinguish between positional isomers of PIP₂, the majority is expected
211 to be PI(4,5)P₂. In hiPSCs, a significant increase was seen in the total PIP (Fig. 4A) and PIP₂ (Fig. 4B)
212 mass in LSPH004 compared to control (Fig. 4A-B). In experiments with NSCs, we compared D149
213 with LSPH004 and found no difference in the total PIP mass (Fig. 4C) or PIP₂ mass (Fig. 4D) between
214 these two lines. Thus, loss of OCRL results in a modest change in PI4P and PI(4,5)P₂ mass in LS
215 patient derived hiPSC.

216

217 **Elevated plasma membrane PI(4,5)P₂ levels in Lowe NSC**

218 Since we did not observe a significant change in the total mass of PI(4,5)P₂ in the LS patient NSC, we
219 wondered whether the OCRL enzyme might control a relatively small but functional pool of PI(4,5)P₂
220 at a specific endomembrane in NSC and changes in this small pool might not be reflected in

221 measurements of total PI(4,5)P₂ mass measurements. In cells, a key sub-cellular membrane where
222 PI(4,5)P₂ is enriched is the plasma membrane. To measure plasma membrane PI(4,5)P₂ levels, we used
223 the reporter lines expressing mCherry::PH-PLC- δ . Live cell imaging was performed on D149 and
224 LSPH004 NSC expressing mCherry::PH-PLC- δ . We estimated PI(4,5)P₂ levels at the plasma
225 membrane by imaging the biosensor expressing cells in a monolayer and calculating the plasma
226 membrane to cytosolic (PM/Cyt) fluorescence ratio of the probe (Fig. 4E). Quantification of these data
227 revealed that the PI(4,5)P₂ levels were significantly higher at the plasma membrane of the patient cell
228 line LSPH004 compared to D149 (Fig. 4F). Thus, loss of OCRL alters the pool of PI(4,5)P₂ at the
229 plasma membrane in NSC.

230

231 **Compensatory mechanisms for loss of OCRL function**

232 *OCRL* is part of a large family of lipid 5-phosphatases in the human genome (Ramos et al., 2019) and
233 the function of this gene family in neurodevelopment has not been studied. Loss of *OCRL* function in
234 LS patients might result in compensatory changes in the expression of other 5-phosphatase family
235 members during brain development, thus leading to modest or no changes in PI4P and PI(4,5)P₂ levels.
236 To determine the expression pattern of these phosphatases during neural development, we performed
237 qRT-PCR analysis for all the ten 5-phosphatases encoded in the human genome (Ramos et al., 2019)
238 in hiPSC, NSC and 30 DIV neuronal cultures of D149. This analysis revealed an interesting and
239 variable pattern of expression for each gene at these specific stages of neural differentiation *in vitro*.
240 While some 5-phosphatases such as *SYNJ1*, *SYNJ2*, *INPP5B* and *INPPL1* were expressed at similar
241 levels across all three stages, *INPP5D* appeared downregulated during neuronal differentiation. By
242 contrast, a set of 5-phosphatases including *OCRL*, *INPP5F*, *INPP5K*, *INPP5E* and *INPP5J* all showed
243 upregulation during neuronal differentiation (Fig. 5A). We then compared expression of all ten
244 phosphatases in each of the three developmental stages hiPSC, NSC and 30 DIV neurons between
245 D149 and LSPH004. At the hiPSC stage, five of ten 5-phosphatases that we assayed were upregulated
246 in the patient line LSPH004; these were *INPP5D*, *INPP5E*, *INPP5F*, *INPP5K*, *INPPL1*. By contrast,
247 *SYNJ1* and *INPP5J* were downregulated; as expected, *OCRL* was significantly down regulated in the
248 patient hiPSC (Fig. 5B). In NSC, we observed five out of ten 5-phosphatases were upregulated in the
249 patient line namely, *INPP5J*, *INPP5E*, *INPP5F*, *SYNJ2*, *INPPL1* and the only 5-phosphatase
250 downregulated was *OCRL* (Fig. 5C). We also differentiated the NSC into neurons and compared
251 expression levels of the phosphatases at 30 DIV between D149 and LSPH004; this revealed that except
252 for a modest downregulation of *INPP5K*, there were no compensatory changes in LSPH004 (Fig. 5D).
253 Thus, loss of *OCRL* results in distinctive patterns of compensatory changes in 5-phosphatase gene
254 expression at various stages of neurodevelopment (Fig. 5E).

255 Transcriptomic changes in LS cells

256 To characterize the gene expression changes resulting from OCRL loss of function that may lead to
257 neurodevelopmental defects, we performed transcriptomic analysis from hiPSC and NSC of D149 and
258 LSPH004. At the iPSC stage, corresponding to the earliest stages of human embryonic development,
259 we found ca. 475 genes upregulated and ca. 400 genes downregulated (Fig. 6A). Analysis of this set of
260 altered genes using Gene Ontology revealed a number of categories of highly enriched downregulated
261 genes suggestive of altered brain development (GO terms: generation of neurons, neurogenesis, neuron
262 differentiation, nervous system development) (Fig. 6C-D, Supplementary Table 2). These included
263 *SLITRK1*, *DCX*, *MAP2*, *BRINP1*, *CEP290*, *PAX7*, *NCAM1*, *SEMA6D* and *CNTN2*.

264 Likewise, a comparative analysis of D149 and LSPH004 NSC revealed more than 750 downregulated
265 and 250 upregulated genes (Fig. 6B). Gene Ontology analysis of this gene set also revealed strong
266 enrichment of GO terms suggesting altered neural development with the most highly significant GO
267 terms being neurogenesis and nervous system development and function (Fig. 6E, Supplementary Table
268 3). These include *SEMA3F*, *PLXND1*, *SOX8*, *LRRC7*, *HAPLN2*, *NTRK2*, *NRG1* and *SYT4*. These
269 findings suggest a role for OCRL in the development of the brain, starting with the earliest cellular
270 stages of embryogenesis, that can be analysed in this experimental system.

271

272 Discussion

273 Although several sub-cellular functions have been described for OCRL (De Matteis et al., 2017; Mehta
274 et al., 2014), the mechanism by which mutations in this gene result in human neurodevelopmental
275 defects remains unknown. A particular challenge in understanding the brain phenotype in LS patients
276 arises from the inability to obtain conventional biopsy samples from the human brain. This is in contrast
277 to a limited number of studies where renal biopsies (De Leo et al., 2016) and skin samples (Wenk et
278 al., 2003) have been used to address some specific aspects of the LS phenotype. In addition, since LS
279 is neurodevelopmental in origin, understanding brain phenotypes present at birth requires the ability to
280 study the cellular and physiological changes during brain development. To achieve this goal, it is
281 necessary to have a model in which the development of human brain cells can be studied *in vitro*. A
282 suitable model must recapitulate key aspects of brain development *in vitro* and also the key biochemical
283 effects seen in LS patient cells.

284 We have identified a family with LS (Ahmed P et al., 2021) and in this study, report the generation of
285 hiPSC from patients in this family as a resource to analyze the cellular and molecular basis of brain
286 development in LS. Since *OCRL* encodes a PI(4,5)P₂ phosphatase, it is expected that loss-of-function

287 mutations in this gene should result in elevation of PI(4,5)P₂ levels and a drop in PI4P levels. Our
288 measurements of the mass of PIP₂ and PIP in hiPSC cultures of the patient lines showed an elevation
289 of total PIP₂ and PIP in LSPH004 cells. This parallels observations on cultured human fibroblast cell
290 lines from LS patients that have been previously reported (Wenk et al., 2003). In this study, we also
291 engineered the LSPH004 line to express a fluorescent reporter for plasma membrane PI(4,5)P₂ levels
292 and found using this approach that the levels of this lipid at the plasma membrane of the patient line
293 were elevated in comparison to control. Thus, the *in vitro* model system presented here recapitulates
294 the key biochemical defect in phosphoinositide metabolism reported in LS patient tissues.

295

296 We differentiated these hiPSC lines using protocols based on the known principles of developmental
297 neurobiology to generate 2D cultures of human neural tissue, primarily neurons *in vitro*. This
298 developmental process *in vitro* recapitulates key aspects of brain development *in vivo* (Mertens et al.,
299 2016). These hiPSC derived neural cultures show progressive increase of neural activity as a function of
300 the age of the culture (DIV) (Fig. 2D, E). A comparison of neural activity development from LS patient
301 derived cell lines reported here compared to those from control lines will provide an insight into the
302 altered physiological development in brain tissue of LS patients. During metazoan development, gene
303 expression is a key process that determines cell fate specification and differentiation. To understand
304 gene expression changes that underlie the neurodevelopment phenotype in LS, we compared
305 transcriptomes between control and LSPH004 at both the iPSC and NSC stages. Large differences in
306 transcriptome were seen at both of these developmental stages (Fig. 6) revealing substantial changes in
307 the expression of genes annotated as having roles related to nervous system development. These findings
308 imply that the use of the hiPSC lines described here are likely to be valuable in understanding the
309 mechanism by which loss of OCRL leads to altered brain development. Altogether, the model system
310 described here allows an analysis of the physiological development in LS patients as well as the
311 biochemical and molecular correlates of this process.

312

313 In addition to neuronal differentiation, the LS patient derived hiPSCs can be differentiated into glia to
314 study the contribution of OCRL function in these non-neuronal cell types to the altered brain
315 development in LS patients. Hyperechoic periventricular lesions have been described in MRI scans of
316 patients with LS with features suggestive of enhanced gliosis (Sener, 2004) and evidence suggestive of
317 enhanced gliosis in a fish model of LS has been reported (Ramirez et al., 2012). However, its relevance
318 in the brain tissue of human LS patients remains unknown and the hiPSC lines generated here will also
319 allow this question to be studied. In addition, the use of co-cultures of one neural cell type (e.g neurons)

320 from LS patients with glial cells derived from controls and vice versa (G. Nadadhur et al., 2019) will
321 allow an assessment of the cell autonomous and non-cell autonomous function of *OCRL* in supporting
322 normal brain development. The developing brain is a complex 3D tissue and 2D cultures may not
323 recapitulate all aspects of the cell-cell interactions or cortical layer formation that impact normal brain
324 development. However, hiPSC can be used to generate 3D organoids of the developing brain (Di Lullo
325 and Kriegstein, 2017); the use of the LS patient lines described in this study will also allow the role of
326 *OCRL* in supporting the 3D architecture during brain development to be investigated. Finally, a recent
327 study has proposed the use of the phosphoinositide 3-kinase inhibitor, alpelisib, for correcting renal
328 defects in a mouse model of LS (Berquez et al., 2020). The availability of the hiPSC lines described in
329 this study can help evaluate the effectiveness of such inhibitors in alleviating the phenotypes of LS in
330 brain tissue.

