Dominant spinal muscular atrophy linked mutations in the cargo binding domain of BICD2 result in altered interactomes and dynein hyperactivity.

Hannah Neiswender[1], Rajalakshmi Veeranan-Karmegam[1], Jessica E. Pride[1], Phylicia Allen[1], Grace Neiswender[1], Avneesh Prabakar[1], Caili Hao[1], Xingjun Fan[1], and Graydon B. Gonsalvez[1]

[1]Cellular Biology and Anatomy
Medical College of Georgia, Augusta University
1460 Laney Walker Blvd
Augusta, GA, 30912, USA

[*]Address correspondence to
Graydon Gonsalvez (ggonsalvez@augusta.edu)
Cellular Biology and Anatomy
Medical College of Georgia, Augusta University
1460 Laney Walker Blvd. CB2917
Augusta, GA 30912, USA
Tel: (706) 721-1756

ABSTRACT

Cytoplasmic dynein-1 (dynein) is responsible for the transport of most cellular cargo towards the minus end of microtubules. Dynein activation requires the multi-subunit dynactin complex and an activating cargo adaptor. The adaptors serve to link dynein with cargo and to fully activate the motor. Mutations in one of these activating adaptors, Bicaudal-D2 (BICD2), are associated with a neurodegenerative disease called Spinal Muscular Atrophy with Lower Extremity Predominance (SMALED2). The molecular defect that underlies SMALED2 is largely unknown. In addition to interacting with dynein, BICD2 has also been shown to associate with KIF5B, a plus-end directed microtubule motor. We hypothesized that interactome changes associated with mutant versions of BICD2, and the resulting differences in cargo transport, might underlie the etiology of SMALED2. To test our hypothesis, we first defined the interactome of wild-type BICD2. This led to the identification of known BICD2 interacting proteins in addition to potentially novel cargo such as components of the HOPS complex, a six-subunit complex involved in endo-lysosomal trafficking. We next determined the interactome of three SMALED2 linked mutants in BICD2, two of which reside in the cargo binding domain. Interestingly, all three mutations resulted in BICD2-mediated dynein hyper-activation. Furthermore, all three mutants were associated with interactome changes. One of these mutants, BICD2_R747C, was deficient in binding to HOPS complex components and the nucleoporin RANBP2. In addition, this mutant also resulted in a gain of function interaction with GRAMD1A, a protein localized to the endoplasmic reticulum. This gain of function interaction resulted in mis-localization of GRAMD1A in BICD2_R747C expressing cells. Collectively, our results suggest that dynein hyperactivity, interactome changes, and the resulting cargo transport defects likely contribute to the symptoms associated with SMALED2.

INTRODUCTION

The intracellular transport of mRNAs, proteins, vesicles, and organelles is facilitated by microtubule motors. These motors operate along microtubules, which are polarized cytoskeletal filaments. Typically, kinesin family motors carry cargo toward the microtubule plus end (Yildiz, 2025), whereas transport toward the minus end relies primarily on a single motor protein, cytoplasmic dynein-1 (dynein) (Canty and Yildiz, 2020; Reck-Peterson et al., 2018). While motor-driven transport is vital across various cell types, it holds particular importance in large, polarized cells like neurons. For instance, the axon of some neurons can extend over a meter in length, making them highly reliant on efficient transport systems. Even minor disruptions in motor-based transport can impair neuronal function and lead to disease (Franker and Hoogenraad, 2013). As a result, mutations affecting motor proteins, their activators, or adaptors are linked to a variety of neurological disorders (Sleigh et al., 2019).

The isolated dynein motor is present in an inhibited conformation and has a limited capacity to traverse the microtubule cytoskeleton (Torisawa et al., 2014; Zhang et al., 2017). Processive movement by dynein requires the multi-subunit dynactin complex and an activating cargo adaptor (McKenney et al., 2014; Schlager et al., 2014; Splinter et al., 2012). Bicaudal-D (BicD), initially identified in *Drosophila*, is one of the best characterized activating cargo adaptors (Hoogenraad and Akhmanova, 2016) (Mohler and Wieschaus, 1986). Mammals encode four orthologous proteins, BICD1, BICD2, BICDR1 and BICDR2. The closest mammalian ortholog of *Drosophila* BicD is BICD2. BICD2 contains three coiled-coil regions designated CC1, CC2, and CC3 (Fig. 1A) (Olenick and Holzbaur, 2019). The first coiled-coil, CC1, is responsible for binding to dynein and dynactin (Chowdhury et al., 2015; Urnavicius et al., 2015), whereas the third coiled-coil, CC3, mediates cargo binding (Hoogenraad and Akhmanova, 2016). In the absence of cargo, an intramolecular interaction between the CC1 and CC3 regions of BICD2 results in the protein folding into an inhibited conformation (Liu et al., 2013; Terawaki et al., 2015; Wharton and Struhl, 1989). In this state, BICD2 is unable to bind dynein (Fig. 1B). Cargo binding to CC3 competes with the intramolecular interaction, effectively opening up BICD2 and enabling the cargo-bound adaptor to interact with and activate dynein for motility (Goldman et al., 2019; Huynh and Vale, 2017; Liu et al., 2013; McClintock et al., 2018; Sladewski et al., 2018). This type of regulation ensures that the motility of dynein is tightly coupled to cargo binding.

The importance of BICD2 in dynein-mediated transport is highlighted by the fact that mutations in this gene are associated with a type of spinal muscular atrophy (SMA). SMA refers to a group of disorders characterized by progressive muscle weakness and atrophy caused by loss of motor neurons. Most often, SMA is autosomal recessive, and in the majority of these cases, causative mutations are linked to the SMN (survival of motor neurons) gene (Angilletta et al., 2023). Autosomal dominant SMA, caused predominantly by mutations in BICD2, is associated with weakness and atrophy of muscles in the feet and legs (Koboldt et al., 2020; Neveling et al., 2013; Oates et al., 2013; Peeters et al., 2013). Thus, this type of SMA is referred to as Spinal Muscular Atrophy with Lower Extremity Predominance (SMALED2). In some, the disease is relatively mild, whereas in others it is more severe, presenting with contractures, hip dysplasia, brain abnormalities, cognitive impairment, and even death (Koboldt et al., 2020).

Although the link between BICD2 and SMALED2 has been recognized, we have a limited understanding of the molecular basis of the disease. Three SMALED2 associated mutations within the dynein binding region of BICD2 have been characterized. These mutations have been shown to increase the interaction between BICD2 and dynein/dynactin (Huynh and Vale, 2017). Consequently, dynein displayed hyperactivity in motility assays (Huynh and Vale, 2017). However, numerous additional SMALED2 mutations have also been identified within the cargo binding domain of BICD2 (Koboldt et al., 2020; Martinez-Carrera and Wirth, 2015). The mechanism by which these mutations result in SMALED2 is unknown. Given that BICD2 is a cargo adaptor for dynein, we hypothesized that mutations within the cargo binding domain likely result in interactome changes. As such, cargo transport defects caused by altered BICD2 interactomes, might underlie the etiology of SMALED2. The goal of this study was to define the interactome of wild-type and mutant alleles of BICD2 and to identify disease relevant interactome changes.

RESULTS

Defining the wild-type BICD2 interactome.

In order to test our hypothesis that SMALED2 associated mutations in the cargo binding domain of BICD2 are associated with interactome changes, it was first critical for us to define the interactome of wild-type BICD2. Although BICD2 has been known to function as a dynein cargo adaptor, only a few proteins have been shown to interact with BICD2 (Hoogenraad and Akhmanova, 2016; Olenick and Holzbaur, 2019). A more comprehensive interactome of BICD2 was defined by Redwine and colleagues using proximity biotin ligation (Redwine et al., 2017). However, the goal of that study was to more broadly define the dynein interactome and potential novel BICD2 interacting proteins were not validated by follow up studies.

To begin our analysis, we generated stable cell lines expressing either RFP tagged mini-TurboID (RFP-mTrbo) or full length BICD2 tagged on the C-terminus with mini-TurboID (BICD2-mTrbo). TurboID is a proximity biotin ligase that was generated by selecting mutants of BioID that displayed much higher levels of activity in comparison to the original enzyme (Branon et al., 2018). mini-TurboID is a smaller version of TurboID, the biotinylation activity of which is more tightly coupled to the addition of exogenous biotin (Branon et al., 2018). HEK293 FLP-In T-Rex cells were used for this experiment because this enabled us to integrate the constructs at the same genomic locus across all cell lines. In addition, expression of the fusion proteins was inducible upon the addition of tetracycline. This prevents any toxic effects that might arise from constitutive expression of SMALED2 alleles of BICD2.

Cells expressing either RFP-mTrbo or BICD2_WT-mTrbo were grown to scale and proximal proteins were labeled using biotin. Biotinylated proteins were purified using streptavidin magnetic beads and identified using mass spectrometry, with the entire experiment being conducted in triplicate. Proteins enriched in the BICD2_WT-mTrbo pellet at least two-fold in comparison to the RFP-mTrbo pellet and having a p value of at least 0.05 were considered potential BICD2 interacting partners (Fig. 1C, C', Supplemental table 1). This list included several dynein components and known BICD2 interacting proteins such as RANBP2, RANGAP1, and PLK1 (Fig. 1D) (Gallisa-Sune et al., 2023; Splinter et al., 2010). In addition, four components of the HOPS complex were also specifically enriched in the BICD2_WT-mTrbo pellet (Fig. 1D). The HOPS complex consists of six subunits and is involved in the fusion of late endosomes with lysosomes (van der Beek et al., 2019). Consistent with known BICD2 functions, a cellular component GO analysis indicated an enrichment of terms such as "cytoplasmic dynein complex", "nuclear pore" and "vesicle tethering complex" (Fig. 1E).

Surprisingly, we failed to identify RAB6A in our interactome analysis. RAB6A was the first cargo identified for BICD2 using a yeast-two hybrid screen (Matanis et al., 2002). Previous studies have shown that GTP bound RAB6A is the preferred binding partner for BICD2 (Matanis et al., 2002). Thus, in order to determine whether RAB6A is capable of associating with full length BICD2-mTrbo, we transfected cells expressing either RFP-mTrbo, full length BicD2-mTrbo, or a version of BICD2-mTrbo lacking the CC3 domain along with a GTP locked mutant of RAB6A (GFP-RAB6A Q72L). Consistent with published results, GFP-RAB6A Q72L specifically associated with full length BICD2-mTrbo (Supplemental Fig. 1A). Based on this, we conclude that although a GTP

141 locked version of RAB6A is capable of interacting with BICD2-mTrbo, the interaction between
142 endogenous RAB6A and BICD2 might not be that prevalent under physiological conditions.

143 **Subunits of the HOPS complex associate with BICD2 in vivo.**
144 Four components of the six member HOPS complex were found to associate with BICD2 in our
145 interactome analysis (Fig. 1C', D). We therefore chose to validate these interactions using co-
146 immunoprecipitation. Strains expressing either RFP-mTrbo, full length BICD2-mTrbo, or the delta
147 CC3 domain construct (BICD2_delCC3-mTrbo) were used for this analysis. The TurboID
148 constructs contain a V5 tag. Thus, V5 trap beads were used to immunoprecipitate the tagged
149 proteins. Bound proteins were eluted and analyzed by western blotting using antibodies against
150 VPS41 (Fig. 2A), VPS18 (Fig. 2B) or VPS16 (Fig. 2B). Consistent with our interactome analysis,
151 all three HOPS components specifically co-immunoprecipitated with full length BICD2-mTrbo (Fig.
152 2A, B). Thus, we conclude that BICD2 associates with VPS41, VPS18, and VPS16 in vivo and
153 that these interactions require the BICD2 cargo binding domain.

154 Previously identified BICD2 cargo such as RANBP2 and RAB6A have been shown to interact with
155 the isolated CC3 domain of BICD2 (Matanis et al., 2002; Splinter et al., 2010). In order to
156 determine whether the same conditions apply for the HOPS complex components, we repeated
157 the experiment using the same three strains mentioned above in addition to a strain expressing
158 the isolated CC3 domain fused to mTrbo (BICD2_CC3-mTrbo) (Fig. 2C). Unexpectedly, although
159 RANBP2 was able to interact with both full length BICD2 and the isolated CC3 domain, VPS41,
160 VPS18 and VPS16 were only capable of associating with full length BICD2 (Fig. 2D,
161 Supplemental Fig. 1B). Thus, the minimal cargo binding domain of BICD2 is not sufficient for
162 mediating the interaction with the HOPS complex components. We also attempted the binding
163 experiment using an extended BICD2 C-terminal construct (residues 442-825), but even this
164 construct failed to efficiently bind the HOPS complex components (data now shown). Thus,
165 although the CC3 domain of BICD2 is required for interaction with the HOPS complex, it is not
166 sufficient. Our inability to map the minimal domain of BICD2 required for interaction with the HOPS
167 complex components precluded our ability to test for their direct interaction. Thus, although our
168 results indicate that BICD2 associates with these proteins in vivo, we cannot conclude whether
169 the interaction is direct or indirect.

170 **The proper localization of VPS41 and LAMP1 vesicles requires BICD2.**
171 We next examined the localization of the HOPS complex in control versus BICD2 depleted HeLa
172 cells. Although antibodies are available that can detect endogenous VPS41, VPS18, and VPS16
173 by western blot, these antibodies were not suitable for detecting the native protein by
174 immunofluorescence. Thus, we used a GFP-tagged VPS41 construct for this analysis. In HeLa
175 cells, microtubule minus ends are present at the perinuclear centrosome and plus ends extend
176 towards the cortex. As expected, given the role of the HOPS complex in late endosome-lysosome
177 fusion, GFP-VPS41 positive vesicles were enriched in a perinuclear area. Dispersed vesicles and
178 vesicles present closer to the cortex were also observed in cells transfected with a control siRNA
179 (Fig. 3A, D, E, data not shown). As expected, depletion of Dynein heavy chain (DHC, DYNC1H1)
180 resulted in a reduction of perinuclear clustering and an accumulation of vesicles close to the cell
181 cortex (Fig. 3B, D, E, Supplemental Fig. 2A). Because BICD2 is a cargo adaptor for dynein, we
182 expected that depletion of BICD2 would produce a similar phenotype. Surprisingly however,

183 BICD2 depletion resulted in perinuclear clustering of GFP-VPS41 vesicles (Fig. 3C, D, E,
184 Supplemental Fig. 2A). A similar phenotype was also observed using a different siRNA targeting
185 BICD2 (Supplemental Fig. 2B).

186 In addition to the HOPS complex, late endosomes and lysosomes are also positive for LAMP1.
187 We therefore used a validated antibody to detect LAMP1 vesicles under these same experimental
188 conditions. Consistent with what was observed for GFP-VPS41, LAMP1 was present in more
189 peripheral vesicles upon DHC depletion and was clustered closer to the nucleus in BICD2
190 depleted cells (Fig. 3F-H, Supplemental Fig. 2C).

191 Although we were able to visualize GFP-VPS41 for immunofluorescence experiments using a
192 GFP antibody to boost signal intensity, the native fluorescence of GFP-VPS41 was consistently
193 very low, precluding our ability to examine the motility of these vesicles in living cells. We therefore
194 examined lysosome motility using a SiR lysosome kit. This reagent detects cathepsin D present
195 in mature lysosomes. As expected, although lysosomes were present throughout the cells, they
196 were enriched in a perinuclear area and a large fraction of these particles were motile (Video 1).
197 Depletion of BICD2 caused an even more pronounced perinuclear enrichment of lysosomes,
198 consistent with the localization of GFP-VPS41 and LAMP1. However, in contrast to cells treated
199 with the control siRNA, very few motile particles were detected in BICD2 depleted cells (Video 2;
200 Supplemental Fig. 2D). Thus, depletion of BICD2 results in perinuclear enriched lysosomes that
201 are largely immotile.

202 The localization of GFP-VPS41 and LAMP1 vesicles in BICD2 depleted cells is similar to the
203 phenotype obtained upon depletion of KIF5B, a plus-end directed Kinesin-1 motor (Guardia et al.,
204 2016). Although BICD2 is primarily regarded as a cargo adaptor for dynein, it has also been shown
205 to interact with KIF5B (Grigoriev et al., 2007). In addition, *Drosophila* BicD was recently shown to
206 activate the motility of Kinesin-1 (Ali et al., 2025). In order to more closely examine the role of
207 BICD2 in LAMP1 vesicle localization, we over-expressed either GFP or BICD2 in cells. In contrast
208 to cells over-expressing GFP, LAMP1 vesicles were peripherally scattered in BICD2 over-
209 expressing cells. This phenotype was observed in HeLa and Cos7 cells (Fig. 3I, J, L, M and
210 Supplemental Fig. 2E, E'). Depletion of KIF5B in BICD2 over-expressing cells reverted this
211 phenotype and restored the perinuclear localization of LAMP1 vesicles (Fig. 3K-M, and
212 Supplemental Fig. 2A). These results suggest that BICD2 likely functions to link VPS41 and
213 LAMP1 vesicles with KIF5B for plus-end directed transport. Our studies do not reveal the
214 mechanism of this linkage, however, and further studies will be needed to fully understand the
215 role of BICD2 in this process.

216 **SMALED2 mutations in the BICD2 cargo binding domain result in dynein hyperactivation.**
217 Several SMALED2 associated mutations have been identified in the BICD2 cargo binding domain
218 (Koboldt et al., 2020). For this initial analysis, we chose to examine BICD2_R694C and
219 BICD2_R747C. These mutants, which result in the substitution of arginine for cystine at the
220 indicated residues, were particularly intriguing because the R747C mutation was predicted to
221 disrupt the interaction between BICD2 and RANBP2 (Terawaki et al., 2015). By contrast, previous
222 studies suggest that the R694C mutation results in a higher level of interaction between BICD2
223 and RANBP2 (Yi et al., 2023). In addition to these mutations in the cargo binding domain, we also
224 chose to analyze an additional mutant, BICD2_N188T (substitution of asparagine for threonine).

225 This mutation is present within the first coiled coil domain and has been shown to result in dynein
226 hyperactivation (Huynh and Vale, 2017). No studies have thus far addressed the global
227 interactome of these mutants and how they might differ from the wild-type protein.

228 Stable cell lines capable of expressing BICD2_N188T, R694C and R747C with a C-terminal
229 mTrbo tag were generated in HEK293 FLP In T-Rex cells. We first determined whether the cargo
230 binding domain mutants retained their ability to interact with dynein. Wild-type and mutant
231 versions of BICD2-mTrbo, containing a V5 tag, were immunoprecipitated using V5 trap beads.
232 The co-precipitated proteins were analyzed by western blotting using antibodies against
233 Dynactin1/p150 Glued (DCTN1) and Dynein intermediate chain (DYNC1I1). Interestingly, not only
234 did BICD2_R694C and BICD2_R747C retain their interaction with dynein, both mutants interacted
235 with dynein and dynactin at a higher level than the wild-type protein (Fig. 4A). In this regard, they
236 were similar to the BICD2_N188T mutant which had previously been shown to interact with dynein
237 at a higher level and to hyperactivate the motor (Fig. 4A) (Huynh and Vale, 2017).

238 We next examined the localization of wild-type and mutant BICD2 in HeLa cells. Wild-type BICD2
239 was localized throughout the cell with only a small fraction co-localized with a centrosomal marker
240 (Fig. 4B and F). By contrast all three mutants were enriched at varying degrees within the
241 centrosome (Fig. 4C-F). Notably, the centrosomal enrichment was highly significant for
242 BICD2_R694C and BICD2_R747C (Fig. 4F). These results suggest that the cargo binding domain
243 mutants might hyperactivate dynein. To test this more directly, we monitored the distribution of
244 peroxisomes using a well-characterized tethering assay (Kapitein et al., 2010; Passmore et al.,
245 2021). Peroxisomes are relatively immotile but can be transported towards microtubule minus
246 ends in a dynein dependent manner if they are tethered to an activating adaptor (Fig. 4G). Thus,
247 centrosomal clustering of peroxisomes is an indicator of dynein activity. Peroxisomes were
248 tethered to wild-type or mutant alleles of BICD2. Consistent with the higher level of dynein
249 interaction, peroxisomal clustering was increased in cells expressing all three SMALED2 mutants
250 (Fig. 4H and Supplemental Fig.3A-D). Collectively, these results suggest that SMALED2
251 mutations within the cargo binding domain of BICD2 are also capable of hyperactivating dynein.

252 In contrast to HeLa cells, microtubules are organized differently in neurons. In these cells,
253 microtubule minus ends are localized within the cell body and plus ends extend towards the axon
254 (van Beuningen and Hoogenraad, 2016). Thus, anterograde movement towards the axon tip is
255 driven by a plus end motor, whereas retrograde transport towards the cell body involves dynein.
256 Primary hippocampal neurons were established from E18 rat brains and after 3 days in vitro,
257 plasmids containing either wild-type or mutant alleles of BICD2 were transfected into these cells.
258 Consistent with BICD2 being a cargo adaptor for dynein, most of the signal for the wild-type
259 protein was detected within the cell body (Fig. 5A). However, wild-type BICD2 could also be
260 detected within the axon. A similar pattern was noted for BICD2_N188T (Fig. 5B). By contrast,
261 axonal signal for BICD2_R694C and BICD2_R747C was reduced, reflected as a relative increase
262 in cell body enrichment (Fig. 5C, D). The difference in localization was statistically significant for
263 the R747C mutant (Fig. 5E). These results are consistent with the cargo binding domain mutants
264 resulting in dynein hyperactivation. Huynh and Vale found that expression of certain SMALED2
265 associated BICD2 mutants, including BICD2_N188T, in hippocampal neurons results in reduced
266 neurite growth (Huynh and Vale, 2017). We obtained similar results and found that this phenotype

267 was also shared by the cargo binding domain mutations, BICD2_R694C and BICD2_R747C (Fig.
268 5F).

269 **SMALED2 mutations result in altered BICD2 interactomes.**
270 We next determined the interactomes of the three SMALED2 mutations and compared these
271 interactomes to that of wild-type BICD2. As before, the entire experiment was done in triplicate.
272 Proteins that displayed a two-fold increased or decreased interaction with the mutant allele in
273 comparison to the wild-type and had a p value of at least 0.05 were considered significant (Fig.
274 6A-C, Supplemental tables 2, 3, 4). All three mutations, including N188T, which is present in the
275 CC1 domain of BICD2, displayed an altered interactome. The most dramatic difference, however,
276 was observed for BICD2_R747C (Fig. 6C).

277 In order to validate the proteomics results, we repeated the experiment in triplicate and analyzed
278 the pellets by western blotting. Consistent with the proteomics data, and with published results
279 using a mutation in mouse BicD1 (Terawaki et al., 2015), BICD2_R747C displayed a reduced
280 interaction with RANBP2 (Fig, 6D, E, Supplemental table 4). BICD2 has been shown to associate
281 indirectly with RANGAP via its direct interaction with RANBP2 (Splinter et al., 2010). Thus, as
282 expected, a reduced level of RANGAP was detected in pellets from BICD2_R747C in comparison
283 to the wild-type (Supplemental table 4). A further analysis of the proteomics results indicated a
284 reduced association of BICD2_R747C with nuclear import receptors such as importin beta
285 (Supplemental table 4). This result was confirmed by repeating the binding experiment and
286 analyzing the eluate by western blotting (Supplemental Fig. 4A). We hypothesized that the
287 association between BICD2 and import receptors is likely indirect and mediated via the direct
288 interaction between BICD2 and RANBP2. Consistent with this notion, the interaction of wild-type
289 BICD2 with importin beta was greatly reduced in cells depleted of RANBP2 (Supplemental Fig.
290 4B). By contrast, knock-down of RANBP2 had no effect on the BICD2-VPS41 interaction
291 (Supplemental Fig. 4B). Based on these results, we conclude that BICD2_R747C is disrupted for
292 interaction with RANBP2, and consequently also displays a reduced association with additional
293 nuclear import factors.

294 An unexpected finding was that the BICD2_R747C mutant also displayed a reduced interaction
295 with HOPS complex components. The proteomics result indicated a reduced interaction with
296 VPS41, VPS18 and VPS33 (Supplemental table 4). However, the mass spectrometry result
297 indicated that the association of BICD2_R747C with VPS16 was unaffected. In order to validate
298 these results, the experiment was repeated and analyzed by western blotting. Indeed, the
299 association of BICD2_R747C with VPS41 and VPS18 was greatly reduced in comparison to the
300 wild-type protein (Fig. 6D, F and Supplemental Fig. 4C). However, using this approach we
301 consistently also observed a significant reduction in the association between BICD2_R747C and
302 VPS16 (Supplemental Fig. 4C). Based on these results, we conclude that this SMALED2 mutant
303 is most likely compromised for interacting with the entire HOPS complex.

304 In addition to interactions that were lost or reduced, all three mutants were also associated with
305 changes in the positive direction. For instance, the mass spectrometry results revealed that the
306 mutants showed a stronger interaction with the centrosomal protein CSPP1 compared to wild-
307 type BICD2 (Supplemental Tables 2, 3, 4). Validation experiments confirmed this finding,
308 demonstrating that the cargo-binding domain mutants had significantly elevated interactions with

309 CSPP1, exceeding that observed with either wild-type BICD2 or BICD2_N188T (Fig. 6D, G).
310 Since BICD2_R694C and BICD2_R747C are enriched at the centrosome (Fig. 4D-F), their altered
311 localization most likely accounts for their increased association with centrosomal proteins.

312 **BICD2_R747C exhibits a gain of function interaction with GRAMD1A.**
313 Among the three mutants analyzed, BICD2_R747C exhibited the most distinct interactome. One
314 particularly notable interaction was with GRAMD1A, a protein absent from the wild-type BICD2
315 interactome but highly enriched in the BICD2_R747C pull-down, indicating a gain-of-function
316 interaction. GRAMD1 encodes three isoforms: A, B, and C, with GRAMD1A being highly
317 expressed in the central nervous system. To validate this observation, the experiment was
318 repeated in triplicate and analyzed by western blotting. Consistent with the mass spectrometry
319 result, GRAMD1A showed only background binding to the control, wild-type BICD2,
320 BICD2_N188T, or BICD2_R694C. In contrast, GRAMD1A strongly associated with
321 BICD2_R747C (Fig. 7A, B).

322 We next examined the localization of GRAMD1A in Cos7 cells co-expressing GRAMD1A-
323 mScarlet3 with wild-type or mutant alleles of BICD2 tagged with mNeon. GRAMD1A localizes to
324 the endoplasmic reticulum (ER) and concentrates at sites of ER-plasma membrane contact
325 (Besprozvannaya et al., 2018). Cos7 cells are typically used for examining the localization of
326 GRAMD1A due to their large and flattened appearance (Besprozvannaya et al., 2018; Naito et
327 al., 2019). As expected, GRAMD1A displayed a typical ER localization pattern in cells expressing
328 wild-type BICD2 or BicD2_N188T (Fig. 7C, D). Much like what was observed in HeLa cells,
329 BICD2_R694C was often enriched at the centrosome in Cos7 cells (Fig. 7E, Fig. 4D). The
330 localization of GRAMD1A was essentially unchanged in these cells, although a small amount of
331 the protein co-localized with BICD2_R694C in the centrosomal region (Fig. 7E, G). By contrast,
332 consistent with a gain of function interaction, a significant fraction of GRAMD1A co-localized with
333 BICD2_R747C at the centrosome (Fig. 7F, G). This effect was specific for GRAMD1A because a
334 general ER maker remained correctly localized in cells expressing BICD2_R747C (Supplemental
335 Fig. 4D, D', E, E').