331

332 It has been noted that although LS is a monogenic disorder, there is considerable variability in the brain
333 phenotypes of individual patients. It has been proposed that such variability in clinical features between
334 patients may arise from the impact of background mutations in the genomes of individual patients
335 carrying functionally equivalent mutations in *OCRL*. However, the cellular and developmental basis of
336 this has not been tested. The unique genetic structure and clinical features of the LS family we have
337 studied (Ahmed P et al., 2021) provides a unique opportunity to address this scientific question. The
338 three patients in this study, LSPH002, LSPH003 and LSPH004 vary clinically in their
339 neurodevelopmental phenotype with LSPH004 showing much severe brain phenotype than LSPH002
340 and LSPH003 who are identical twins. In this study, we have generated hiPSC from all three patients;
341 these cell lines carry the identical mutation in *OCRL* in the background of the genome of the individual
342 patient from whom they were derived. By differentiating these hiPSC into neural tissue and comparing
343 LSPH004 with LSPH002 and LSPH003, it is likely that we will discover the cellular and
344 developmental correlates of the variable brain phenotype. Since the whole genome sequence of each line
345 has been determined, it is also possible to experimentally test the importance of specific variants in the
346 patient genome by CRISPR/Cas9 genome editing of specific variants (Paquet et al., 2016) followed by
347 phenotypic analysis.

348

349 Although we observed changes in the levels of PIP and PIP₂ in patient derived hiPSC from LS patient,
350 several key aspects of altered phosphoinositide biochemistry in LS patient cells remain to be understood:
351 (i) Changes were relatively modest and the changes in PIP and PIP₂ mass seen in hiPSC were no longer
352 evident at the NSC stage. These observations indicate a plasticity in the control of PI(4,5)P₂ levels,

353 perhaps by other enzymes that may also regulate PI(4,5)P₂ levels that needs to be understood. (ii) In
354 addition to elevated PIP₂ levels in LS hiPSC, PIP levels were also elevated; this is unexpected though
355 it has also previously been reported in fibroblasts from LS patients (Wenk et al., 2003). (iii) It is unclear
356 if the elevated PIP₂ levels, PIP levels or both lead to cellular effects leading to patient phenotypes. In
357 order to address these questions, the system described here offers many advantages. Our genome
358 engineering approach using the GSH in these lines offers the opportunity for controlled expression of
359 enzymes to modulate the levels of phosphoinositides acutely (Idevall-Hagren and De Camilli, 2014;
360 Varnai et al., 2006) to test specific hypotheses related to the role of individual lipids in altered cellular
361 and developmental phenotypes in developing neural cells. One likely mechanism underlying plasticity
362 in the control of PI(4,5)P₂ levels in LS patient cells is changes in the expression of other genes encoding
363 members of the 5-phosphatase family of enzymes. Indeed, RT-PCR analysis of the ten members of the
364 5-phosphatase in LSPH004 revealed that there were distinctive patterns of up or down regulation in
365 the expression of the 5-phosphatase gene family members in wild type cells during development. Such
366 patterns of 5-phosphatase expression and compensatory changes of these in LS patient cells during
367 development may underlie the specific nature of the neurodevelopmental defects in LS patients. In
368 summary, the hiPSC resources and their engineered derivatives described here offer powerful tools for
369 understanding the regulation of PI(4,5)P₂ to PI4P balance in the developing nervous system by OCRL
370 and the mechanism by which loss of this activity leads to neurodevelopmental defects.

371

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377 facilities for support. We thank Dr. O. Mukherjee and M. Rao for advice in stem cell generation.

378

379 **Materials and Methods**

380 **Cell Lines and culture conditions**

381 **hiPSCs:** D149 (Iyer et al., 2018), NIH5 (Baghbaderani et al., 2015), LSPH002, LSPH003, LSPH004
382 (generated in this study). When grown on a mouse embryonic fibroblast (MEF) feeder layer, all hiPSC
383 were grown in standard HuES (Human embryonic Stem cell) media. When transitioned to feeder-free
384 extra cellular matrix Matrigel (hESC-qualified Matrigel Corning, #354277) coated surface, the cells

385 were grown in E8 complete media (E8 basal + E8 supplement). Cultures were maintained at 37°C and
386 5% CO₂ throughout.

387 NSCs: D149, LSPH004, D149-GSH, LSPH004-GSH, D149-PIP_{2R}, LSPH004-PIP_{2R} (generated in
388 this study). All Neural Stem Cells (NSCs) were grown on Matrigel coated tissue culture plasticwares
389 in neural expansion media at 37°C and 5% CO₂.

390

391 **Mycoplasma testing**

392 Mycoplasma contamination was checked using spent media from sub-confluent hiPSC and NSC dishes
393 after 48 h in culture. MycoAlert™ (Lonza, #LT07-418) was used per the manufacturer's protocol.

394

395 **Karyotyping**

396 Overall chromosomal integrity of hiPSCs and NSCs was confirmed by karyotyping. For metaphase
397 preparation, cells were arrested in log phase by treating with 0.1 µg/mL Colcemid™ (Gibco, #15212-
398 012) treatment for 45 min at 37°C. Cells were harvested in fresh Carnoy's fixative (Methanol:Glacial
399 Acetic Acid at 3:1) and G-banding karyotype analysis performed at a National Accreditation Board
400 for Testing and Calibration Laboratories, India (NABL) accredited facility.

401

402 **Generation of hiPSC lines**

403 Blood was drawn from donors after informed consent and under aseptic conditions following IRB
404 regulations. A peripheral blood mononuclear cell fraction was obtained by density gradient
405 centrifugation and transformed into Lymphoblastoid Cell Lines (LCL) by Epstein Barr virus (EBV)
406 transformation using previously established protocol (Hui-Yuen et al., 2011). These LCLs were
407 reprogrammed by electroporation of plasmids containing Yamanaka factors to generate hiPSCs as
408 previously described (Iyer et al., 2018). On-feeder hiPSC cultures were gradually transitioned to feeder-
409 free conditions by weaning off from the standard HuES media to Essential 8™ medium (Gibco,
410 #A1517001) and maintained on hESC-qualified Matrigel coated surface. hiPSCs were frozen at a
411 density of 1x10⁶ in 500µL PSC cryomix (Gibco, #A26444-01). The plasmid footprint in feeder-free
412 iPSC lines LSPH002 (passage 7), LSPH003 (passage 7) and LSPH004 (passage 8) was tested using
413 PCR analysis with relevant primer sets (Supplementary Table 5), with 3% DMSO as a PCR additive.
414 Plasmid pCXLE-hUL was used as a positive control while iPSC NIH5 was used as negative control.
415 To ascertain differentiation potential, embryoid bodies (EB) were generated by transferring hiPSCs to

416 a non-adherent surface in standard HuES media without bFGF for 48 h, and cDNA probed for primers
417 specific for the three lineages.

418

419 **Generation of NSCs**

420 NSCs were generated as previously described (Mukherjee et al., 2019) with slight modifications. Briefly,
421 hiPSCs were differentiated to form embryoid bodies in E6 medium (Gibco, #A1516401). Primary
422 neural rosettes formed by this method were selected and manually passaged to obtain secondary and
423 tertiary rosettes that were eventually triturated and plated as NSC monolayer in Neural Expansion
424 Medium (NEM). As a measure to eliminate any non-NSC cells and obtain a reliable homogeneous
425 NSC culture, the generated NSCs were subjected to CD133+ selection as previously described via
426 fluorescence-activated cell sorting (FACS) (Peh et al., 2009). A sub-confluent NSC culture maintained
427 in NEM was enzymatically dissociated using Stempro Accutase (Gibco, #A11105-01) and following a
428 wash in PBS, cells were immunolabelled using CD133-PE conjugated primary antibody (Abcam,
429 #ab253271) at 10 μ l antibody/million cells and incubated at room temperature for 30 min in dark. Cells
430 were washed with PBS and resuspended in 1mL sorting media [(1x DMEM/F12-without phenol red
431 (Gibco, #21041025), 1% FBS (Gibco, #16000-044) + Penicillin-Streptomycin (Gibco, #15140-122)]
432 keeping the concentration at 2 million cells per mL to obtain efficient sorting. The cells were sorted
433 using FACS- Aria-III instrument (BD Biosciences) (Supplementary Fig.1D-E). Forward and side
434 scatter parameters were adjusted so as to eliminate cell clumps and debris. Cells with highest
435 fluorescence intensity were collected and plated on Matrigel at a concentration of 0.5 million cells per
436 well of 12-well tissue culture plate in NEM and expanded. Cells were periodically checked for bacterial
437 or mycoplasma contamination. NSCs were further characterized by immunofluorescence and neuronal
438 differentiation.

439

440 **Reporter line generation**

441 For generation of D149 and LSPH004 NSC safe harbor lines, cells growing in Neural Expansion
442 Medium (NEM) without Penicillin-Streptomycin were harvested by enzymatic dissociation using
443 Stempro Accutase for 5 min at 37 °C. The cells were spun down at 1000g for 3 min to remove Accutase
444 post neutralization at room temperature. After a wash with PBS, cells were incubated in buffer-R
445 (included in Invitrogen, #MPK1025) with 2 μ g/ μ L each of plasmids: AAVS-1-TALEN-R (ZYP017),
446 AAVS-1-TALEN-L (ZYP018) and ZYP037 (Pei et al., 2015) for 3 min at a concentration of 1 million
447 cells per hit. The cell-plasmid mixture was electroporated using the Neon Electroporation System

448 (Invitrogen, USA. #MPK1025) as per manufacturer's protocol under the following standardized
449 parameters (1100 volts, width-20, pulses-2) and plated in 1 well of 12well plate coated with Matrigel
450 containing pre warmed NEM without Penicillin-Streptomycin. A complete media change was
451 performed 4 h post electroporation to remove dead cells and debris which could potentially cause
452 cytotoxicity in the culture if not removed. After 24 h, cells were checked under an epifluorescence
453 microscope for expression of GFP and upon confirmation, the cells were subjected to Puromycin
454 (Gibco, #A1113803) selection at a concentration of 0.4 $\mu\text{g}/\text{mL}$ for 7-9 days with daily media change.
455 After a week, large GFP positive colonies were visible in the plate, at this point the culture was subjected
456 to Fluorescence-activated cell sorting (FACS) to remove any GFP-negative cell. The mixed cell culture
457 was enzymatically dissociated using Stempro Accutase, washed once with PBS and resuspended in
458 sorting media [(1x DMEM/F12-without phenol red (Gibco, #21041025), 1% FBS + Penicillin-
459 Streptomycin] at a concentration of 2 million cells per mL. The cells were sorted using FACS- Aria-
460 III (BD Biosciences). Forward and side scatter parameters were adjusted so as to eliminate cell clumps
461 and debris. The gating parameter threshold for GFP-positive cells was set using non-electroporated
462 control NSCs. The cells were collected in NEM and plated in pre-incubated Matrigel coated plate at a
463 concentration of 0.5 million cells per well of 12-well tissue culture plate in NEM and expanded for
464 cryopreservation.