336 Collectively, our results suggest that SMALED2 associated mutations in BICD2 hyperactive
337 dynein and result in gain of function and loss of function interactions with cargo. Cargo trafficking
338 defects that arise because of this likely contribute to the symptoms associated with SMALED2.

339

340

DISCUSSION

Mutations in the dynein cargo adaptor BICD2 have been linked to spinal muscular atrophy with lower extremity predominance (SMALED2) (Koboldt et al., 2020). Mutations in the heavy chain of the dynein motor have also been implicated in this disorder (Chan et al., 2018; Das et al., 2018), suggesting that defects in dynein-mediated transport contribute to its etiology. However, the molecular and cellular mechanisms underlying SMALED2 pathogenesis remain poorly understood. Previous studies have characterized mutations within the first coiled-coil domain of BICD2, a region responsible for interactions with dynein and dynactin. These analyses elegantly demonstrated that mutants such as BICD2_N188T result in dynein hyperactivity (Huynh and Vale, 2017). In addition to these mutants, however, recent studies have identified several SMALED2-associated alleles within the C-terminal cargo-binding domain of BICD2 (Ravenscroft et al., 2016; Synofzik et al., 2014). Given BICD2's role as a dynein cargo adaptor, these findings raise two important questions: (1) Is dynein hyperactivity a common feature of SMALED2-associated BICD2 mutations? and (2) Do these mutations alter the interactome of BICD2 relative to the wild-type protein? The goal of this study was to address these questions and elucidate the molecular consequences of SMALED-2 associated BICD2 mutations.

BICD2 is one of the best characterized dynein cargo adaptors. However, most studies involving BICD2 have focused on the mechanism by which this adaptor activates dynein for processive motility. Relatively little is known regarding the cargo that is linked to dynein by BICD2. In *Drosophila*, BicD links the RNA binding protein Egalitarian (Egl) with dynein for transport of specific mRNAs in the oocyte and embryo (Dienstbier et al., 2009; Goldman et al., 2021; Goldman et al., 2019; Mach and Lehmann, 1997; McClintock et al., 2018). Loss of either BicD or Egl compromises transport of these mRNAs and consequently results in defective oogenesis or embryogenesis. The first definitive cargo identified for mammalian BICD2 was the small GTP binding protein, RAB6A (Matanis et al., 2002). Despite the ability of BICD2 to directly bind RAB6A, most vesicles containing RAB6A move towards the plus end of microtubules, suggesting that their transport is not dependent on dynein (Grigoriev et al., 2007). The other "cargo" that has been shown to directly bind BICD2 is the nucleoporin RANBP2. However, in this case, the purpose of the BICD2-RANBP2 interaction is to target BICD2 to the nuclear envelope, rather than for transport of RANBP2 (Splinter et al., 2010). As such, loss of BICD2 does not appear to compromise the localization of RANBP2 (Splinter et al., 2010). Thus, despite many years of study, the full BICD2 interactome remains unknown.

In order to determine whether SMALED2 alleles of BICD2 are associated with interactome changes, it was therefore critical for us to determine the interactome of wild-type BICD2. This was done using the promiscuous biotin ligase miniTurboID (mTbro). In comparison to an RFP-mTrbo control, BICD2-mTrbo resulted in the biotinylation and purification of numerous known interacting partners including RANBP2, as well as several components of the dynein motor. One interesting group of potentially novel interacting proteins were components of the HOPS complex, a six-subunit complex of proteins involved in endocytic trafficking (Spang, 2016). Four of the six HOPS components were identified in the wild-type BICD2 interactome. Although, we were able to validate the in vivo association between BICD2 and VPS41, VPS16 and VPS18, we were not able to conclude whether BICD2 is capable of directly interacting with these proteins. Unlike RANBP2 and RAB6A, both of which are able to bind the isolated BICD2 cargo binding domain (Matanis et

384 al., 2002; Splinter et al., 2010), the HOPS complex components were only able to bind full-length
385 BICD2. The BICD2 cargo binding domain was therefore necessary but not sufficient for interaction
386 with HOPS components. To the best of our knowledge, this is the first example of BICD2
387 interacting proteins that display this binding characteristic. The ScaC protein from the intracellular
388 pathogen *O. tsutsugamushi* was recently also shown to interact with BICD2 and although the
389 binding site of ScaC was different from that used by RANBP2 or RAB6A, it was still able to interact
390 with the isolated cargo binding domain of BICD2 (Manigrasso et al., 2024).

391 Another unusual aspect of the BICD2-HOPS complex interaction is that it does not appear to be
392 linked to dynein-mediated trafficking. Depletion of dynein heavy chain resulted in the peripheral
393 distribution of GFP-VPS41 and LAMP1 vesicles, indicative of a reduction in minus end transport,
394 and a net gain in plus end directed transport. By contrast, depletion of BICD2 resulted in the
395 perinuclear accumulation of lysosomal vesicles that were mostly immotile. Interestingly, however,
396 over-expression of BICD2 caused the outward spreading of LAMP1 vesicles, a process that
397 depends on KIF5B (Guardia et al., 2016). Previous studies have shown that BICD2 is also able
398 to interact with KIF5B via a central coiled coil domain (Grigoriev et al., 2007; Hoogenraad and
399 Akhmanova, 2016). A recent report suggests that *Drosophila* BicD is capable of interacting with
400 and activating the motility of Kinesin-1, the fly homolog of KIF5B (Ali et al., 2025). Consistent with
401 the notion that BICD2 might link late endosomal vesicles with KIF5B, depletion of KIF5B in BICD2
402 over-expressing cells restored the normal localization of LAMP1 vesicles. Additional studies will
403 be required to determine whether BICD2 is capable of directly interacting with these vesicles and
404 whether these vesicles are directly linked to KIF5B by BICD2.

405 As noted earlier, mutations in the CC1 region of BICD2 hyperactivate dynein (Huynh and Vale,
406 2017). Our findings indicate that this property is also shared by BICD2_R694C and
407 BICD2_R747C, mutations present within the C-terminal cargo binding domain. In the absence of
408 cargo, BICD2 is thought to exist in an inhibited conformation due to intramolecular interactions
409 between the N and C termini of the protein (Fig. 1B) (Liu et al., 2013; Terawaki et al., 2015;
410 Wharton and Struhl, 1989). Cargo binding to the C-terminus of BICD2 counteracts the
411 intramolecular interaction, enabling N-terminal residues within BICD2 to bind the dynein/dynactin
412 complex (Goldman et al., 2019; Huynh and Vale, 2017; Liu et al., 2013; McClintock et al., 2018;
413 Sladewski et al., 2018). How might mutations in BICD2 result in dynein hyperactivation? One
414 possibility is that these mutations disrupt the autoinhibited state of BICD2, effectively causing
415 BICD2 to be present in a more open and uninhibited conformation that promotes dynein/dynactin
416 binding. Molecular dynamics simulations suggest that the R747C substitution causes a registry
417 shift in the coiled coil, likely destabilizing this domain and thus disrupting the intramolecular
418 interaction between the N and C termini of BICD2 (Cui et al., 2020).

419 In addition to hyperactivating dynein, all three mutations, including BICD2_N188T, alter the BICD2
420 interactome. This finding was unexpected for BICD2_N188T because this mutation is not within
421 the cargo binding domain. One possible explanation for this phenotype is that BICD2_N188T is
422 present in a more open conformation and this change affects its binding properties. Another
423 possibility that is not mutually exclusive is that the different binding profile results from the altered
424 localization of BICD2_N188T within the cell. In comparison to wild-type BICD2, we generally
425 observed greater centrosomal enrichment of BICD2_N188T. In comparing the three mutants, the

general trend was that more proteins displayed a reduced interaction with the SMALED2 mutants in comparison to wild-type BICD2. Among the three mutants analyzed, BICD2_R747C displayed the most drastically altered interactome. This mutant displayed reduced association with RANBP2, importin beta, and HOPS complex components. Interestingly, this mutant also displayed numerous gain-of-function interactions. For instance, although minimal binding was observed between wild-type BICD2 and GRAMD1A, this protein abundantly interacted with BICD2_R747C. GRAMD1A is normally localized within the ER and is often concentrated at sites of plasma membrane-ER contact (Besprozvannaya et al., 2018; Sandhu et al., 2018). However, in cells expressing BICD2_R747C, this localization pattern was disrupted and GRAMD1A co-localized with BICD2_R747C at the centrosome.

Our study is the first to comprehensively examine the interactome of wild-type BICD2 and to identify changes that occur in SMALED2 linked mutant alleles of BICD2. We find that not only are mutations within the cargo binding domain associated with interactome changes, but that these mutations are additionally capable of hyperactivating dynein. Collectively, our results suggest that cargo trafficking defects are likely to underlie the etiology of SMALED2.

In the current study, we chose to determine the BICD2 interactome in HEK FLP-In cells (embryonic kidney cells). These cells were chosen because it enabled us to precisely integrate wild-type and mutant alleles of BICD2 at a specific locus. It also enabled us to expand cultures of these cells to levels that were sufficient for proteomic analysis. However, the main cell type affected in patients with SMALED2 are motor neurons. Primary motor neurons are much harder to culture to scale and to genetically manipulate to express the desired wild-type or mutant BICD2 transgenes. Thus, although motor neurons were not used in our study, the next significant challenge will be to perform these types of experiments using motor neurons.

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MATERIALS AND METHODS

Reagents used

The full list of DNA constructs, antibodies and reagents used in this work is found in Supplemental table 5.

DNA constructs

Synthesized DNA fragments were generated by either Twist biosciences or Genewiz/Azenta. All final plasmids used in this work were sequenced by Plasmidsaurus. Plasmid sequences as well as detailed cloning strategies will be provided upon request.

DNA encoding wild-type BICD2, generated by gene synthesis, was cloned into the Kpn1 and Xho1 sites of the pCDNA-FRT-TO vector (Thermo Fisher Scientific). Next, a fragment encoding mTrbo was cloned downstream of BICD2_wild-type using High fidelity assembly (NEB). A similar strategy using a synthesized fragment was used to clone mRFP-mTrbo into the pCDNA-FRT-TO vector. BICD2 mutants fused to mTrbo were generated by swapping in a synthesized fragment containing the desired mutation into the appropriate site of the BICD2_wild-type-mTrbo construct. Stable cell lines were created using these FRT constructs and the pOG44 vector (Thermo Fisher Scientific). For localization studies, PCR sub-cloning was used to move the appropriate wild-type or BICD2 mutans into the pLVX-Neo vector (Takara Bio). A fragment encoding mNeon-green along with the V5 epitope tag was cloned downstream of wild-type or mutant BICD2. The GFP-VPS41 construct was generated by cloning a synthesized fragment encoding human VPS41 into the Kpn1 and Not1 sites of the GFP-BICD2 plasmid (Addgene plasmid #161626; (Bonet-Ponce et al., 2020)). This removed the BICD2 cDNA from this vector and inserted the cDNA for VPS41 in its place. For the dynein activity experiment described in Fig. 4, a construct containing the PEX3 peroxisome targeting sequenced fused to mGreenLantern and the FRB dimerization sequence was cloned into the pLVX neo vector (Takara). The BICD2_wild type and mutant construct described above were modified by replacing the mTrbo sequence with the FKBP dimerization domain. The GRAMD1A-mScarlet3 construct was generated by cloning fragments corresponding to human GRAMD1A and mScarlet3 into the pLVX neo vector.

Cell culture

HeLa and Cos7 cells were obtained from ATCC and were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific). The HEK Flp-In T-REx 293 Cell Line was obtained from Thermo Fisher Scientific and was cultured according to the instructions provided by the manufacturer. Stable cells were generated by culturing the transfected HEK Flp-In T-REx 293 in 100ug/ml Hygromycin B (Gibco) for 12 to 14 days, with fresh media changes every 3 days. The E18 rat hippocampus culturing kit was obtained from Transnetyx. Primary neurons were prepared according to the instructions provided by the manufacturer. 40,000 cells were seeded onto poly Lysine coated coverslips (Neuvitro Corporation) in each well of a 12-well plate. The cells were transfected after 3 to 4 days in culture using Lipofectomine 2000 (Thermo Fisher scientific).

Stable HEK Flp-In T-REx 293 expressing mGreenLantern tagged peroxisomes were generated by infection with lentivirus. Lentiviral particles were produced in HEK293T cells following a previously published protocol (Wei et al., 2024). Culture medium containing the viral particles were passed through a 0.2 micron filter to remove cell debris. Next, the HEK Flp-In T-REx 293 cells were infected with freshly prepared viral particles for 48 hours. After infection, puromycin (1ug/mL) was added to the culture medium to select for stable cell lines. Uniformly expressing mGreenLantern clones were selected and used to integrate BICD2_wt or mutant containing the FKBP dimerization motif at the FRT locus. Stables cells containing the integrated BICD2 constructs were selected using Hygromycin B as described above.