465 The Safe Harbor NSCs; D149-GSH and LSPH004-GSH were enzymatically dissociated using
466 Stempro Accutase for 5 min at 37 °C. The cells were spun down at 1000g for 3 min at room temperature
467 to remove Accutase. After a wash with PBS they were resuspended in Buffer-R (included in Invitrogen,
468 #MPK1025) with 2 $\mu\text{g}/\mu\text{L}$ each of plasmids: LoxP-CAG-mCherry::PH-PLC δ (ZYP070-PIP $_{2R}$) and
469 Cre-Recombinase (ZYP073) at a concentration of 1 million cells per hit of electroporation which was
470 performed using Neon Electroporation System (Invitrogen, USA. #MPK1025) as per manufacturer's
471 protocol under the following standardized parameters (1100 volts, width-20, pulses-2) and plated in 1
472 well of 12-well plate coated with Matrigel containing pre warmed NEM without Penicillin-
473 Streptomycin. A complete media change was performed 4 h post electroporation to remove dead cells
474 and debris which could potentially cause cytotoxicity in the culture if not removed. After 24 h, cells
475 were checked under epifluorescence microscope for expression of mCherry and upon confirmation, the
476 cells were subjected to G418 (Gibco, #10131035) selection at a concentration of 400 $\mu\text{g}/\text{mL}$ for 15-20
477 days with daily media change. After about two weeks under selection, distinct mCherry positive colonies
478 were observed, at this point the culture was subjected to Fluorescence-activated cell sorting (FACS) to
479 remove GFP-positive cells. The cells were sorted at a concentration of 2 million cells per mL using
480 FACS- Aria-III (BD Biosciences). Forward and side scatter parameters were adjusted so as to eliminate
481 cell clumps and debris. The gating parameter threshold for mCherry-positive cells was set using GFP-
482 positive and non-electroporated control NSCs. Post FACS cells were collected in NEM and plated in

483 pre-incubated Matrigel coated plate at a concentration of 0.5 million cells per well of 12-well tissue
484 culture plate in NEM and expanded to freeze down additional stock vials.

485

486 qRT-PCR

487 Isolation of RNA and cDNA synthesis

488 Total RNA was extracted from well characterized hiPSCs and NSCs of patient and control lines using
489 TRIzol (Ambion, Life Technologies, #15596018) as per manufacturer's protocol in 6 biological
490 replicates and quantified using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).
491 Following treatment with 1U of DNase I (amplification grade, Thermo Fisher Scientific, #18068-015),
492 1 µg of the RNA from each replicate was used for cDNA synthesis in a reaction mixture containing 10
493 mM DTT and 40U of RNase inhibitor (RNaseOUT, Thermo Fisher Scientific, #10777-019). The
494 reaction mixture of 45.5 µl was incubated at 37°C for 30 min followed by heat inactivation at 70°C for
495 10 min, following which 200U of Superscript II Reverse Transcriptase (Invitrogen, #18064-014) was
496 added to the reaction volume along with 2.5 µM of random hexamers, and 0.5 mM of dNTPs making
497 the final volume to 50 µl. The reaction was then incubated at 25°C for 10 min, followed by 42°C for 60
498 min and then heat inactivated at 70°C for 10 min on ProFlex PCR Systems (Life Technologies).

499

500 Real-Time Quantitative PCR

501 The primers used for qRT-PCR were designed using Primer-BLAST, NCBI
502 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). With a condition of spanning exon-exon junction
503 of their respective genes, their details are provided in supplementary Table 5. Real-Time qRT-PCR
504 was performed in a volume of 10µl with Power SYBR Green Master mix (Applied Biosystems,
505 #4367659) on an Applied Biosystems ViiA7 system. It was performed with technical triplicates from
506 the patient and control lines with primers for genes of interest and GAPDH was used as a house-
507 keeping gene control. A no-reverse transcriptase control was also set up. The reaction was run under
508 the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 30 sec
509 (denaturation), 60°C for 30 sec (annealing) and 72°C for 45 sec (extension). The C_t values obtained for
510 individual genes were normalized to those of GAPDH from the same sample. The relative expression
511 levels were calculated using ΔC_t method, whereas the fold change between patient and control was
512 calculated using $\Delta\Delta C_t$ method.

513

514 **Junction PCR**

515 Junction PCR was performed using the genomic DNA of the edited cells, extracted using QIAamp
516 DNA Mini Kit (Qiagen, #51304) using manufacturer's protocol and quantified using a Nanodrop 1000
517 spectrophotometer (Thermo Fisher Scientific). Insertion of the ZYP037 donor template was confirmed
518 using two pairs of primers; one of the primers in each pair annealed outside the region spanned by the
519 homology arm, in the AAVS1 locus, while the other annealed within the inserted template, to avoid
520 false detection of residual plasmid if any. 50 ng of genomic DNA was used as the template in 20 μ l
521 PCR reaction consisting Phusion Pol (0.02 U/ μ l), 25 mM DNTPs, 5X HF-buffer and appropriate
522 primers. The reaction was run as a touchdown PCR in two steps as follows: 98°C for 3 min (initial
523 denaturation), step-1 [98°C for 20 sec-denaturation, 66°C to 61°C (-0.5°C per cycle) for 30 sec-
524 annealing, 72°C for 2 min-extension] x10 cycles, step-2 [98°C for 20 sec-denaturation, 55°C for 30 sec-
525 annealing, 72°C for 2 min-extension] x25 cycles, 72°C for 5 min (final extension).

526

527 **List of Primers and Antibodies used in this study is provided as Supplementary Table 5**

528

529 **Immunocytochemistry**

530 hiPSCs were characterized for pluripotency markers SOX2 and SSEA4 using the PSC
531 immunocytochemistry kit (Invitrogen, #A24881) as per manufacturer's instructions.

532 NSC cultures were fixed using 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min,
533 permeabilized using 0.1% Tx-100 for 5 min and incubated at room temperature for 1 h in a blocking
534 solution of 5% BSA in PBS. Primary antibodies at respective dilutions were added and incubated
535 overnight at 4°C in blocking solution, followed by incubation with secondary antibodies in blocking
536 solution (Invitrogen) for 1 h.

537 Confocal images were recorded by collecting a range of z-stack using an Olympus FV 3000 confocal
538 microscope. Epifluorescence images were captured using EVOS® FL Cell Imaging System (Thermo
539 Fisher Scientific). The image stack was merged using Z-project (maximum intensity projection)
540 function using ImageJ (National Institute of Health, USA, <http://imagej.nih.gov/ij>).

541 **Quantitative FACS analysis**

542 hiPSCs were characterized quantitatively for pluripotency markers OCT4 and SSEA4 by flow
543 cytometry. Briefly, the cells were detached using Stempro Accutase for 5 min at 37°C and washed with
544 standard HuES media. The cells were then washed once with PBS (Phosphate-buffered saline) and

545 fixed with 4% PFA solution at a cell density of 1×10^6 cells per mL at room temperature for 15 min.
546 The cells were then permeabilized with 0.1% v/v Triton X-100 in HBSS for 15 min at room
547 temperature, washed once with HBSS and incubated with 1% BSA in HBSS for 30 min. Following
548 this, the cells were incubated with primary antibodies in 1% BSA in combination at the following
549 dilutions, Rabbit Anti-OCT4 (Thermo Fisher Scientific, #A24867) at 1:100 and Mouse IgG3 Anti-
550 SSEA4 (Thermo Fisher Scientific, #A24866) at 1:100 and incubated for 3 h at room temperature with
551 gentle mixing. The cells were then washed 2-3 times in HBSS and incubated with appropriate
552 secondary antibodies, Alexa Fluor™ 594 donkey anti-rabbit Antibody (Thermo Fisher Scientific,
553 #A24870) and Alexa Fluor™ 488 goat anti-mouse IgG3 (Thermo Fisher Scientific, #A24877) for 1 h
554 at room temperature. Before use, the secondary antibodies were diluted at 1:250 in 1% BSA. The cells
555 were washed twice and then analyzed using a BD FACSVerser cytometer. (Supplementary Fig. 1A-C)

556

557 Western Blotting

558 NSCs and 30 DIV neurons were harvested using Stempro Accutase and pelleted at 1000g for 5 min
559 then washed thrice with ice-cold PBS. The pelleted cells were homogenized in 1X RIPA lysis buffer
560 containing freshly added phosphatase and protease inhibitor cocktail (Roche). To remove cellular
561 debris, crude RIPA lysates were centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant was
562 transferred to a new tube and quantified with a Pierce BCA protein assay (Thermoscientific, #23225).
563 Thereafter, the samples were heated at 95°C with Laemmli loading buffer for 5 min and 20 ug protein
564 was loaded onto Bolt™ 4 to 12%, Bis-Tris SDS gel (Invitrogen, #NW04120BOX). The proteins were
565 then transferred onto a nitrocellulose membrane and incubated overnight at 4°C with indicated
566 antibodies. The blots were then washed thrice with Tris Buffer Saline containing 0.1% Tween-20 (0.1%
567 TBS-T) and incubated with 1:10,000 concentration of appropriate HRP-conjugated secondary
568 antibodies (Jackson Laboratories, Inc.) for 45 min. After three washes with 0.1% TBS-T, blots were
569 developed using Clarity Western ECL substrate (Biorad) on a GE ImageQuant LAS 4000 system.

570

571 Calcium imaging

572 Calcium imaging was performed in 30 DIV D149 and LSPH004 neurons, according to our previously
573 published protocol with minor modifications (Sharma et al., 2020). Briefly, neurons were washed with
574 Tyrode's buffer solution (5 mM KCl, 129 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 30 mM glucose and
575 25 mM HEPES, pH 7.4) for 10mins and later incubated with 4 uM fluo-4/AM (1 mM, Molecular
576 probes, #F14201) and 0.002% pluronic F-127 (Sigma, #P2443) in the Tyrode's buffer solution in dark
577 for 30-45 min at room temperature. Following dye loading, the cells were washed again with the buffer

578 thrice, each wash for 5 min. Finally, cells were incubated for an additional 20 min at room temperature
579 to facilitate de-esterification. Ca^{2+} imaging was performed for 8 min with a time interval of 1 second
580 using CellSens Dimension software (Olympus, build 16686) at 20X objective of wide-field fluorescence
581 microscope Olympus IX-83. A four-minute baseline measurement was recorded to visualize calcium
582 transients, followed by the addition of TTX to abolish calcium transients for another 4 min. Calcium
583 traces were obtained using the CellSens Dimensions software by drawing a region of interest (ROI)
584 manually around each neuronal soma. To plot the calcium traces, the raw fluorescence intensity values
585 from each neuron were normalized to the first fluorescence intensity signal of the baseline recording.
586 GraphPad Prism 5.0 was used to plot calcium traces.

587

588 Electrophysiology

589 Whole-cell patch clamp recordings were performed at 10, 20, 30 and 40 DIV following initiation of
590 neuronal differentiation of NSC. Recording micropipettes (5-7M Ω) were filled with internal solution
591 composed of (in mM): 130 K-gluconate, 0.1 EGTA, 1 MgCl₂, 2 MgATP, 0.3 NaGTP, 10 HEPES,
592 5 NaCl, 11 KCl, and Na²⁻-phosphocreatinine (pH 7.4). Recordings were made at room temperature
593 using a Multiclamp 700B amplifier (Molecular devices, USA). Signals were amplified and filtered at 10
594 Hz and 3 Hz, respectively. Voltage was corrected for liquid junction potential (-14 mV). The bath was
595 continuously perfused with oxygenated artificial cerebrospinal fluid (ACSF) composed of (in mM): 110
596 NaCl, 2.5 KCl, 2 CaCl₂, 10 glucose and 1 NaH₂PO₄, 25 NaHCO₃, and 2 MgCl₂ (pH 7.4) (Gunhanlar
597 et al., 2018). For current-clamp recordings, voltage responses were evoked from a holding potential of
598 -60mV to -70mV using 500 ms steps ranging from -5 to +70 pA in 5 pA intervals. Action potential
599 (AP) properties were calculated from the first evoked AP in response to a depolarizing step. Repetitively
600 firing neurons were defined as those capable of firing >3 APs in response to a depolarizing current step.

601

602 Mass spectrometry

603 Lipid standards

604 17:0-20:4 PI(4,5)P₂ (Avanti Lipids – LM1904), 17:0-20:4 PI(4)P (Avanti Lipids – LM1901), 17:0-
605 14:1 PE (Avanti Lipids – LM 1104)

606

607 Solvent mixtures

608 **Quench mixture** –MeOH/CHCl₃/1M HCl in the ratio 484/242/23.55 (vol/vol/vol).

609 **Lower Phase Wash Solution (LPWS)** –MeOH/1M HCl/CHCl₃ in the ratio 235/245/15 (vol/vol/vol).

610 **Post derivatization Wash Solution (PDWS)** – CHCl₃/MeOH/H₂O in the ratio 24/12/9 (vol/vol/vol).
611 Shake the mixture vigorously and allow settling into separate phases and use the upper phase only for
612 washes.

613

614 **Lipid Extraction**

615 Cells in culture were washed with DMEM/F12 to remove debris and then harvested using Stempro
616 Accutase over a few minutes at 37°C. The Accutase was then neutralized and the cell suspension was
617 then centrifuged at 1000g (for iPSCs) or 2500g (for NSCs) to pellet down the cells. The supernatant
618 was discarded and the cell pellet was resuspended in 1 mL of 1X Phosphate Buffer saline (PBS) for a
619 wash and transferred to a 2 mL low-bind tube. The tubes were centrifuged at the previously indicated
620 speeds to pellet the cells again. The supernatant was discarded and the cell pellet was resuspended in
621 340 µl of 1X PBS and divided into two aliquots of 170 µl each for subsequent processing.

622

623 To each tube, 750 µl of ice-cold quench mixture, followed by 15 µl of a pre-mixed ISD mixture
624 containing 25 ng of 37:4 PIP, 25 ng of 37:4 PIP₂ and 50 ng of 31:1 PE was added. The tubes were
625 vortexed for 2 min at about 1500 rpm. Thereafter, 725 µl of CHCl₃ and 170 µl of 2.4 M HCl was
626 added. The tubes were again vortexed for 2 min and kept at room temperature on the bench for 5 min.
627 Two separate phases can be seen with a whitish precipitate at this stage.

628

629 All tubes were then spun at 1500 rpm in a benchtop centrifuge for 3 min to clearly separate the phases.
630 In fresh 2 mL low-bind tubes, one for each sample, 708 µl of LPWS was added and kept aside. Once
631 the phases were observed to be separate in the original tubes upon centrifugation, 900 µl of the lower
632 organic phase was pipetted out by piercing through the upper phase and the protein precipitate at the
633 interface and added to the tubes containing the LPWS. The tubes were then vortexed for 2 min and
634 spun at 1500 rpm in a benchtop centrifuge for 3 min to separate the phases. The lower phase was
635 pipetted out to extent possible taking care not to aspirate any of the upper phase and collected into a
636 fresh tube.

637

638 **Lipid Derivatization**

639 The following steps were performed inside a chemical hood, while wearing appropriate respirator mask.
640 50 µl of TMS-Diazomethane was added to each sample and incubated on a shaker at 600 rpm for 10
641 min at room temperature. At the end of 10 min, TMS-Diazomethane in each tube was quenched using
642 10 µl of Glacial Acetic acid. The tubes were inverted a few times to complete the quenching and
643 carefully snapped open once to let the Nitrogen released during quenching to escape. At this point, the
644 samples were moved out of the hood.

645

646 500 μ l of the upper phase of PDWS was added to each sample. Tubes were then vortexed for 2 min
647 and spun at 1500 rpm in a benchtop centrifuge for 3 min to separate the phases. 400 μ l of the upper
648 phase was discarded and another 500 μ l of upper phase of PDWS was added to each tube and the vortex
649 and spin steps were repeated as done earlier to separate the phases. Finally, the entire upper phase was
650 discarded from each sample. 45 μ l MeOH and 5 μ l H₂O was added to each tube, mixed and spun
651 down. All the samples were then dried in a SpeedVac at 500 rpm for around 2 h till only about 10-20
652 μ l of solvent was remaining. 90 μ l of MeOH was added to reconstitute the sample to a final volume of
653 about 100-110 μ l and taken for injection and analysis.

654

655 **Liquid Chromatography and Mass Spectrometry:**

656 Samples were injected in duplicates. We performed chromatographic separation on an Acquity UPLC
657 BEH300 C4 column (100 X 1.0 mm; 1.7 μ m particle size - Waters Corporation, USA) using a Waters
658 Aquity UPLC system connected to an ABSCIEX 6500 QTRAP mass spectrometer for ion detection.

659 The flow rate was set to 100 μ l /min.

660 Solvent gradients were set as follows –

661 Solvent A (Water + 0.1% Formic Acid)

662 Solvent B (Acetonitrile + 0.1% Formic acid)

663 0-5 min: 55% Solvent A + 45% Solvent B

664 5-10 min: Solvent B increased from 45% to 100%,

665 10-15 min: Solvent B at 100%,

666 15-16 min: Solvent B reduced from 100% to 45%,

667 16-20 min: 55% Solvent A + 45% Solvent B.

668 On the mass spectrometer, we first employed Neutral Loss Scans during pilot standardization
669 experiments on biological samples and searched for parent ions that would lose neutral fragments
670 corresponding to 490 a.m.u and 382 a.m.u indicative of PIP₂ and PIP species respectively and likewise
671 155 a.m.u for PE species as described in (Clark et al., 2011). Thereafter, we quantified PIP, PIP₂ and
672 PE species in biological samples using the selective Multiple Reaction Monitoring (MRM) method in
673 the positive ion mode (Supplementary Table 4). For each sample, PE levels measured were used to
674 normalize for total cellular phospholipid content on the assumption that PE levels are not likely to be
675 different between control and Lowe syndrome cell lines based on previous studies. The Sciex
676 MultiQuant software was used to quantify the area under the peaks. For each run, the area under curve
677 for each species of PIP, PIP₂ and PE was normalized to PIP, PIP₂ and PE internal standard peak
678 respectively to account for differences in loading and ion response. Thereafter, the sum of normalized
679 areas for all the species of PIP or PIP₂ was then divided by the sum of normalized areas for all the

680 species of PE in each of the biological samples to account for differences in total phospholipid extracted
681 across samples.

682

683 **Live cell imaging of PI(4,5)P₂ probe**

684 5-10,000 NSC of LSPH004- PIP_{2R} and the control line D149- PIP_{2R} were seeded on 35mm glass
685 bottom 15mm bore confocal dishes (Biostar LifeTech LLP #BDD011035) in NEM and cultured for
686 48 h to reach a uniform confluency. Prior to imaging, NEM was aspirated out of the dishes and cells
687 were incubated with Hoechst (Invitrogen, USA. #H3570) at 5 μM final concentration in NEM for 10
688 min to stain the nucleus. The cells were then washed with PBS and NEM was added prior to imaging.
689 Confocal images were recorded by collecting a z-stack using an Olympus FV 3000 confocal microscope.
690 This was performed in 3 independent biological replicates.

691

692 **Image Analysis:**

693 The quantification of mCherry::PH-PLCδ probe was performed manually by generating the
694 maximum z-projections of middle few planes of cells from confocal slices. Thereafter, line profiles were
695 drawn across clearly identifiable plasma membrane regions and their adjacent cytosolic regions and the
696 ratios of mean intensities (PM/Cyt) for these line profiles were calculated for each cell and used to
697 generate statistics (Sharma et al., 2019).

698

699 **Transcriptomics**

700 **NGS sequencing**

701 Total RNA was extracted from well characterized hiPSCs and NSCs of patient and control lines using
702 TRIzol (Ambion, Life Technologies, #5596018) as per manufacturer's protocol in 2 biological
703 replicates. The RNA was quantified using Qubit4 dsDNA HS Assay Kit (Thermo Fisher Scientific,
704 #Q32854) and run on a Bio-analyzer chip (Agilent High Sensitivity DNA Chip, #5067-4626) to assess
705 integrity. Post NEB Next Poly(A) mRNA Magnetic Isolation Module (#E74906), 150 ng of total RNA
706 (RIN values > 9) was used per sample for the library preparation using the NEB Next Ultra II
707 Directional RNA Library Prep Kit for Illumina (New England Biolabs, #E7760L). The libraries were
708 then sequenced on Illumina HiSeq 2500 sequencing platform using 2x100 bp sequencing format.

709

710 **Bioinformatics Analysis**

711 Illumina sequenced paired-end reads were obtained from sequencing as mentioned above. The quality
712 of processed reads (adapter removal and trimming) were evaluated using FastQC (version: 0.11.9). The
713 RNA-seq reads were then mapped onto to the human reference genome (hg38) using HISAT2
714 (version: 2.1.0), the resulting BAM files containing the aligned reads were provided to HTSeq (version:
715 0.12.4) to obtain gene-level read count table using the reference annotation file (GTF format). We
716 further utilized the iDEP (0.91)(Ge et al., 2018) to transform the read counts data using the regularized
717 log (rlog) transformation method that is originally implemented in the DESeq2
718 [<https://bioconductor.org/packages/release/bioc/html/DESeq2.html>]. Differential expression,
719 Enrichment analysis and Gene Ontology (Biological Process) were then conducted using DESeq2 and
720 default settings within the iDEP (0.91). Genes with \log_2FC greater than +1.5 and lesser than -1.5 were
721 considered as up and down regulated respectively while adhering to p-value < 0.05. Such filtered genes
722 were used for further downstream analysis which included gathering enriched Gene Ontology
723 (Biological Process) terms, for which the filtered gene list was provided to ShinyGO v0.66 (Ge et al.,
724 2020) (<http://bioinformatics.sdstate.edu/go/>) with default filtering parameters. To remove redundant
725 GO terms, we evaluated the gene lists for statistical significance by Fisher's test using PANTHER (Mi
726 et al., 2021) (<http://pantherdb.org/>). With a FDR <0.05 cut-off, we obtained the enrichment list which
727 was further submitted to REViGO (Supek et al., 2011) (<http://revigo.irb.hr/>) which refines the number
728 of redundant functional terms based on semantic similarity between the ontology terms. Using a tight
729 filter of dispensability < 0.05, the Gene Ontology (Biological Process) terms were obtained.

730

731 DNA constructs

732 ZYP017- AAVS1-TALEN-R (XCell Science. Novato, CA); ZYP018- AAVS1- TALEN-L (XCell
733 Science. Novato, CA); ZYP037- AAVS1P-CAG-copCFP iRMCE (XCell Science. Novato, CA);
734 ZYP070- DCX-GFP donor (XCell Science. Novato, CA); ZYP073- AAV-PGK-Cre (XCell Science.
735 Novato, CA). To drive the probe expression in neural stem cells we replaced neuron specific DCX
736 promoter in ZYP070 with CAG promoter by using the SalI and BsrGI restriction sites. mCherry::PH-
737 PLC δ was cloned into ZYP070 at the GFP site by overlapping primers using GIBSON assembly.