DNA and siRNA transfections

The Qiagen Effectene reagent was used for transfecting DNA into HeLa, HEK Flp-In T-REx 293, and Cos7 cells using the directions provided by the manufacturer. For transfecting cells in 6 well dishes, 0.4ug of DNA was used along with 10ul of Effectene. Primary neurons grown on glass coverslips in a 12 well dish were transfected using Lipofectamine 2000 (Thermo Fisher Scientific). 0.5-0.8ug of DNA and 2ul of Lipofectamine 2000 was used in each transfection. Expression of the transgenes was induced using 0.3 ug/ml of Doxycycline 24 hours after transfection. The cells were fixed and processed for immunofluorescence the following day. Lipofectamine RNAimax (Thermo Fisher Scientific) was used for the transfection of siRNA according to the instructions provided by the manufacturer.

Purification and analysis of biotinylated proteins

For small scale experiments (Figure 2, 6, 7, Supplemental figures 1 and 4), HEK Flp-In T-REx 293 cells expressing the desired constructs by Tet-based induction (1ug/ml, 24 hours), were incubated with biotin (500uM) for 40 minutes. The cells were then harvested, and lysates were prepared by resuspending the cells in RIPA buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% NP-40, 1 mM EDTA) containing a Halt Protease inhibitor cocktail (Thermo Fisher Scientific). 1mg of total protein was used in the binding experiment with 15ul of High-Capacity Streptavidin Agarose beads (Thermo Fisher Scientific) in RIPA buffer. The binding was performed overnight at 4^0C. The samples were washed four times using RIPA buffer, bound proteins were eluted in Laemmli buffer and analyzed by western blotting. All western blot images were acquired on a Bio Rad ChemiDoc MP.

For proteomics experiments, the same cells were grown in 10cm dishes and 5mg of total protein was used in each purification. Biotionylated proteins were purified using 70ul of Streptavidin magnetic beads (Thermo Fisher Scientific), incubated at 4^0C overnight. The following day, the samples were extensively washed using 1ml of the following: 3 times with RIPA buffer, 3 times with high salt RIPA buffer (50 mM Tris-Cl [pH 7.5], 1M NaCl, 1% NP-40, 1 mM EDTA), 3 times with RIPA buffer and 4 times with PBS. The entire experiment was done in triplicate. After the final wash, the beads were resuspended in 70ul of PBS and shipped to the Emory Integrated Proteomics Core on dry ice.

Mass spectrometry

The mass spectrometry was performed at the Emory Integrated Proteomics Core.

548 On bead digestion: A published protocol was followed for on bead digestion of proteins
549 (Soucek et al., 2016). A digestion buffer containing 50 mM NH_4HCO_3 was added to the
550 beads. The mixture was then incubated with 1 mM dithiothreitol (DTT) at room
551 temperature for 30 minutes, followed by the addition of 5 mM iodoacetimide (IAA). This
552 mixture was incubated at room temperature for an additional 30 minutes in the dark.
553 Proteins were digested with 1 μg of lysyl endopeptidase (Wako) at room temperature
554 overnight and were further digested overnight at room temperature with 1 μg trypsin
555 (Promega). The resulting peptides were desalted using an HLB column (Waters) and
556 were dried under vacuum.

557 LC-MS/MS: The data acquisition by LC-MS/MS was adapted from a published procedure
558 (Seyfried et al., 2017). Derived peptides were resuspended in the loading buffer (0.1%
559 trifluoroacetic acid, TFA) and were separated on a Water's Charged Surface Hybrid
560 (CSH) column (150 μm internal diameter (ID) x 15 cm; particle size: 1.7 μm). The samples
561 were run on an EVOSEP liquid chromatography system using the 15 samples per day
562 preset gradient and were monitored on a Q-Exactive Plus Hybrid Quadrupole-Orbitrap
563 Mass Spectrometer (ThermoFisher Scientific). The mass spectrometer cycle was
564 programmed to collect one full MS scan followed by 20 data dependent MS/MS scans.
565 The MS scans (400-1600 m/z range, 3 x 10^6 AGC target, 100 ms maximum ion time)
566 were collected at a resolution of 70,000 at m/z 200 in profile mode. The HCD MS/MS
567 spectra (1.6 m/z isolation width, 28% collision energy, 1 x 10^5 AGC target, 100 ms
568 maximum ion time) were acquired at a resolution of 17,500 at m/z 200. Dynamic exclusion
569 was set to exclude previously sequenced precursor ions for 30 seconds. Precursor ions
570 with +1, and +7, +8 or higher charge states were excluded from sequencing.

571 MaxQuant: Label-free quantification analysis was adapted from a published procedure
572 (Seyfried, Dammer et al. 2017). Spectra were searched using the search engine
573 Andromeda, integrated into MaxQuant, against 2022 human UniProtKB/Swiss-Prot
574 database (20,387 target sequences). Methionine oxidation (+15.9949 Da), asparagine
575 and glutamine deamidation (+0.9840 Da), and protein N-terminal acetylation (+42.0106
576 Da) were variable modifications (up to 5 allowed per peptide); cysteine was assigned as
577 a fixed carbamidomethyl modification (+57.0215 Da). Only fully tryptic peptides were
578 considered with up to 2 missed cleavages in the database search. A precursor mass
579 tolerance of ±20 ppm was applied prior to mass accuracy calibration and ±4.5 ppm after
580 internal MaxQuant calibration. Other search settings included a maximum peptide mass
581 of 6,000 Da, a minimum peptide length of 6 residues, 0.05 Da tolerance for orbitrap and
582 0.6 Da tolerance for ion trap MS/MS scans. The false discovery rate (FDR) for peptide
583 spectral matches, proteins, and site decoy fraction were all set to 1 percent.

584 Quantification settings were as follows: re-quantify with a second peak finding attempt
585 after protein identification has completed; match MS1 peaks between runs; a 0.7 min
586 retention time match window was used after an alignment function was found with a 20-
587 minute RT search space. Quantitation of proteins was performed using summed peptide
588 intensities given by MaxQuant. The quantitation method only considered razor plus
589 unique peptides for protein level quantitation.

590 This work was supported by the Emory University Emory Integrated Proteomics Core
591 Facility (RRID:SCR_023530).

592
593 **Co-Immunoprecipitation**
594 HEK293 FLP-in T-Rex cells expressing the desired constructs by Tet-based induction
595 (1ug/ml, 24 hours), were harvested and lysates were prepared using RIPA buffer. 1mg of
596 total protein was used in the binding experiment with 15ul of V5-Trap agarose beads
597 (ChromoTek, ProteinTech) in RIPA buffer. The binding was performed at 4^0C for 2 hours.
598 The samples were then washed four times using RIPA buffer, bound proteins were eluted
599 in Laemmli buffer and analyzed by western blotting using the indicated antibodies.
600
601 **Immunofluorescence**
602 Cells (HeLa, HEK293 FLP In T-Rex, Cos7, and primary rat neurons) adhered to glass
603 coverslips were fixed using either 4% formaldehyde for 5 minutes at room temperature
604 (Figures 5, 7, Supplemental figure 3, 4) or with methanol at -20^0C for 10 minutes (Figures
605 3, 4, Supplemental figure 2). After fixation, cells were permeabilized by incubating with
606 PBST (PBS containing 0.1% TritonX-100) for 5 minutes. Next the samples were blocked
607 for 1 hour at room temperature using 5% normal goat serum (Thermo Fisher Scientific).
608 Cells were incubated overnight at 4^0C with primary antibody in blocking solution. The next
609 day, the coverslips were washed three times with PBS. The cells were then incubated
610 with secondary antibody diluted in blocking solution for 1 hour at room temperature.
611 Following this, the cells were washed three times with PBS, stained with DAPI (Thermo
612 Fisher Scientific), and mounted onto slides using Prolong Diamond antifade reagent
613 (Thermo Fisher Scientific).
614
615 **Microscopy**
616 All imaging experiments were performed at the Augusta University Cell Imaging Core
617 (**RRID:SCR_026799**). Fixed images were captured on either an inverted Leica Stellaris
618 confocal microscope or an inverted Zeiss LSM780 equipped with Airyscan. Live imaging
619 of SiR labeled lysosomes was performed on an inverted Nikon AXR confocal microscope
620 equipped with the NSPARC detector. Images were processed for presentation using Fiji,
621 Adobe Photoshop, and Adobe Illustrator.
622
623 **Quantifications**
624 The clustering phenotype of GFP-VPS41 and LAMP1 vesicles (Fig. 3) was quantified
625 using Imaris 10.2. The nucleus was defined using the “surface” feature of Imaris and the
626 vesicles were defined using the “spots” feature. The percentage of vesicles that were less
627 than or equal to 10 microns from the nucleus and the percentage of vesicles that were
628 present a distance greater than 10 microns from the nucleus was determined using object
629 to object classification and the filtering feature in Imaris. The quantification of lysosome
630 motility in live cells (Supplemental figure 2D) was also performed in Imaris. Vesicles were
631 defined using the “spots” feature and were tracked over time. Motile vesicles were defined
632 as those that displayed movement over at least a 3 second interval and had at least
633 0.1microns of displacement over the course of the imaging experiment. This enabled us
634 to quantify the percent of motile particles per cell and the velocity of these vesicles. The
635 peroxisome clustering phenotype (Fig. 4H) was quantified using the “cells” module of
636 Imaris. Within this module, the nucleus was defined using the DAPI channel and the cell
637 boundaries were determined using phalloidin staining of F-actin. Next, the peroxisomes

638 were defined using the “spots” feature built into this module. This enabled us to calculate
639 the average distance of the peroxisomes to the nucleus on a cell-by-cell basis. The cell
640 body enrichment of BICD2 signal (Fig. 5E) was determined using the “filaments” module
641 in Imaris. This was used to define the cell body and the axon. The mean intensity of signal
642 in the cell body was compared to the mean intensity of signal in the axon. The co-
643 localization between GRAMD1A and BICD2 (Fig.7) was determined using the “co-
644 localization” module in Imaris. Axon length (Fig. 5F) was quantified using the Simple
645 neurite tracer plugin for FIJI. Densitometry for western blot images captured on the
646 BioRad Chemidoc MP were analyzed using the BioRad Image Lab software. The binding
647 level was compared to the amount of the respective protein detected in the BICD2_wt
648 lane. Graphing of data and statistical analysis was performed using Graphpad Prism10.
649

650 **FIGURE LEGENDS**

651 **Figure 1: Wild-type BICD2 interactome**
652 (A). Schematic of human BICD2, indicating the three coiled-coil domains and sites of interaction
653 with dynein/dynactin, kinesin-1, and cargo.

654 (B). Model of BICD2 binding to dynein/dynactin in a cargo dependent manner.

655 (C, C'). A volcano plot indicating proteins that were enriched with BICD2-mTrbo in comparison to
656 RFP-mTrbo. The dashed line along the x-axis indicates a fold enrichment of 2, whereas the
657 dashed line along the y-axis indicates a p value of 0.05. Specific interacting proteins were
658 considered those that were enriched at least two-fold with BICD2-mTrbo and had a p value of at
659 least 0.05. These candidates are shown in the zoomed in image in C'.

660 (D). Known BICD2 interacting proteins, components of the dynein motor, and components of the
661 HOPS complex that were specifically enriched with BICD2-mTrbo are indicated.

662 (E). A cellular component GO analysis of the BICD2 interactome.

663 **Figure 2: BICD2 interacts with components of the HOPS complex in vivo.**
664 (A). A co-immunoprecipitation experiment was performed with HEK cells expressing the indicated
665 constructs. The lysates were incubated with V5 trap beads to precipitate the tagged proteins. The
666 co-precipitating proteins were analyzed using western blotting with the indicated antibodies.
667 VPS41 specifically co-precipitates with BICD2_wt. Minimal binding was observed with RFP-
668 mTrbo or a BICD2 construct lacking the cargo binding domain.

669 (B). A similar co-precipitation experiment was set up as in panel A. The co-precipitating proteins
670 were analyzed by blotting using antibodies against VPS16, VPS18 and the V5 epitope. VPS16
671 and VPS18 co-precipitate specifically with BICD2_wt-mTrbo.

672 (C). A schematic of the BICD2 constructs used in the binding experiment.

673 (D). Cells expressing either RFP-mTrbo, BICD2_delCC3-mTrbo (lacking the cargo binding
674 domain), BICD2_wt-mTrbo (full length BICD2) or BICD2_CC3-mTrbo (just the cargo binding
675 domain) were used to examine the interaction with VPS41. Lysates were incubated with
676 streptavidin beads to precipitate biotinylated proteins. The precipitated proteins were analyzed by
677 blotting using the indicated antibodies. Although RANBP2 interacts with the isolated cargo binding
678 domain of BICD2, VPS41 does not.

679 **Figure 3: Role of BICD2 in localization of GFP-VPS41 and LAMP1 vesicles.**
680 (A-C). HeLa cells were transfected with either a control-siRNA (A), an siRNA targeting dynein
681 heavy chain (B), or an siRNA targeting BICD2 (C). Two days after the siRNA transfection, the cells
682 were transfected with a plasmid encoding GFP-VPS41. The next day, the cells were fixed and
683 processed for immunofluorescence using an antibody against GFP. The cells were counterstained
684 with DAPI (cyan) and Phalloidin (grey). Depletion of DHC results in an outward spreading of GFP-
685 VPS41 vesicles, whereas depletion of BICD2 results in more perinuclear clustered vesicles.

686 (D, E). The distance of GPF-VPS41 vesicles relative to the nucleus was determined and plotted.
687 Vesicles present within 10 microns of the nucleus are shown in D, and those present at a distance
688 greater than 10 microns are shown in panel E.

689 (F-H). HeLa cells were transfected with the indicated siRNAs. Three days later, the cells were
690 fixed and processed for immunofluorescence using an antibody against LAMP1. As with GFP-
691 VPS41 vesicles, depletion of DHC resulted in peripheral vesicles, whereas depletion of BICD2
692 resulted in perinuclear clustering of LAMP1 vesicles.