738

739 Statistics and Software

740 Two-tailed unpaired student's t-test was used to compare datasets of two. One-way ANOVA with post
741 hoc Tukey's multiple pairwise comparison was used whenever the experiment consisted of more than

742 two biological groups. All statistical analyses were performed on Graph Pad Prism (version. 8).
743 Schematics were created with biorender.com.

744 **Data availability**

745 RNA sequencing data used in this study has been submitted to the Sequence Read Archive database on
746 NCBI under BioProject ID [PRJNA741484](#)

747 **Ethics Approval**

748 This work was carried out under the ethics approval provided by the Institutional Ethics Committee,
749 St. John's Medical College & Hospital, Bangalore (IEC Study Ref. No. 28 / 2017) and the Institutional
750 Ethics Committee, National Centre for Biological Sciences, Bangalore (NCBS/IEC-8/002).
751 Institutional Stem Cell Committee Approval-National Centre for Biological Sciences (01/ICSCR/IX-
752 06.01.2020-RP2).

753 **Competing interests:** The authors declare no competing interests.

754 **Author contributions**

755 Conceptualization: PR, AV

756 Methodology: PR, AV

757 Software: -

758 Validation: BA, PB, SA, SS, YS, KG, ABS, AV

759 Formal analysis: BA, SS

760 Investigation: BA, PB, SA, SS, YS, KG, ABS, AV

761 Resources: AV

762 Data curation: BA, PB, SA, SS, YS, KG, ABS, AV

763 Writing - original draft: BA, PR

764 Supervision: PR

765 Project administration: PR

766 Funding acquisition: PR

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918 Figure Legends

919 Figure 1. Generation and characterization of hiPSCs derived from Lowe syndrome patients

920 (A) i) Pedigree structure of the studied family. Shaded circles indicate heterozygous carrier mothers
921 and shaded squares indicate hemizygous patients. ii) Steps in generation of iPSC, NSC and
922 Neurons from LCL.

923 (B) Immunocytochemistry of hiPSC colonies showing expression of OCRL (green) in control line
924 NIH5 and absent in the patient lines (LSPH002, LSPH003, LSPH004). Nuclear stain DAPI
925 (blue) Scale bar: 50 μ m.

926 (C) Immunocytochemistry of hiPSC colonies showing presence of pluripotency nuclear marker
927 SOX2 (green) and of pluripotency surface marker SSEA4 (red). Scale bar: 200 μ m.

928 (D) Expression of three germ lineage transcripts SOX1 and Nestin (Ectoderm), Nodal
929 (Mesoderm), GATA4 (Endoderm), β -actin as control by RT-PCR from embryoid bodies
930 derived from LSPH002, LSPH003, LSPH004 hiPSC.

931 (E) Karyogram depicting normal karyotype 46(X,Y) of the hiPSC line LSPH004

932

933 Figure 2. Characterization of Neural Stem Cells and Neurons derived from Lowe syndrome hiPSC

934 (A) Immunocytochemistry of Neural Stem Cells (i) D149 (control) (ii) LSPH004 (patient) showing
935 the expression of the NSC markers SOX1, SOX2, PAX-6, Nestin, Mushashi-1 and the
936 proliferation marker Ki-67. Nucleus stained with DAPI. Scale bar: 50 μ m

937 (B) Immunofluorescence images (maximum intensity projections) of D149 and LSPH004 neurons
938 at 30 DIV differentiated from respective NSCs. The cells were stained with the following
939 neuronal markers: (i, ii) DCX (*green*, immature neuronal marker) and MAP2 (*magenta*, mature
940 neuronal marker); (iii, iv) synapsin-1 (*green*) and MAP2 (*magenta*); followed by counterstaining
941 with DAPI (*blue*). Scale bar: 50 μ m.

942 (C) Western blot showing expression of OCRL protein in lysates from 30 DIV Neurons in the
943 control line D149 and its absence in patient line LSPH004. GAPDH was used as a loading
944 control.

945 (D) Calcium transients recorded from 30 DIV D149 (i) and LSPH004 (ii) neurons are shown. Each
946 panel shows $[Ca^{2+}]_i$ traces from individual cells in the dish. Y-axis shows normalized fluorescence
947 intensity $\Delta F/F_0$ and X-axis is time in seconds. The baseline recording for 4 mins, followed by

948 addition of 10 μ M tetrodotoxin (TTX) (as indicated by the arrows).

949 (E) Evoked action potentials (AP) in neurons differentiated from NSC recorded using whole-cell
950 patch clamp electrophysiology at 10, 20, 30 and 40 DIV. The characteristic feature of action
951 potentials recorded from control D149 cells at each time point is shown. (i) immature AP at 10
952 DIV, single action potential at 20 DIV (ii) and (iii) multiple AP on 30 DIV (iv) Most neurons
953 exhibited repetitive firing by 40 DIV.

954

955 **Figure 3. Generation of genomic safe harbour lines for stable expression of protein biosensors**

956 (A) A schematic showing the mechanism for generating the reporter line using Genomic Safe
957 Harbor-RMCE (Recombinase Mediated Cassette Exchange) approach. Abbreviations:
958 TALEN-Transcription activator-like effector nucleases (Left and Right), AAVS1-Adeno-
959 Associated Virus Integration Site 1 (LA-Left Arm, RA-Right Arm).

960 (B) Validation of LoxP-CAG-GFP (ZYP037) insert into AAVS1 genomic locus using junction
961 PCR. Amplicon of the expected size validating the right and left junction are shown.

962 (C) Characterization of Genomic Safe Harbour lines D149-GSH and LSPH004-GSH:
963 Expression of the NSC markers Nestin and SOX2 detected by Immunocytochemistry. GFP
964 expression from the safe harbour construct marker is also shown. Scale bar: 40 μ m.

965 (D) Representative confocal image (maximum z-projection) showing expression and localization of
966 mCherry::PH-PLC δ (magenta) and cytosolic GFP (green) in D149 PIP_{2R} reporter line
967 colonies. Nucleus stained using Hoechst (cyan). Scale bar: 50 μ m.

968 (E) Representative flow cytometry data to illustrate the gating strategy for FACS purification of i)
969 GFP positive cells for Genomic Safe Harbour lines, to select for healthy GFP positive cells,
970 side scatter (SSC-A, log, Y-axis) and signal from the excitation of cells with the 488-nm laser
971 (GFP-A, log, X-axis) are plotted. ii) mCherry positive cells for PIP₂ reporter lines were selected
972 for by plotting signal from the excitation of cells with the 568-nm laser (mCherry-A, log, Y-
973 axis) against signal from the excitation of cells with the 488-nm laser (GFP-A, log, X-axis).

974 (F) Western blot showing OCRL (detected using the OCRL antibody), mCherry::PH-PLC δ
975 (detected using the mCherry antibody) in Neural Stem Cell PIP_{2R} reporter lines. GAPDH was
976 used as a loading control.

977

978 **Figure 4. Mass spectrometry and biosensor estimation of PIP & PI(4,5)P₂**

- 979 (A) Box and Whisker Plot showing total PIP levels using LCMS in whole cell lipid extract from
980 hiPSCs of Control (D149, n=9) and Patient lines (LSPH004, n=10). X-axis denoting samples
981 and Y-axis represents normalized PIP levels. Statistical test: Two-tailed unpaired t-test with
982 Welch's correction. Whiskers at minimum and maximum values and a line at the median.
- 983 (B) Box and Whisker Plot showing total PIP₂ levels using LCMS in whole cell lipid extract from
984 hiPSCs of Control (D149, n=9) and Patient lines (LSPH004, n=10). X-axis denoting samples
985 and Y-axis represents normalized PIP₂ levels. Statistical test: Two-tailed unpaired t-test with
986 Welch's correction. Whiskers at minimum and maximum values and a line at the median.
- 987 (C) Box and Whisker Plot showing total PIP levels using LCMS in whole cell lipid extract from
988 Neural Stem Cells of Control (D149, n=12) and Patient line (LSPH004, n=12). X-axis
989 denoting samples and Y-axis represents normalized PIP levels. Statistical test: Two-tailed
990 unpaired t-test with Welch's correction. Whiskers at minimum and maximum values and a line
991 at the median.
- 992 (D) Total PIP₂ levels using LCMS in whole cell lipid extract from Neural Stem Cells of Control
993 (D149, n=12) and Patient line (LSPH004, n=12). X-axis denoting samples and Y-axis
994 represents normalized PIP levels. Statistical test: Two-tailed unpaired t-test with Welch's
995 correction. Whiskers at minimum and maximum values and a line at the median.
- 996 (E) Representative confocal maximum z-projections of mCherry::PH-PLC δ expressing PIP₂
997 reporter lines used to estimate plasma membrane/cytosolic PIP₂ probe fluorescence ratio
998 (PM/Cyt). Enlarged insert shows mCherry::PH-PLC δ biosensor localization to the plasma
999 membrane in a single cell.
- 1000 (F) Dot plot denoting quantification of PIP₂ levels using mCherry::PH-PLC δ biosensor in
1001 Control (D149) and Patient (LSPH004) PIP₂ reporter neural stem cell lines. X-axis denotes
1002 samples, Y-axis represents plasma membrane/cytosolic fluorescence ratio (PM/Cyt) of
1003 mCherry::PH-PLC δ biosensor. Each dot represents PM/Cyt obtained from a cell. Statistical
1004 test: Two-tailed unpaired t-test with Welch's correction. **** p-value <0.0001. Error bars:
1005 S.E.M.

1006

1007 **Figure 5. Expression analysis of inositol-5-phosphatase genes in hiPSC, NSC and 30 DIV neurons**
1008 **using qRT-PCR.**

1009 (A) Quantitative real time PCR (qRT-PCR) showing mRNA expression of 10 inositol-5 -
1010 phosphatases in the human genome. Data is shown for hiPSC, NSC and 30 DIV neurons in

1011 the D149 control line. Statistical test: one-way ANOVA with post hoc Tukey's multiple
1012 pairwise comparison. * p-value <0.05, ** p-value <0.01, **** p-value <0.0001. Error bars:
1013 Standard Deviation

1014 (B-D) Relative mRNA expression levels of 10 inositol-5 -phosphatases across hiPSC, NSC and 30
1015 DIV neuron in D149 control line and LSPH004 patient line obtained from quantitative real
1016 time PCR (qRT-PCR). The expression levels have been normalized with GAPDH and the
1017 values represented in the terms of $2^{-\Delta Ct}$ on Y-axis. Statistical test: Two-tailed unpaired t-test
1018 with Welch's correction. Error bars: Standard Deviation

1019 (E) Fold Change in expression of 10 inositol-5-phosphatases across hiPSC, NSC and 30 DIV
1020 neurons in LSPH004 patient line relative to D149 obtained from quantitative real time PCR
1021 (qRT-PCR) using the $2^{-\Delta\Delta Ct}$ method with GAPDH as a control gene. Error bars: Standard
1022 Deviation

1023

1024 **Figure 6. RNA-Seq analysis denoting DEGs and enriched Gene Ontology Biological Process terms**
1025 **in patient iPSCs and NSCs.**

1026 (A) Scatter plot showing \log_2 fold change on Y-axis and mean of normalized expression counts of
1027 on X-axis from Differential Expression genes between control (D149) vs patient (LSPH004)
1028 hiPSC RNA-Seq. Red and blue dots indicate statistical significant up and down regulated
1029 genes respectively ($-1.5 > \text{Log}_2\text{FC} > 1.5$, $\text{FDR} \leq 0.05$)

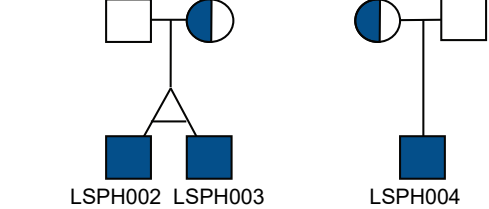
1030 (B) Scatter plot showing \log_2 fold change on Y-axis and mean of normalized expression counts of
1031 on X-axis from Differential Expression genes between control (D149) vs patient (LSPH004)
1032 NSC RNA-Seq. Red and blue dots indicate statistical significant up and down regulated genes
1033 respectively ($-1.5 > \text{Log}_2\text{FC} > 1.5$, $\text{FDR} \leq 0.05$)

1034 (C-E) Dot-plot showing GO (Gene Ontology) Biological Process term enrichment, Y-axis shows
1035 enriched GO terms whereas X-axis shows Fold enrichment of each term in upregulated genes
1036 of hiPSC LSPH004 (C), downregulated genes of hiPSC LSPH004 (D) and downregulated
1037 genes of NSC LSPH004 (E). Colours indicate the $-\log_{10}\text{FDR}$ from Fisher's exact test, and dots
1038 size is proportional to the number of differentially expressed genes (DEG) in the given pathway.

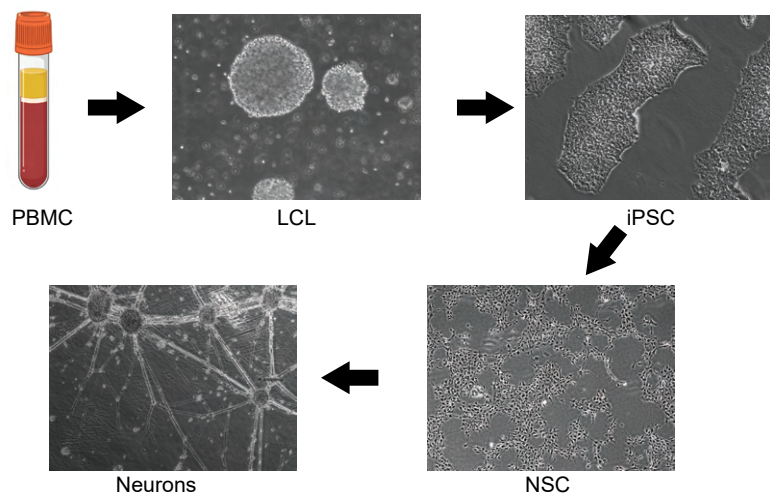
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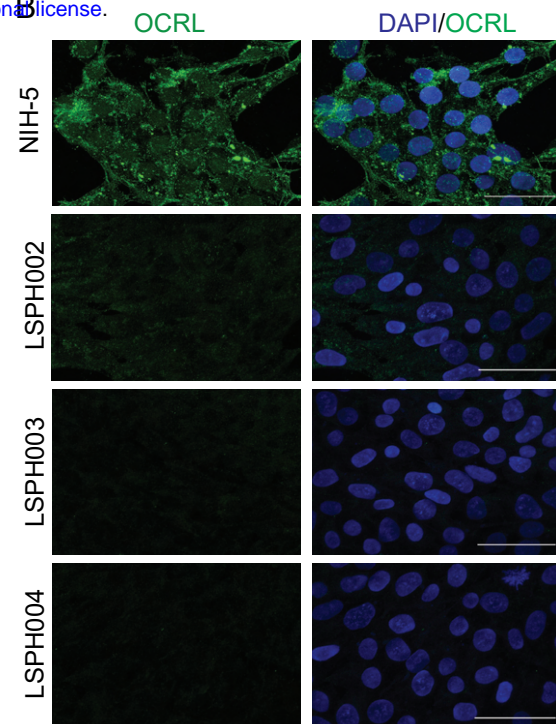
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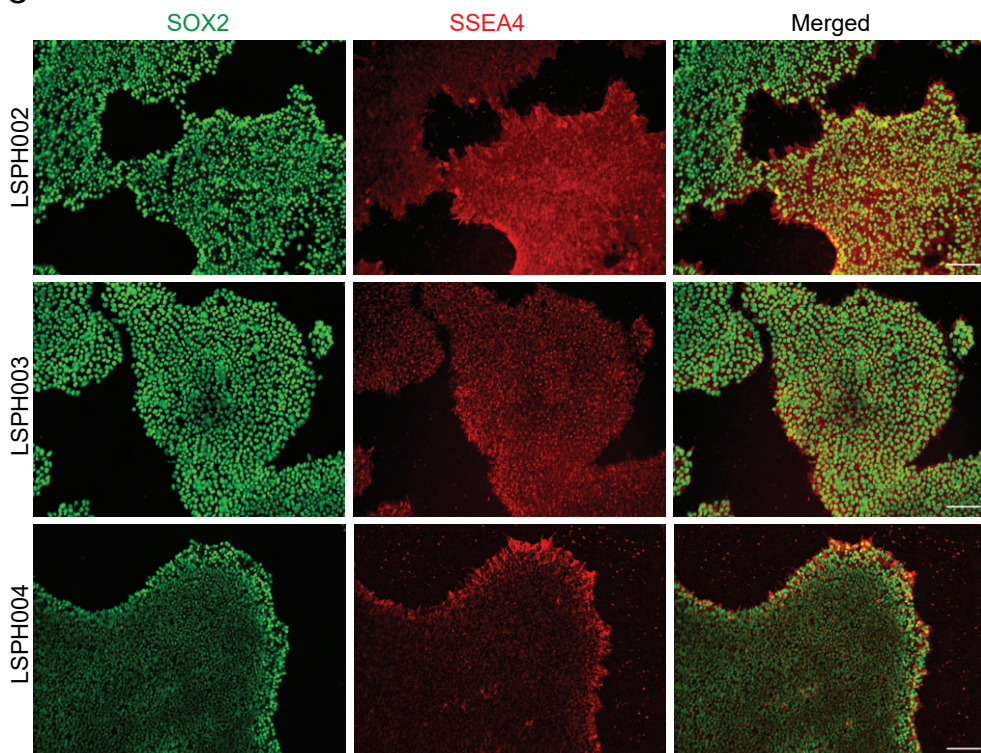
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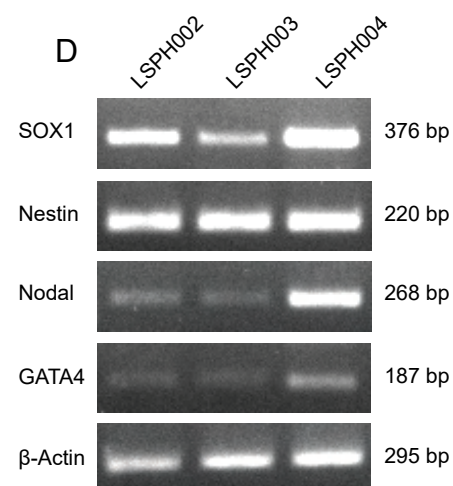
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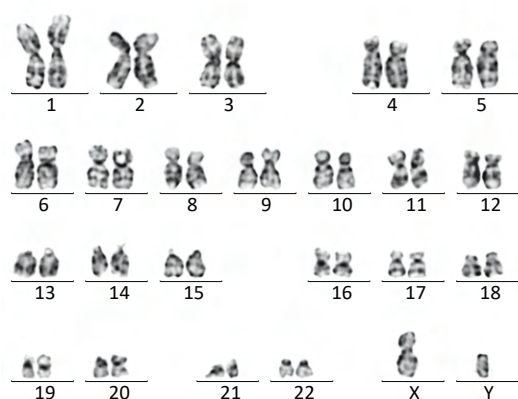
C