693 (I-K). HeLa cells were transfected with either a control siRNA (I, J) or an siRNA targeting KIF5B
694 (K). Two days later the cells were transfected with a plasmid encoding either GFP (I) or BICD2-
695 mNeon (J, K). The cells were fixed on day 4 and processed for immunofluorescence using an
696 antibody against LAMP1. The cells were counterstained with DAPI. Over-expression of BICD2
697 results in the peripheral spreading of LAMP1 vesicles. This phenotype was reversed upon
698 knocking down KIF5B.

699 (L-M). The distance of LAMP1 vesicles relative to the nucleus was determined and plotted.
700 Vesicles present within 10 microns of the nucleus are shown in L, and those present at a distance
701 greater than 10 microns are shown in panel M.

702 The signal for GFP-VPS41 and LAMP1 is displayed using the “red hot” LUT in FIJI. The scale bar
703 is 20 microns. A one-way ANOVA was used for the quantifications shown in panel D, E, L and M
704 with the values compared to the mean of BICD2_wt. ns = not significant, *, $p \leq 0.05$, ***, $p<0.001$.

705 **Figure 4: BICD2 cargo binding domain mutants hyperactivate dynein.**
706 (A). A co-immunoprecipitation experiment was performed using HEK cells expressing the
707 indicated constructs. The tagged proteins were purified using V5 trap beads and the co-
708 precipitating proteins were analyzed by western blotting with the indicated antibodies. A greater
709 amount of DIC and DCTN1 co-purified with mutant BICD2 compared to the wild-type protein.

710 (B-E). HeLa cells expressing BICD2_wt (B), BICD2_N188T (C), BICD2_R694C (D) or
711 BICD2_R747C (E) were fixed and processed for immunofluorescence using antibodies against
712 V5 (cyan) and pericentrin (magenta). Merged images are also shown. All three mutants displayed
713 a centrosomal localization pattern to varying degrees. The scale bar is 20 microns.

714 (F). The centrosomal enrichment of BICD2_wt or mutant was quantified.

715 (G). Schematic of the peroxisome tethering assay.

716 (H). The average distance of peroxisomes to the nucleus was calculated on a cell-by-cell basis.
717 In comparison to wild-type BICD2, all three mutants showed increased clustering of peroxisomes
718 close to the nucleus. A one-way ANOVA was used for the quantifications shown in panels F and
719 H with the values compared to the mean of BICD2_wt. n = not significant, **, $p<0.01$, ***, $p<0.001$,
720 ****, p<0001.

721 **Figure 5: Localization of BICD2 wild-type and mutants in neurons.**
722 (A-D). E18 rat hippocampal neurons were transfected with the indicated constructs. Two days
723 after transfection, the cells were fixed and processed for immunofluorescence using a V5

724 antibody. The axon outline for cells expressing BICD2_R694C and BICD2_R747C are indicated.
725 Signal for wild-type BICD2 could be detected in the cell body and axon. A similar phenotype was
726 noted for BICD2_N188T. By contrast, BICD2_R694C and BICD2_R747C displayed reduced
727 axonal signal. The scale bar is 100 microns. The signal for BICD2 is displayed using the "red hot"
728 LUT in FIJI.

729 (E). Quantification of the cell body enrichment of BICD2 wild-type and mutant.

730 (F). The axon length of neurons expressing either wild-type or mutant alleles of BICD2 was
731 quantified. Expression of BICD2 mutants correlated with shorter axonal lengths. A one-way
732 ANOVA was used for the quantifications shown in panels E and F and the values were compared
733 to the mean of BICD2_wt. n = not significant, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.

734 **Figure 6: BICD2 mutations are associated with altered interactomes.**
735 (A-C). Volcano plots comparing the interactome of BICD2_wt vs BICD2_N188T (A), vs
736 BICD2_R694C (B) and vs BICD2_R747C (C). Interacting proteins that show at least a twofold
737 change in comparison to BICD2_wt and have a p value of at least 0.05 are indicated in the shaded
738 boxes. Red boxes indicate proteins that display a greater interaction with BICD2 mutants vs the
739 wild-type, whereas blue boxes indicate proteins that display a lower interaction vs the wild-type
740 protein.

741 (D) The proteomics results were validated by repeating the experiment and analyzing the bound
742 fractions using the indicated antibodies. Streptavidin beads were used to purify the biotinylated
743 proteins.

744 (E-G). Quantification of binding of BICD2_wt and mutants with RANBP2 (E), VPS41 (F), and
745 CSPP1 (G). The level of binding for the mutants was compared to BICD2_wt. Consistent with the
746 proteomics results, BICD2_R747C displayed reduced binding to RANBP2 and VPS41. All three
747 mutants bound CSPP1 at a greater level than the wild-type. A one-way ANOVA was used for this
748 analysis. ns = not significant, *, $p \leq 0.05$, **, $p<0.01$, ***, $p<0.001$, ****, $p<0.0001$.

749 **Figure 7: BICD2_R747C is associated with a gain of function interaction with GRAMD1A.**
750 (A). Lysates from cells expressing BICD2_wt and mutants were incubated with streptavidin beads
751 to purify biotinylated proteins. Bound proteins were eluted and analyzed by blotting using the
752 indicated antibodies. BICD2_R747C interacted with substantially more GRAMD1A than either the
753 control, BICD2_wt, or the other mutants.

754 (B). Quantification of binding of BICD2_wt and mutants with GRAMD1A. The level of binding for
755 the mutants was normalized to BICD2_wt. A one-way ANOVA was used for this analysis. ns = not
756 significant, ***, $p<0.001$.

757 (C). Cos7 cells were co-transfected with constructs expressing either BICD2_wt or mutant
758 (magenta) along with a plasmid expressing GRAMD1A-mScarlet3 (cyan). Except for cells
759 expressing BICD2_R747C, GRAMD1A was localized to the ER. By contrast, GRAMD1A was
760 highly enriched at the centrosome in cells expressing BICD2_R747C.

761 (D). Quantification of the co-localization between BICD2_wt and mutants with GRAMD1A. A one-
762 way ANOVA was used for this analysis and the values were compared to the mean of BICD2_wt.
763 ns = not significant, ****, p<0.0001.

764 **Supplemental Figure1**
765 (A). HEK cells expressing the indicated constructs were transfected with a plasmid expressing
766 GTP locked RAB6A (GFP-RAB6A Q72L). Lysates were prepared and a co-immunoprecipitation
767 experiment was carried out using V5 trap beads. The co-precipitating proteins were analyzed
768 using the indicated antibodies. GFP-RAB6A Q72L specifically co-precipitated with full-length
769 BICD2.

770 (B). Lysates were prepared from HEK cells expressing the indicated constructs and biotinylated
771 proteins were purified using streptavidin beads. The precipitated proteins were analyzed by
772 blotting using antibodies against VPS16 and VSP18. Both proteins were able to interact with full-
773 length BICD2 but not the isolated cargo binding domain.

774 **Supplemental Figure2**
775 (A). Lysates were prepared from cells transfected with the indicated siRNA. The lysates were
776 analyzed by blotting using antibodies against DHC, BICD2, KIF5B and alpha-Tubulin. The siRNAs
777 were capable of depleting the target protein.

778 (B, C). Representative image of HeLa cells showing the localization of GFP-VPS41 (B) and
779 LAMP1 (C) vesicles in cells transfected with BICD2 siRNA-2.

780 (D). The number of motile lysosome particles were quantified in HeLa cells transfected with either
781 a control siRNA or an siRNA targeting BICD2. The number of motile lysosomes were greatly
782 reduced in BICD2 depleted cells.

783 (E, E'). Cos7 cells were transfected with a plasmid encoding BICD2-mNeongreen. The cells were
784 fixed and immunostained using an antibody against LAMP1. Over-expression of BICD2 results in
785 the outward spreading of LAMP1 vesicles. The outline of the transfected cell is indicated in E'.

786 **Supplemental Figure3**
787 Representative images of HEK cells expressing mGreenLantern positive peroxisomes and co-
788 expressing BICD2_wt (A), BICD2_N188T (B), BICD2_R694C (C), and BICD2_R747C (D) fused
789 to the FKBP dimerization domain. The addition of rapamycin causes the tethering of peroxisomes
790 to the indicated BICD2 construct. BICD2 mutants result in greater perinuclear clustering of
791 peroxisome in comparison to the wild-type protein.

792 **Supplemental Figure4**
793 (A). HEK cells expressing the indicated constructs were used in a binding experiment. Biotinylated
794 proteins were purified using streptavidin beads. Bound proteins were eluted and analyzed by
795 blotting using the indicated antibodies. Importin beta is biotinylated by wild-type BICD2 and
796 BICD2_R694C. However, the binding between the two proteins is reduced in the BICD2_R747C
797 background.

798 (B). HEK cells expressing BICD2_wt were transfected with the indicated siRNA. Three days after
799 the siRNA transfection, lysates were prepared and biotinylated proteins were purified using

800 streptavidin beads. Bound proteins were analyzed by blotting using the indicated antibodies. The
801 ability of Importin beta to be biotinylated by BICD2 depends on RANBP2.

802 (C). Lysates were prepared from HEK cells expressing the indicated constructs. Binding was
803 performed using streptavidin beads and the bound proteins were analyzed by blotting. VPS16
804 and VPS18 displayed reduced binding to BICD2_R747C.

805 (D, E). Cos7 cells were co-transfected with plasmids encoding an ER marker (Addgene plasmid
806 #137805, (Chertkova et al., 2020)) and BICD2_wt (D) or BICD2_R747C (E). In contrast to what
807 was observed with GRAMD1A, expression of BICD2_R747C does not alter the localization of the
808 ER marker.

809 **Supplemental table 1**
810 The wild-type BICD2 interactome.

811 **Supplemental table 2**
812 The interactome of BICD2_N188T vs BICD2_wt.

813 **Supplemental table 3**
814 The interactome of BICD2_R694Cvs BICD2_wt.

815 **Supplemental table 4**
816 The interactome of BICD2_R747Cvs BICD2_wt.

817 **Supplemental table 5**
818 The full list of reagents used in this manuscript.

819 **Supplemental video 1**
820 Live imaging of SiR lysosome labeled vesicles in HeLa cell transfected with a control siRNA.

821 **Supplemental video 2**
822 Live imaging of SiR lysosome labeled vesicles in HeLa cell transfected with an siRNA targeting
823 BICD2.

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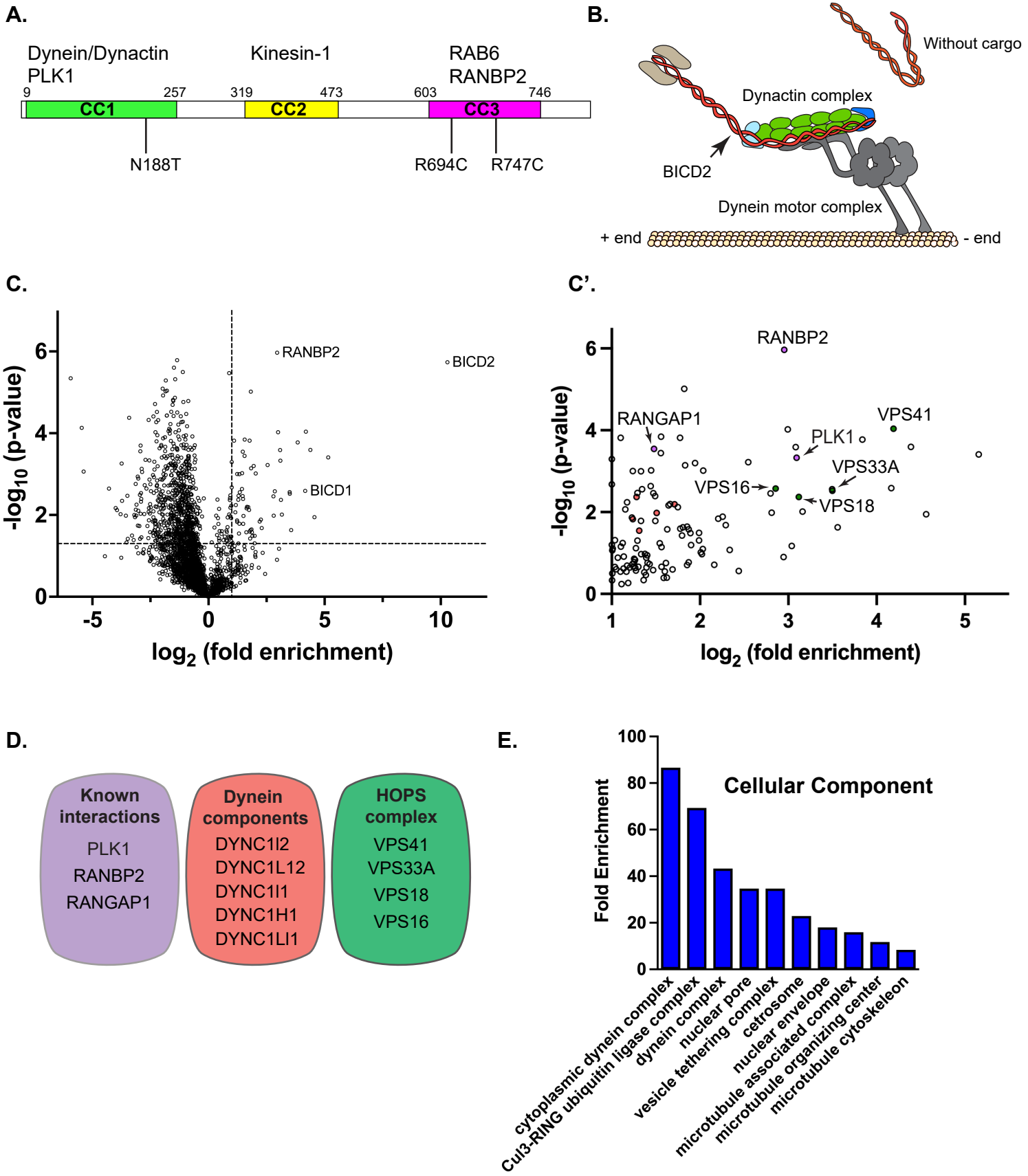