D



E



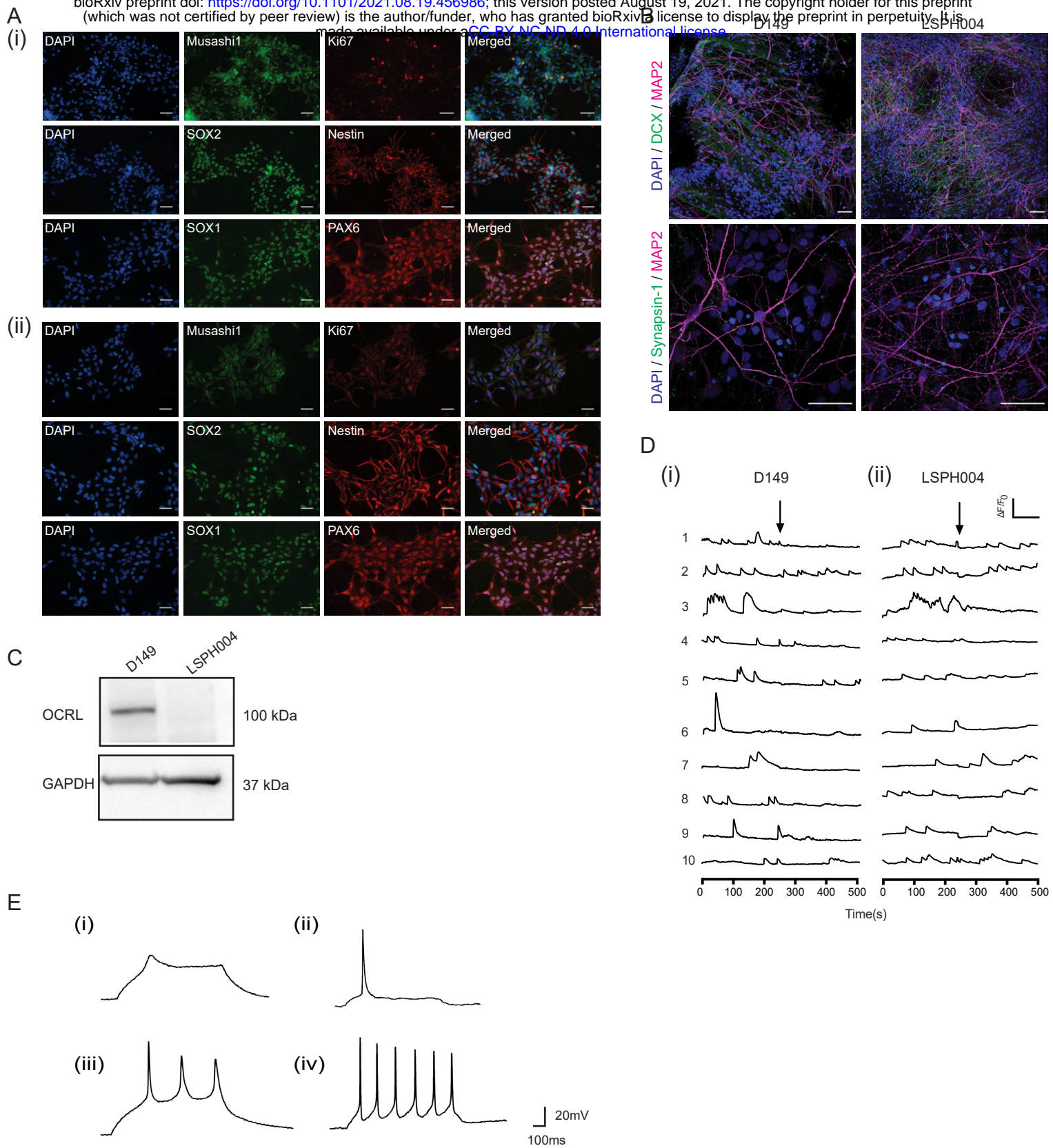
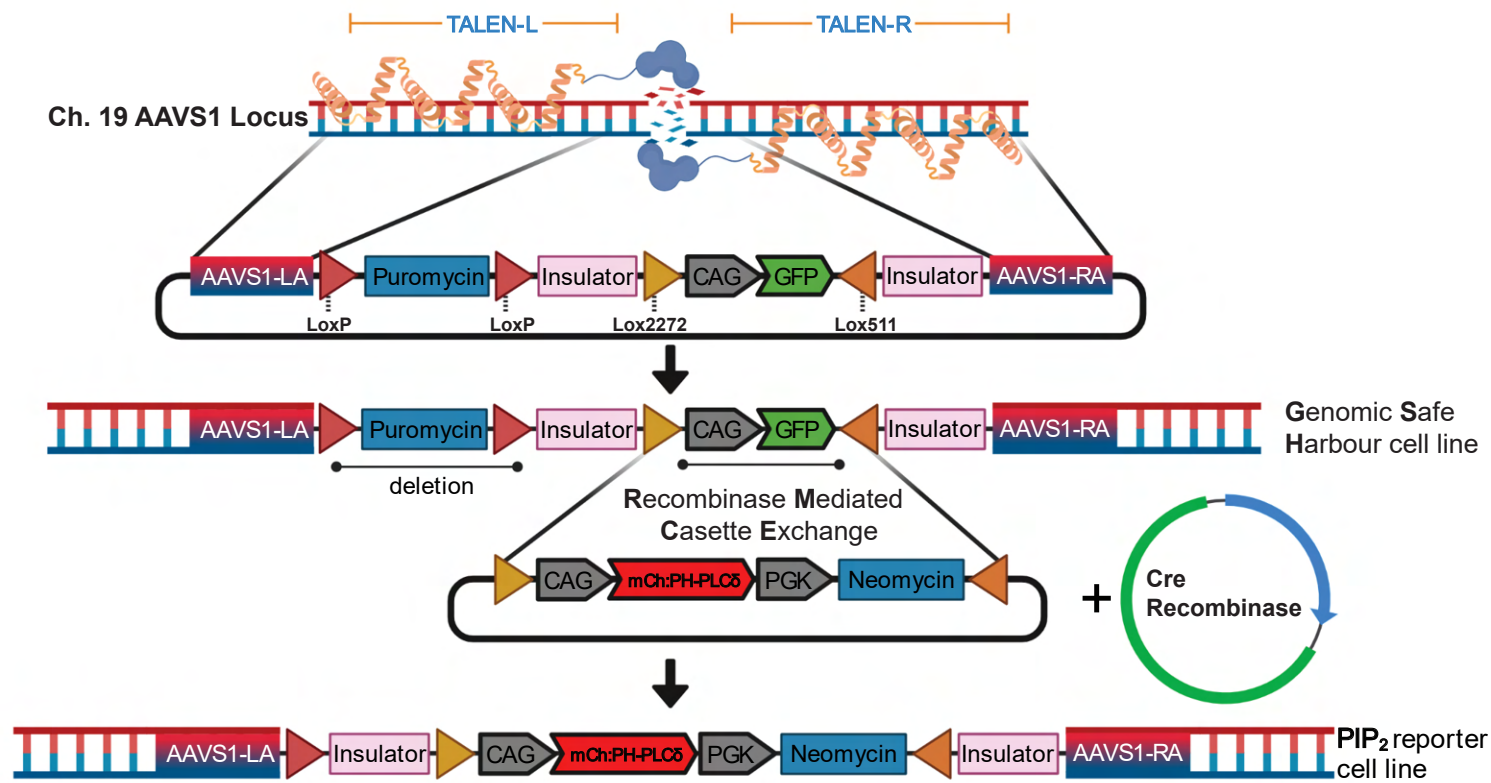
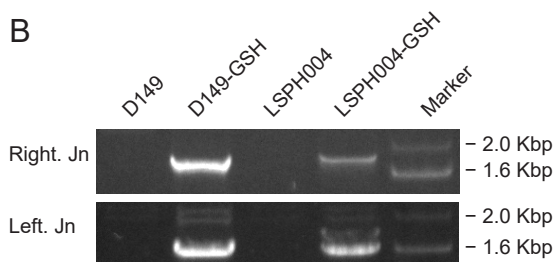


Figure. 2

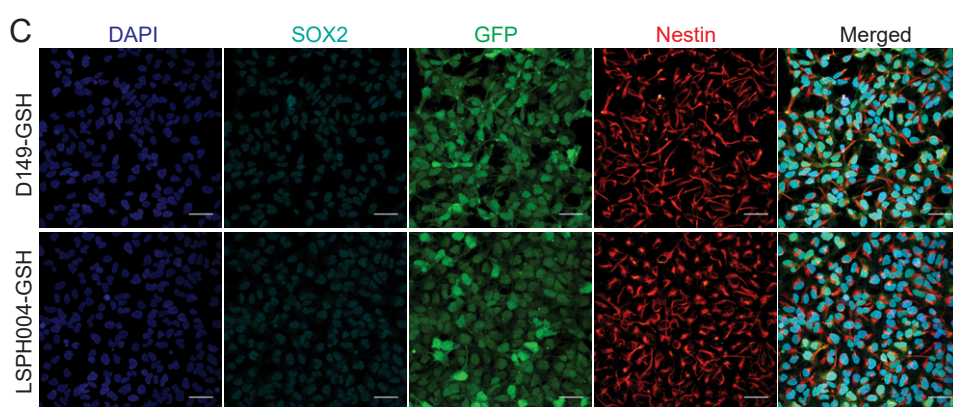
A



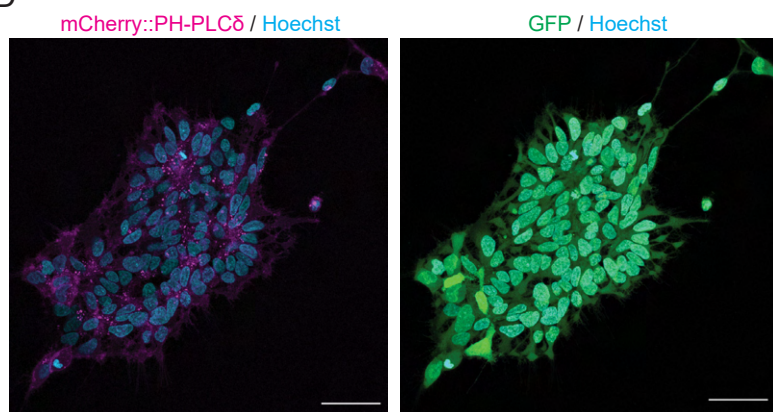
B



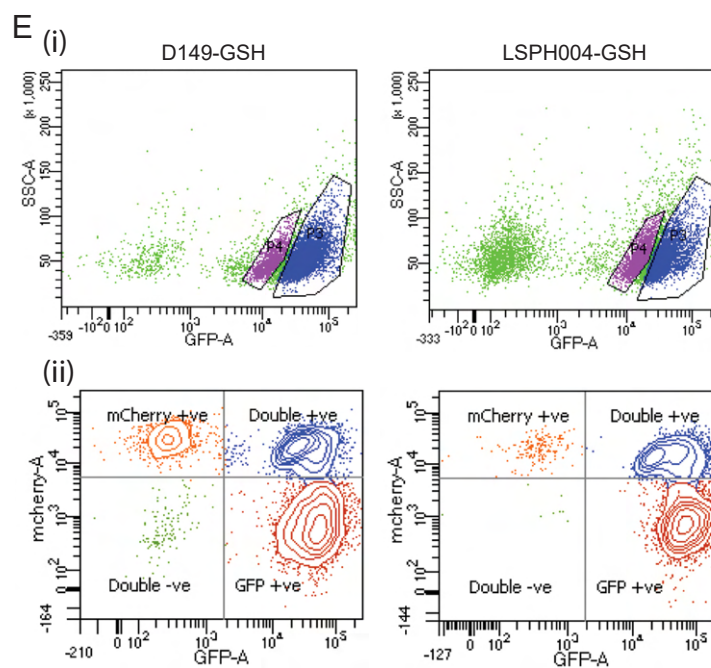
C



D



E



F

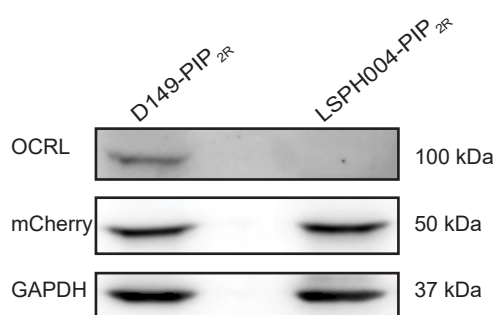
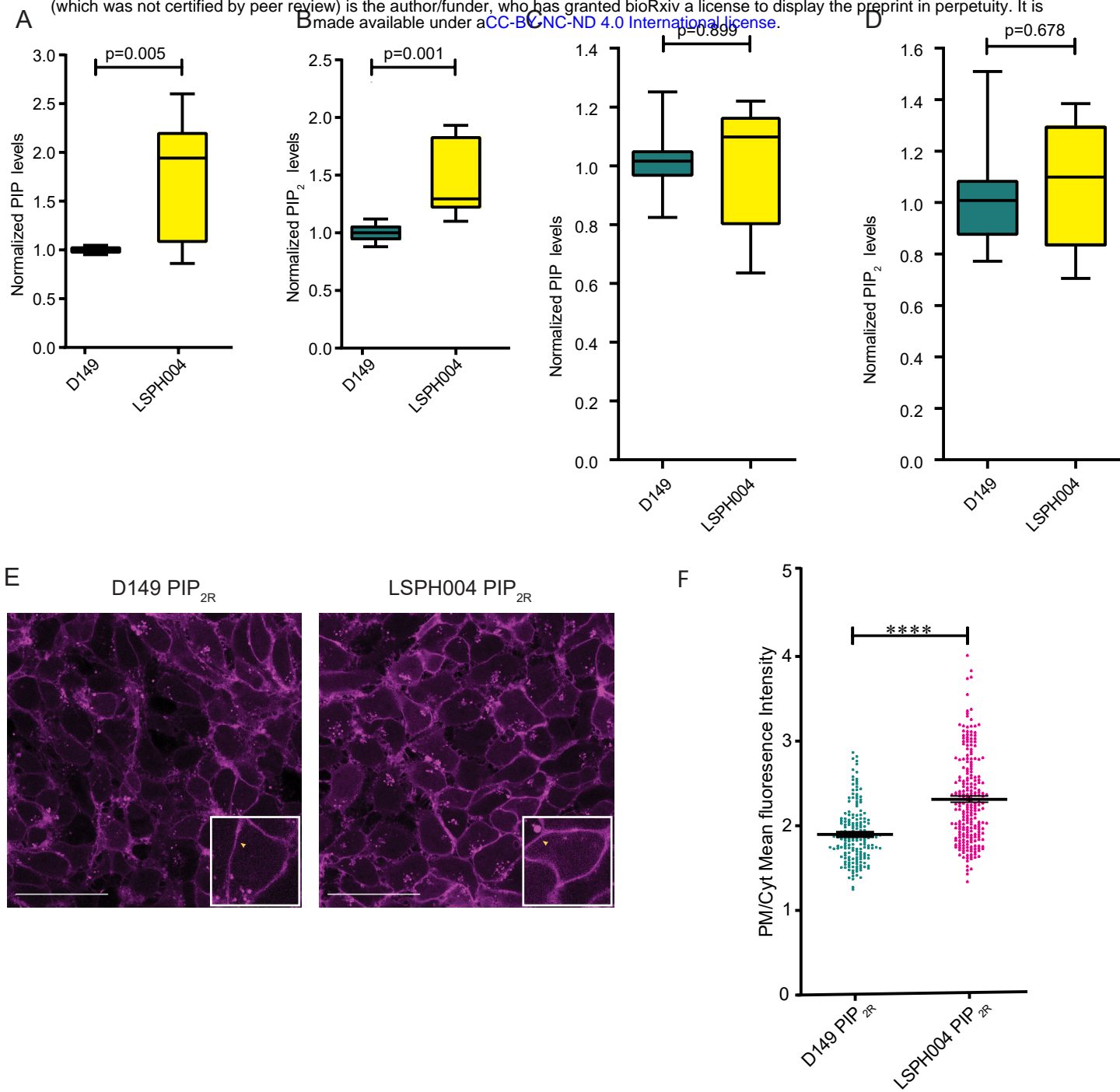


Figure. 3



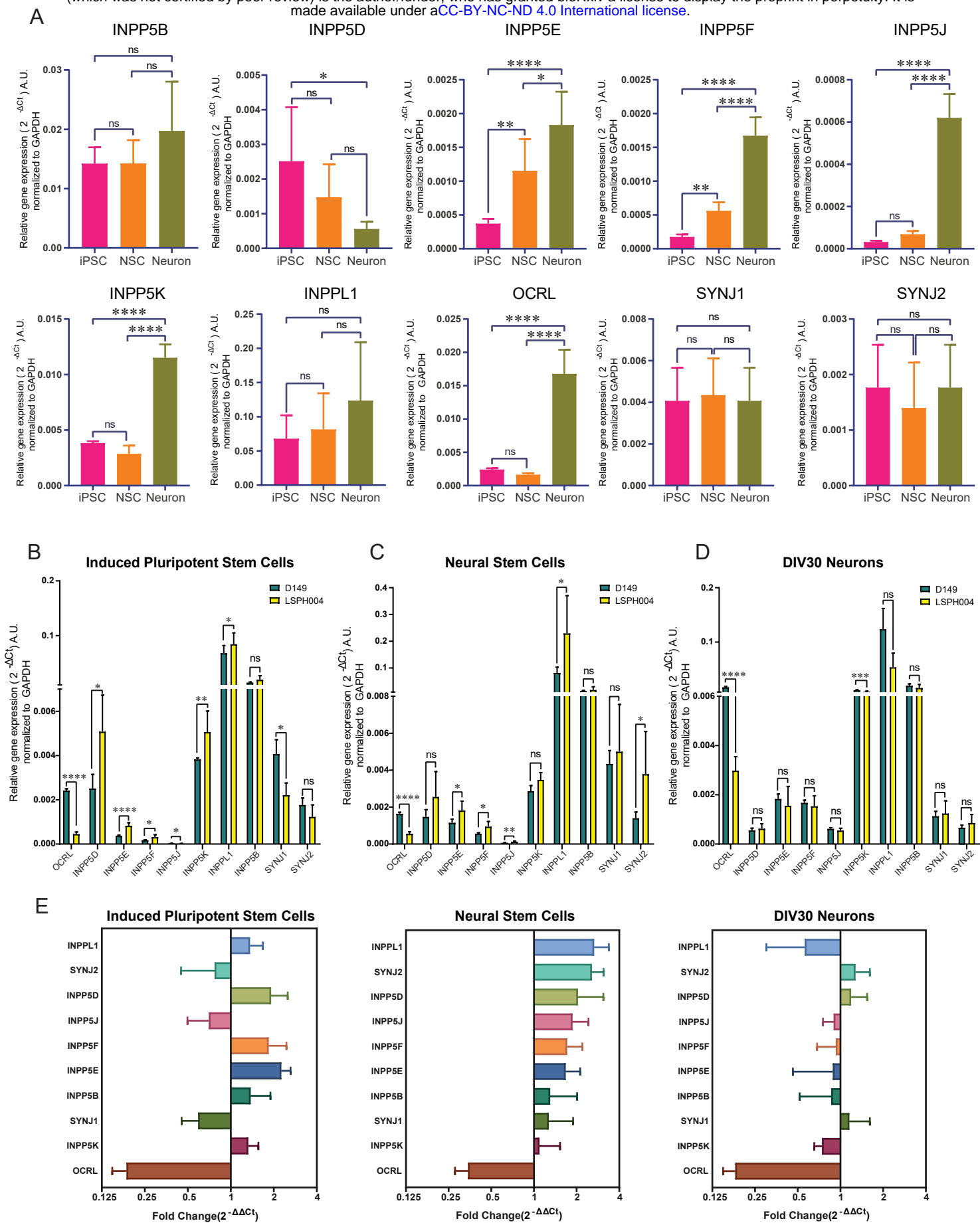
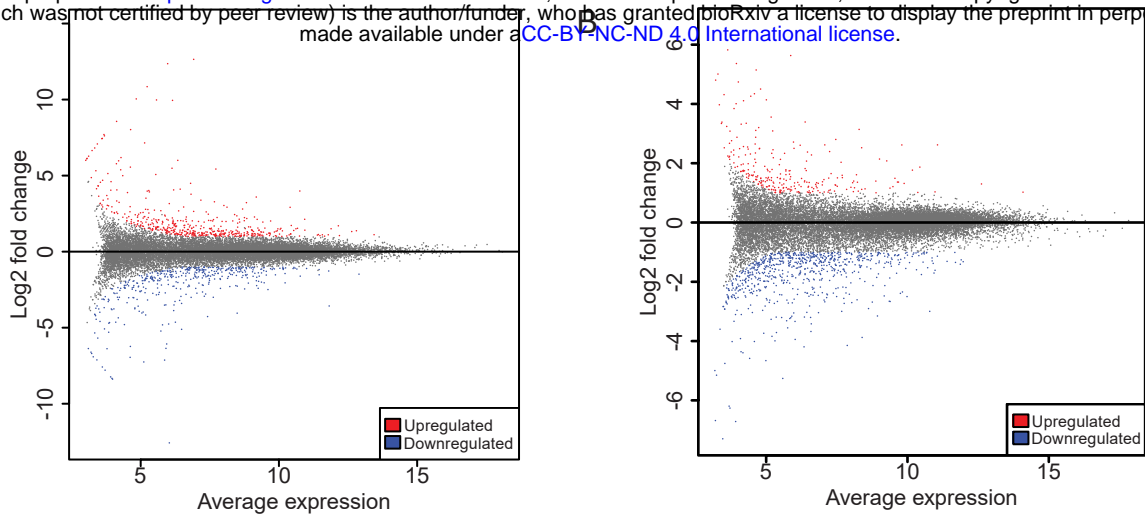
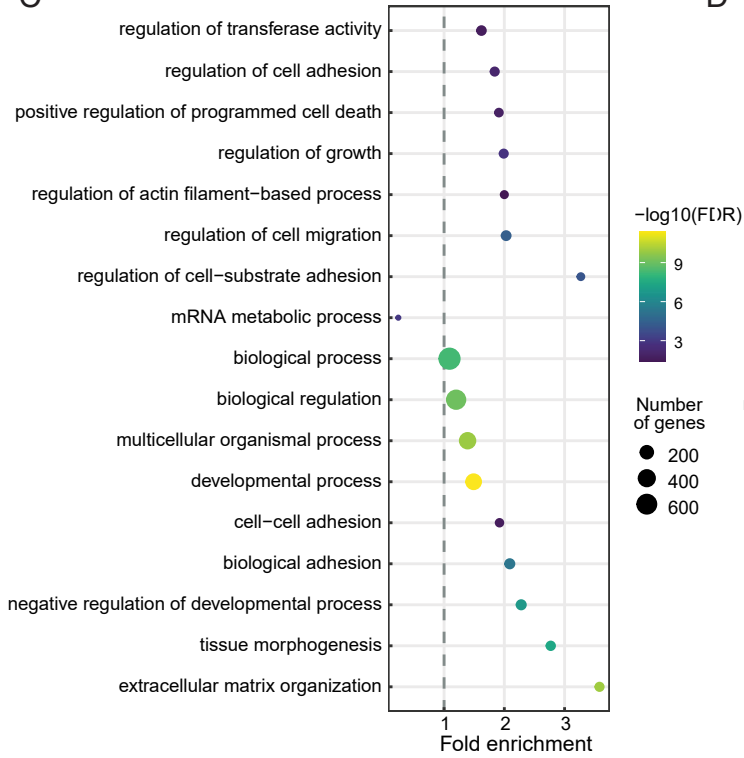


Figure 5

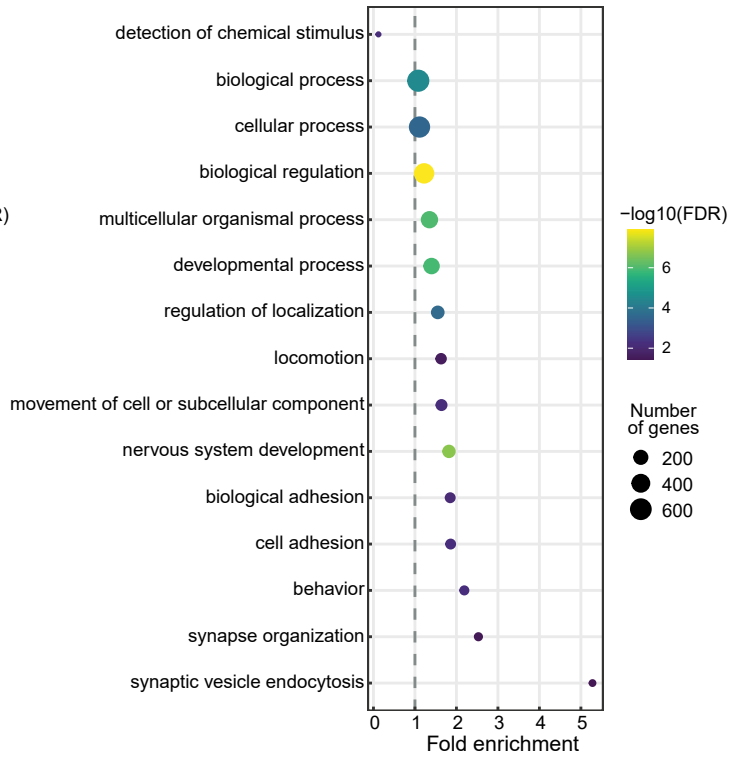
A



C



D



E