A.
Dynein/Dynactin
PLK1
Kinesin-1
RAB6
RANBP2
9
257
319
473
603
746
CC1
CC2
CC3
N188T
R694C
R747C
B.
Without cargo
Dynactin complex
BICD2
Dynein motor complex
+ end
- end
C.
RANBP2
BICD2
BICD1
-log10 (p-value)
log2 (fold enrichment)
C'.
RANBP2
RANGAP1
VPS41
PLK1
VPS33A
VPS16
VPS18
-log10 (p-value)
log2 (fold enrichment)
D.
Known interactions
PLK1
RANBP2
RANGAP1
Dynein components
DYNC1I2
DYNC1L12
DYNC1I1
DYNC1H1
DYNC1LI1
HOPS complex
VPS41
VPS33A
VPS18
VPS16
E.
Cellular Component
Fold Enrichment
cytoplasmic dynein complex
Cul3-RING ubiquitin ligase complex
dynein complex
nuclear pore
vesicle tethering complex
cetrosome
nuclear envelope
microtubule associated complex
microtubule organizing center
microtubule cytoskeleon

Figure 1

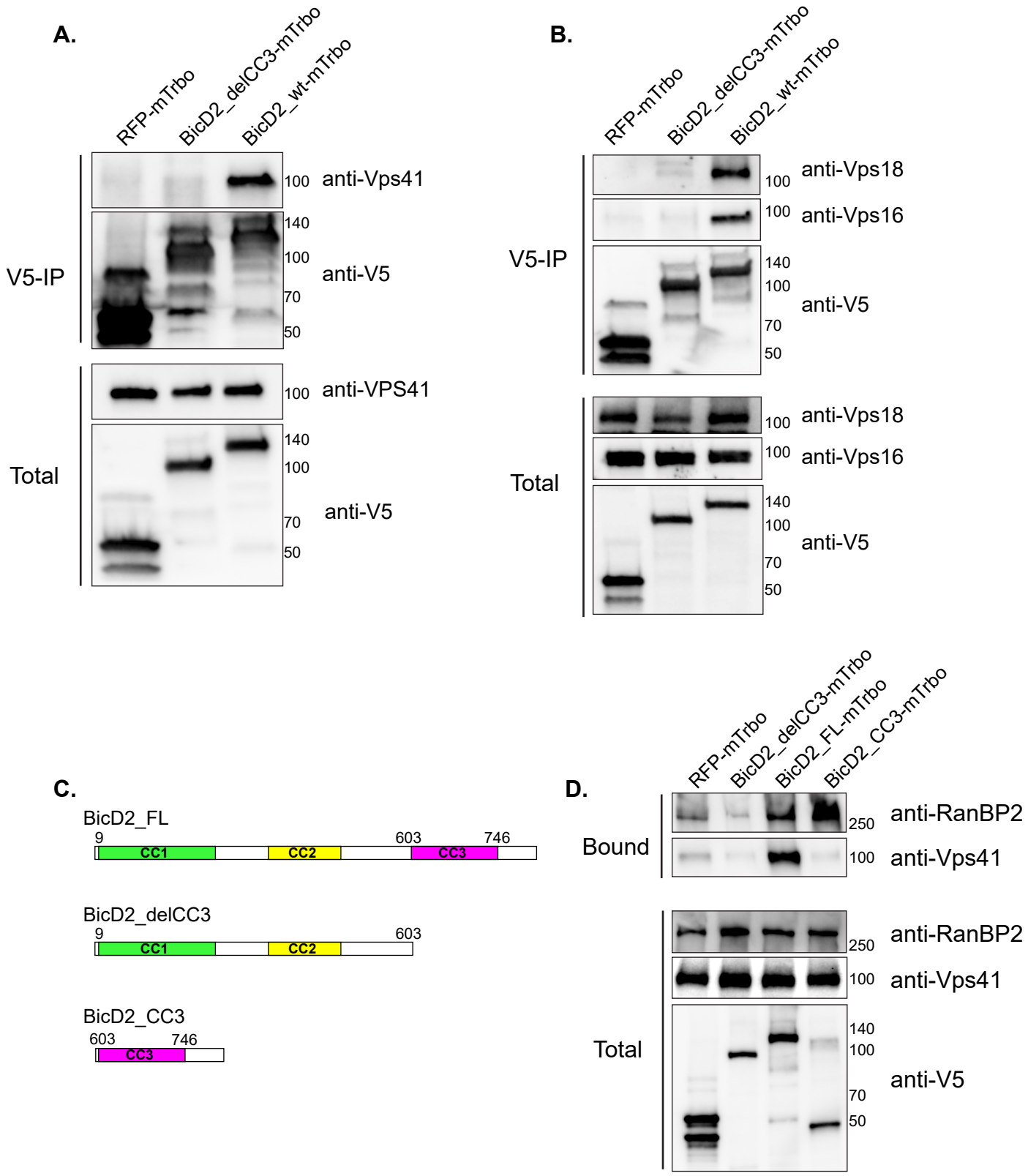
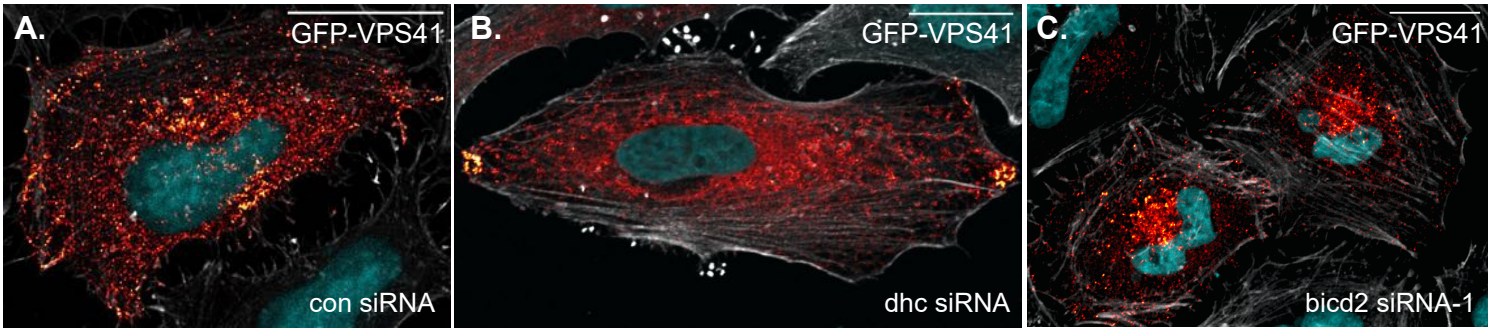
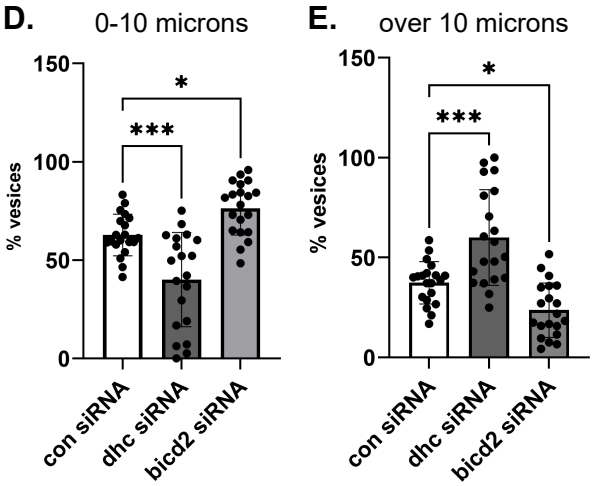
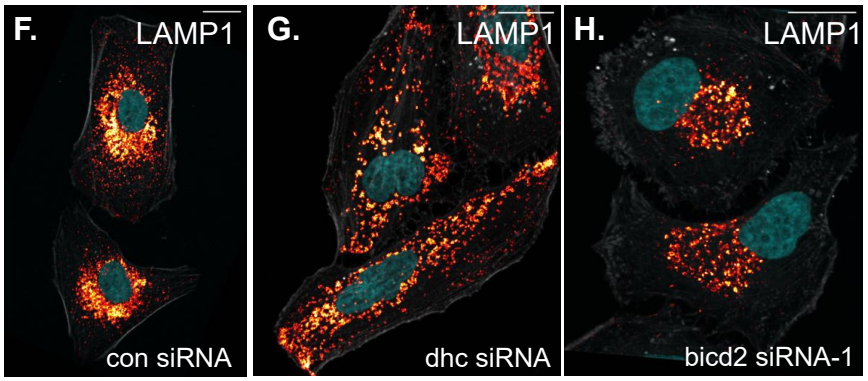
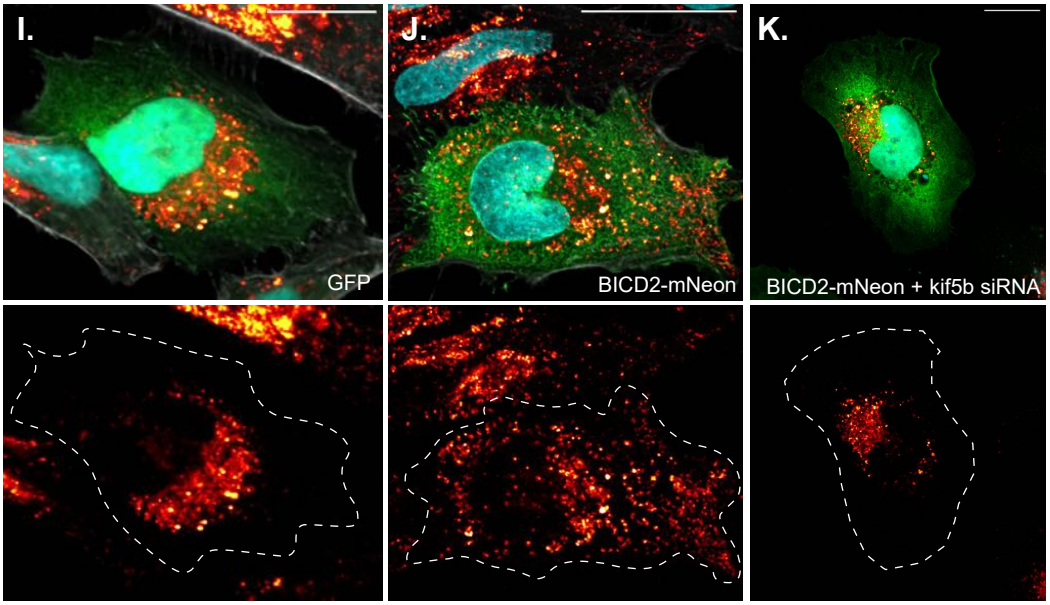

A.
RFP-mTrbo
BicD2_delCC3-mTrbo
BicD2_wt-mTrbo
V5-IP
Total
anti-Vps41
anti-V5
anti-VPS41
anti-V5
100
140
100
70
50
B.
RFP-mTrbo
BicD2_delCC3-mTrbo
BicD2_wt-mTrbo
V5-IP
Total
anti-Vps18
anti-Vps16
anti-V5
100
140
100
70
50
C.
BicD2_FL
9
603
746
CC1
CC2
CC3
BicD2_delCC3
9
603
CC1
CC2
BicD2_CC3
603
746
CC3
D.
RFP-mTrbo
BicD2_delCC3-mTrbo
BicD2_FL-mTrbo
BicD2_CC3-mTrbo
Bound
Total
anti-RanBP2
anti-Vps41
anti-V5
250
100
140
70
50

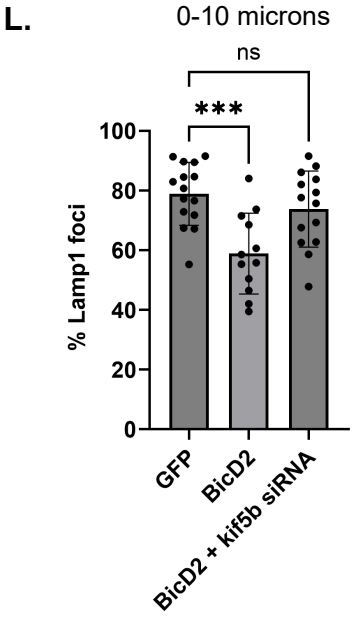
Figure 2


A.
GFP-VPS41
con siRNA
B.
GFP-VPS41
dhc siRNA
C.
GFP-VPS41
bicd2 siRNA-1


D.
0-10 microns
% vesices
150
100
50
0
***
*
con siRNA
dhc siRNA
bicd2 siRNA
E.
over 10 microns
% vesices
150
100
50
0
***
*
con siRNA
dhc siRNA
bicd2 siRNA


F.
LAMP1
con siRNA
G.
LAMP1
dhc siRNA
H.
LAMP1
bicd2 siRNA-1


I.
GFP
J.
BICD2-mNeon
K.
BICD2-mNeon + kif5b siRNA


L.
0-10 microns
ns
***
% Lamp1 foci
100
80
60
40
20
0
GFP
BicD2
BicD2 + kif5b siRNA

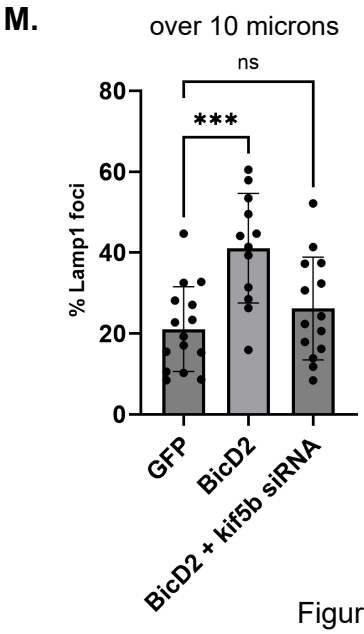
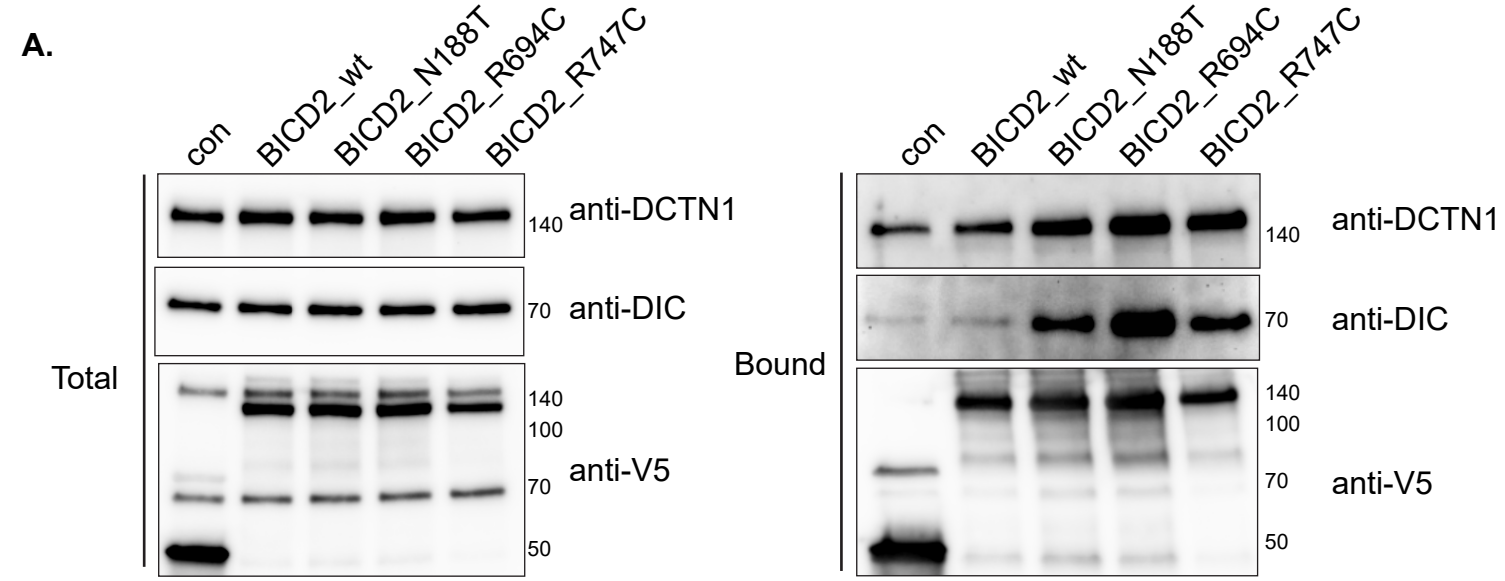
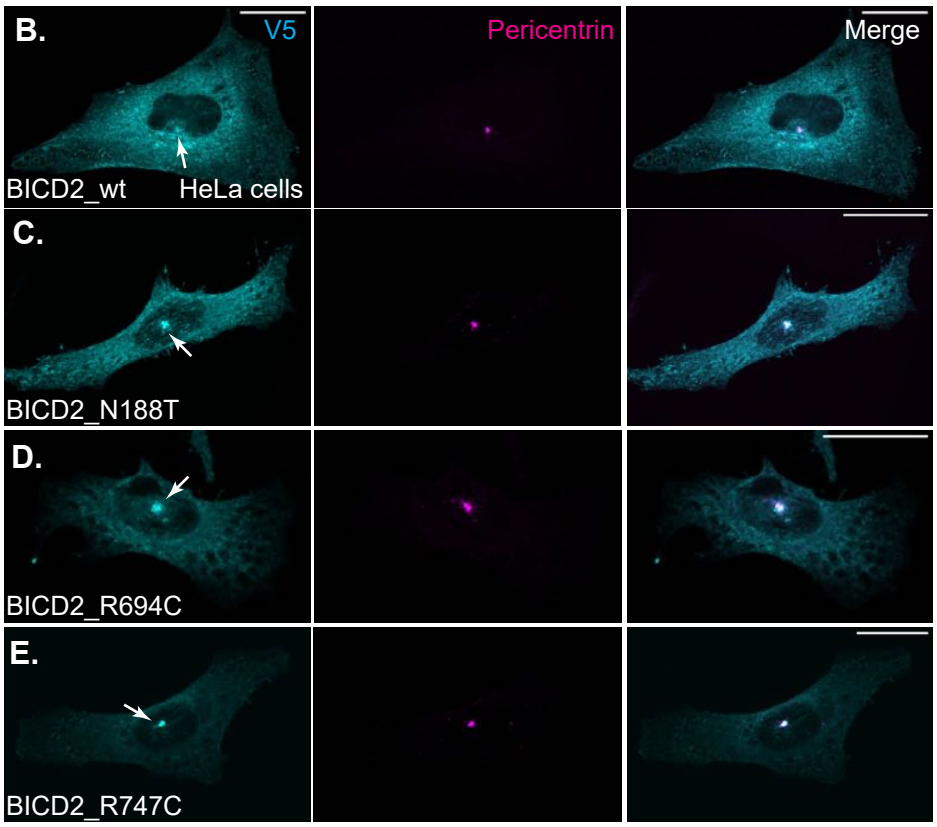
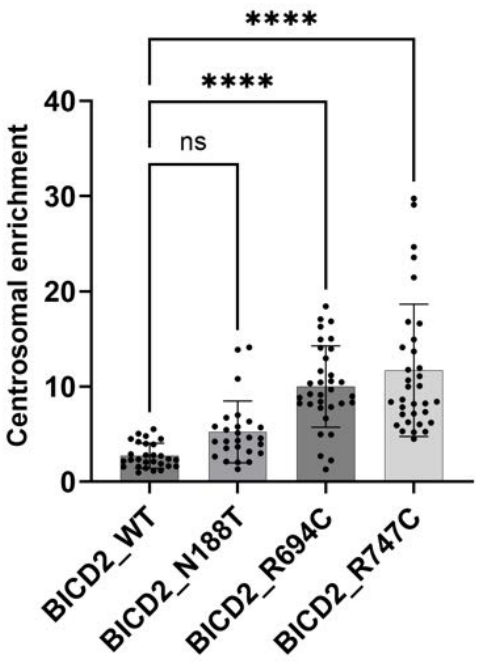
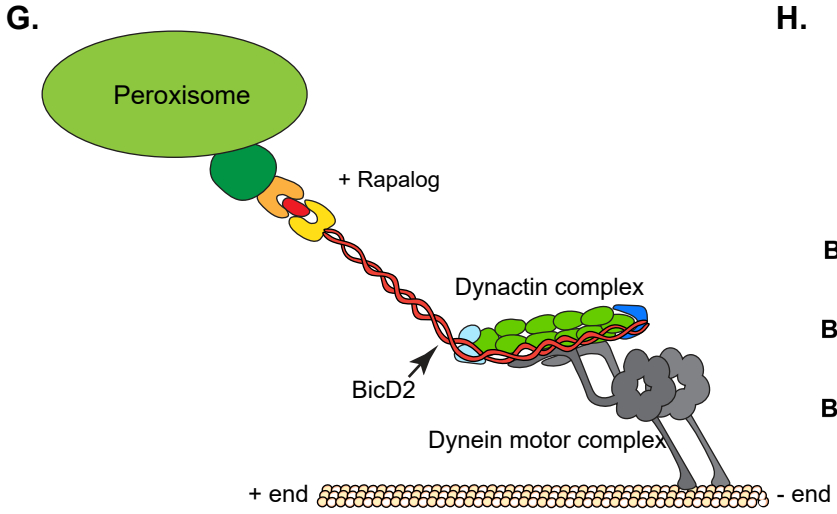

M.
over 10 microns
ns
***
% Lamp1 foci
80
60
40
20
0
GFP
BicD2
BicD2 + kif5b siRNA

Figure 3


A.
con
BICD2_wt
BICD2_N188T
BICD2_R694C
BICD2_R747C
Total
Bound
anti-DCTN1
anti-DIC
anti-V5
140
70
100
50


B.
V5
Pericentrin
Merge
BICD2_wt
HeLa cells
C.
BICD2_N188T
D.
BICD2_R694C
E.
BICD2_R747C


F.
Centrosomal enrichment
0
10
20
30
40
ns
****
****
BICD2_WT
BICD2_N188T
BICD2_R694C
BICD2_R747C


G.
Peroxisome
+ Rapalog
Dynactin complex
BicD2
Dynein motor complex
+ end
- end

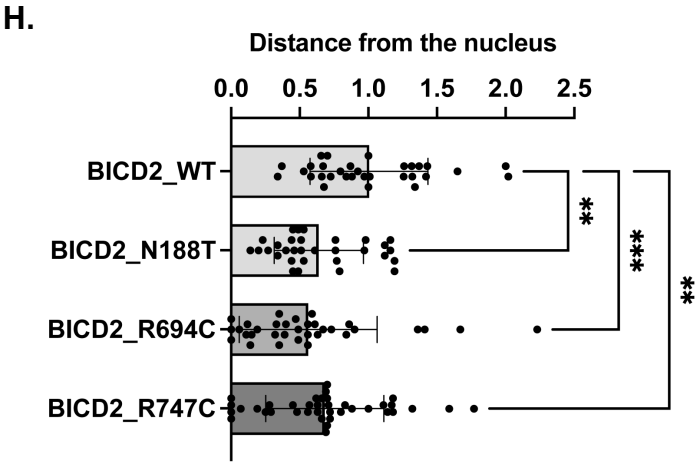
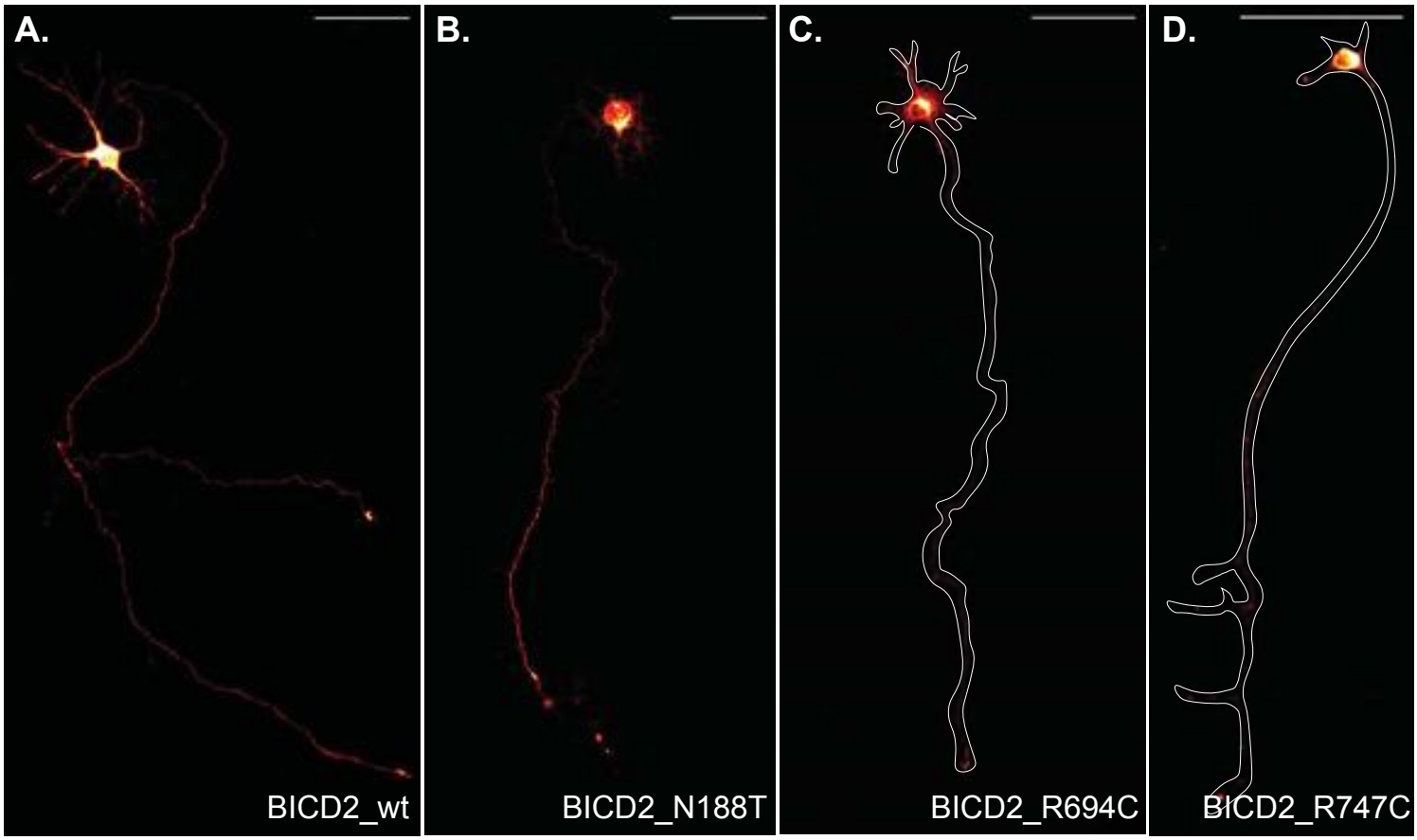
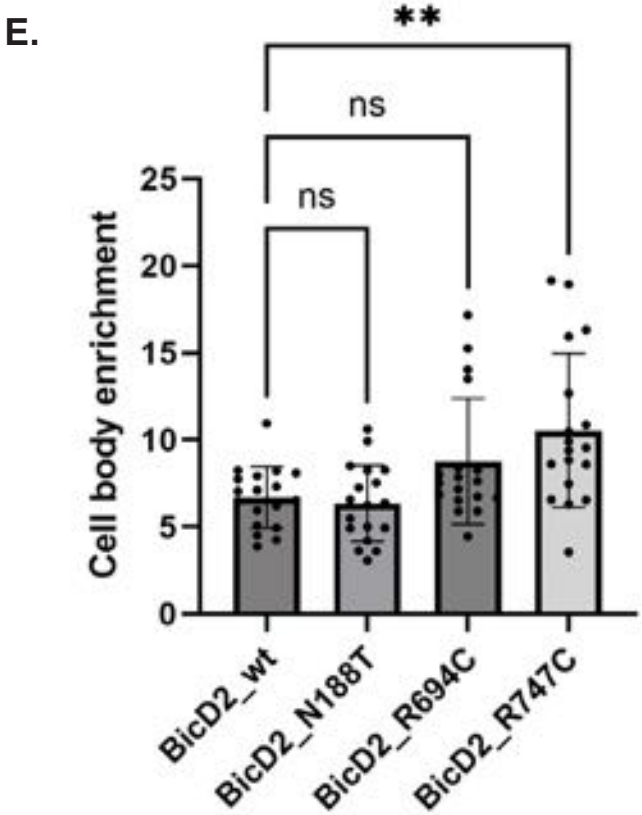

H.
Distance from the nucleus
0.0
0.5
1.0
1.5
2.0
2.5
BICD2_WT
BICD2_N188T
BICD2_R694C
BICD2_R747C
**
***
**

Figure 4


A.
B.
C.
D.
BICD2_wt
BICD2_N188T
BICD2_R694C
BICD2_R747C


E.
**
ns
ns
Cell body enrichment
25
20
15
10
5
0
BicD2_wt
BicD2_N188T
BicD2_R694C
BicD2_R747C

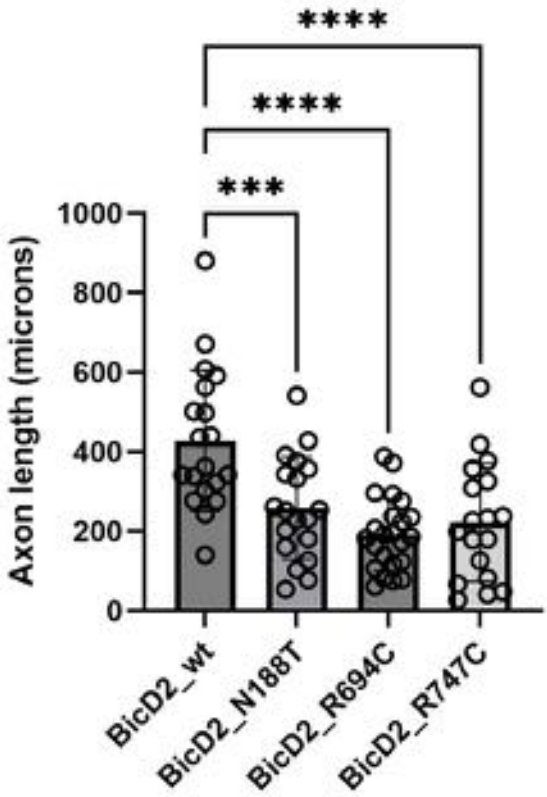

F.
****
****
***
Axon length (microns)
1000
800
600
400
200
0
BicD2_wt
BicD2_N188T
BicD2_R694C
BicD2_R747C

Figure 5

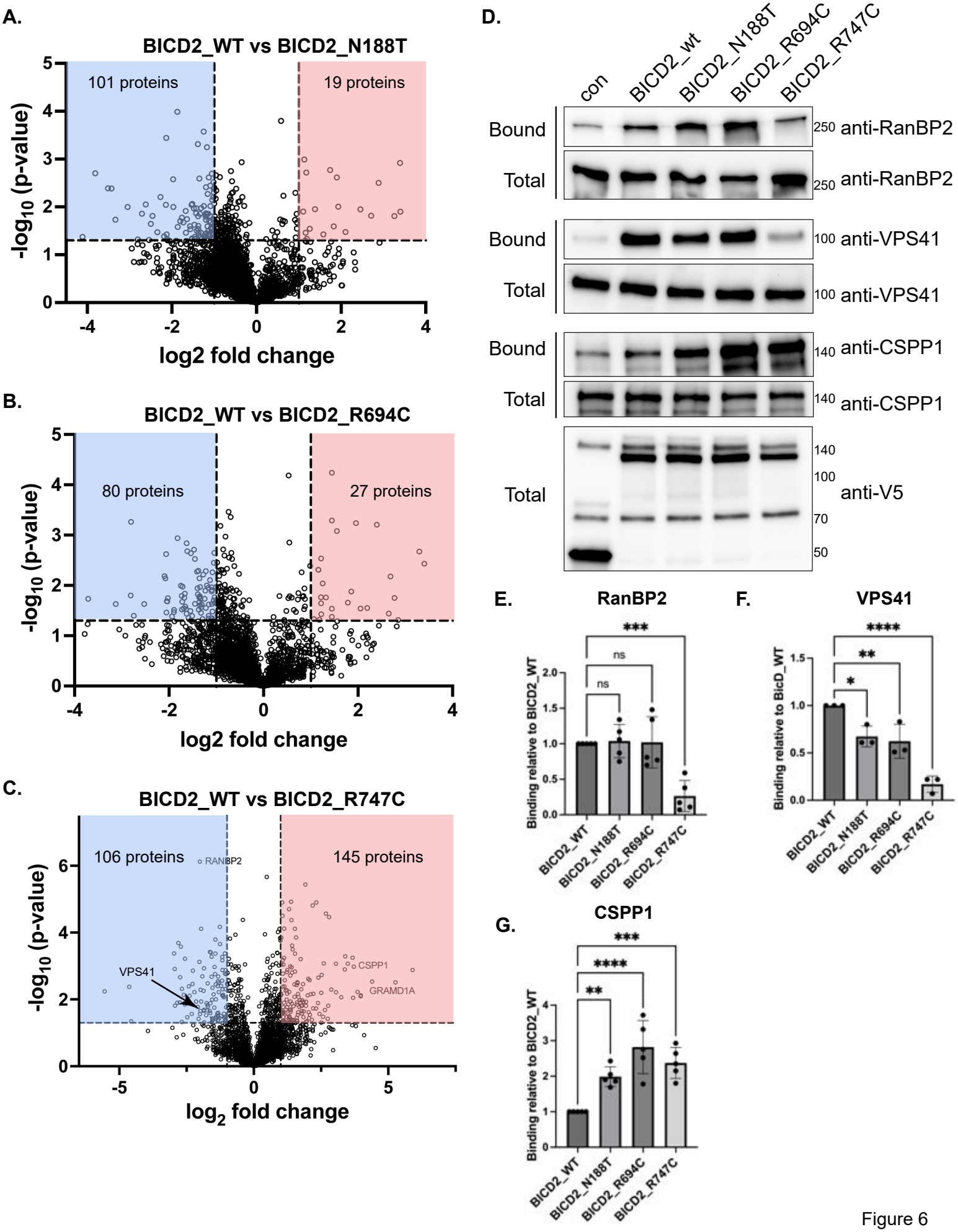

A.
BICD2_WT vs BICD2_N188T
101 proteins
19 proteins
-log10 (p-value)
log2 fold change
B.
BICD2_WT vs BICD2_R694C
80 proteins
27 proteins
-log10 (p-value)
log2 fold change
C.
BICD2_WT vs BICD2_R747C
106 proteins
145 proteins
RANBP2
VPS41
CSPP1
GRAMD1A
-log10 (p-value)
log2 fold change
D.
con
BICD2_wt
BICD2_N188T
BICD2_R694C
BICD2_R747C
Bound
Total
250 anti-RanBP2
250 anti-RanBP2
100 anti-VPS41
100 anti-VPS41
140 anti-CSPP1
140 anti-CSPP1
140
100
70
50
anti-V5
E.
RanBP2
Binding relative to BICD2_WT
ns
ns
***
BICD2_WT
BICD2_N188T
BICD2_R694C
BICD2_R747C
F.
VPS41
Binding relative to BicD_WT
*
**
****
BICD2_WT
BICD2_N188T
BICD2_R694C
BICD2_R747C
G.
CSPP1
Binding relative to BICD2_WT
**
****
***
BICD2_WT
BICD2_N188T
BICD2_R694C
BICD2_R747C

Figure 6

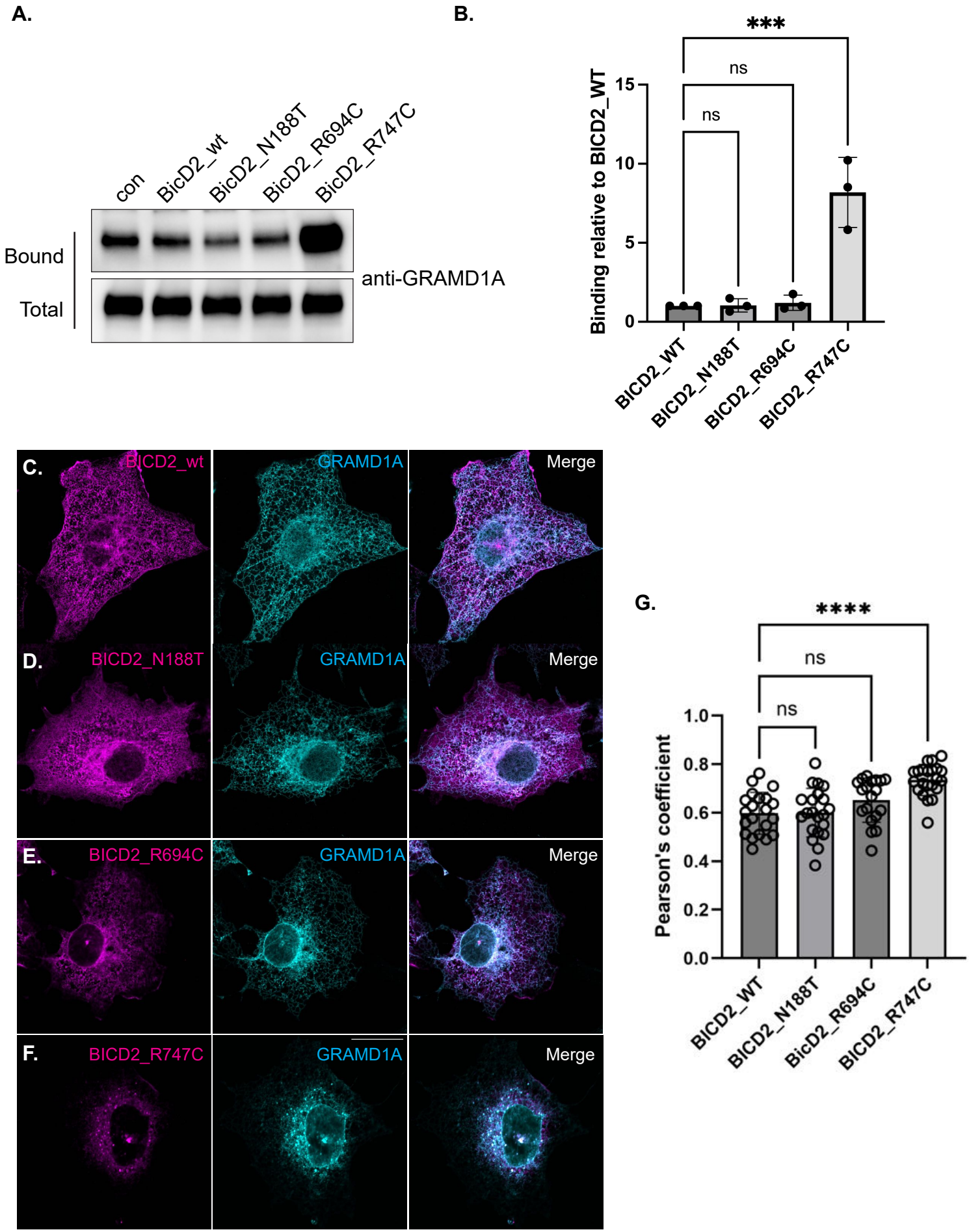

A.
con
BicD2_wt
BicD2_N188T
BicD2_R694C
BicD2_R747C
Bound
Total
anti-GRAMD1A
B.
***
ns
ns
Binding relative to BICD2_WT
15
10
5
0
BICD2_WT
BICD2_N188T
BICD2_R694C
BICD2_R747C
C.
BICD2_wt
GRAMD1A
Merge
D.
BICD2_N188T
GRAMD1A
Merge
E.
BICD2_R694C
GRAMD1A
Merge
F.
BICD2_R747C
GRAMD1A
Merge
G.
****
ns
ns
Pearson's coefficient
1.0
0.8
0.6
0.4
0.2
0.0
BICD2_WT
BICD2_N188T
BicD2_R694C
BICD2_R747C

Figure 7