

Targeting neuronal activity-regulated neuroligin-3 dependency for high-grade glioma therapy

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Abstract:

Neuronal activity promotes high-grade glioma (HGG) growth. An important mechanism mediating this neural regulation of brain cancer is activity-dependent cleavage and secretion of the synaptic molecule and glioma mitogen neuroligin-3 (NLgn3), but the therapeutic potential of targeting NLgn3 in glioma remains to be defined. We demonstrate a striking dependence of HGG growth on microenvironmental NLgn3 and determine a targetable mechanism of secretion. Patient-derived orthotopic xenografts of pediatric glioblastoma, diffuse intrinsic pontine glioma

and adult glioblastoma fail to grow in Nlgn3 knockout mice. Glioma exposure to Nlgn3 results in numerous signaling consequences, including early focal adhesion kinase activation upstream of PI3K-mTOR. Nlgn3 is cleaved from both neurons and oligodendrocyte precursor cells via the ADAM10 sheddase. Administration of ADAM10 inhibitors robustly blocks HGG xenograft growth. This work defines the therapeutic potential of and a promising strategy for targeting Nlgn3 secretion in the glioma microenvironment, which could prove transformative for treatment of HGG.

1 High-grade gliomas (HGG) are a devastating group of tumors, representing the leading cause of
2 brain cancer-related death in both children and adults. Therapies aimed at mechanisms intrinsic
3 to the glioma cell have translated to only limited success, and it is increasingly clear that
4 effective therapeutic strategies will need to also target elements of the glioma microenvironment
5 that promote glioma survival and proliferation. We have recently demonstrated that neuronal
6 activity robustly promotes the growth of a range of molecularly and clinically distinct HGG
7 types, including adult glioblastoma (GBM), anaplastic oligodendroglioma, pediatric GBM, and
8 diffuse intrinsic pontine glioma (DIPG)¹. Mechanisms mediating this growth-promoting effect of
9 neuronal activity on HGG include activity-regulated secretion of the glioma mitogens brain-
10 derived neurotrophic factor (Bdnf) and neuroligin-3 (Nlgn3) into the tumor microenvironment¹.
11 Nlgn3 is a post-synaptic adhesion molecule present chiefly at excitatory synapses^{2,3} and
12 expressed by both neurons and by glial cells⁴. Similar to activity-regulated cleavage of
13 neuroligin-1 (Nlgn1)^{5,6}, we found Nlgn3 to be cleaved at the c-terminal transmembrane domain,
14 resulting in shedding of the large N-terminal ectodomain¹. The released Nlgn3 acts on glioma
15 cells to drive proliferation through the PI3K-mTOR pathway, and also promotes a feed-forward
16 increase in glioma NLGN3 expression¹. Blocking Nlgn3 release into the tumor
17 microenvironment may represent a promising therapeutic opportunity, but the proteolytic
18 mechanism of Nlgn3 cleavage, cell type(s) from which activity-regulated shedding occurs and
19 the relative promise of Nlgn3 as a therapeutic target remain to be clarified.

20 **Neuroligin-3 necessity to *in vivo* glioma growth**

21 Multiple cell-intrinsic and microenvironmental factors could promote glioma growth, and Nlgn3
22 is only one such factor. To test the relative necessity of microenvironmental Nlgn3 to glioma
23 growth *in vivo*, we xenografted patient-derived HGG cells expressing GFP-luciferase into *Nlgn3*

24 knockout mice⁷, back-bred onto an immunodeficient background strain to enable xenografting
25 (*Nlgn3*^{y/-}; NSG). *Nlgn3* knockout mice are healthy and nearly normal neurologically, exhibiting
26 only subtle deficits in behavioral and electrophysiological assessments⁸⁻¹⁰. A patient-derived
27 culture of a pediatric glioblastoma of the frontal lobe expressing GFP and luciferase (SU-
28 pcGBM2-GFP-luc) was xenografted to the frontal cortex at postnatal day 35 (P35) and
29 monitored using *in vivo* bioluminescent (IVIS) imaging over the course of 6 months (Fig. 1a).
30 Initial engraftment was equivalent in both genotypes (*Nlgn3*^{y/-};NSG and *Nlgn3*^{y/+};NSG; Extended
31 Data Fig. 1). A striking stagnation of glioma growth was evident in the *Nlgn3* knockout animals
32 (at 3 months, tumor burden had increased by ~5-10 fold in *Nlgn3*^{y/+};NSG mice yet remained
33 unchanged in *Nlgn3*^{y/-};NSG mice, n=11 WT, n=14 *Nlgn3* KO, P<0.001) up to six months
34 following xenotransplantation (Fig 1a-f and Extended Data Fig. 2). By four and half months, a
35 subset of tumors circumvents this apparent Nlgn3 dependency and began to exhibit growth (Fig.
36 1e, f, Extended Data Fig. 2). Inhibition of growth in the *Nlgn3*^{y/-} mice was demonstrated by IVIS
37 imaging (Fig. 1a, c-f) and confirmed histologically (Fig. 1b)

38 The degree of growth inhibition observed in the Nlgn3-deficient mouse brain was
39 unexpected, particularly given our previous work demonstrating that in addition to Nlgn3, Bdnf
40 also contributes to glioma proliferation following exposure to activity-regulated factors present
41 in conditioned medium (CM) from active brain slices¹. We therefore revisited the necessity of
42 Nlgn3 to glioma cell proliferation in response to active CM *in vitro* to confirm our previous
43 findings regarding a role for other activity-regulated factors. We generated acute cortical slices
44 from optogenetically controllable (Thy1::ChR2), *Nlgn3* knockout mice to enable optogenetic
45 stimulation in the presence or absence of Nlgn3 *in situ*. Acute cortical slices from
46 *Nlgn*^{y/+};Thy1::ChR2 or *Nlgn*^{y/-};Thy1::ChR2 mice were optogenetically stimulated *in situ*, and the

47 surrounding conditioned medium (CM) was placed onto patient-derived HGG cultures (Extended
48 Data Fig. 3). Consistent with our previous findings, CM from *Nlgn3^{y/+};Thy1::ChR2* slices
49 significantly increased the proliferation of HGG cells compared to baseline media, while CM
50 from *Nlgn3^{y/-};Thy1::ChR2* elicited a substantially smaller but still significant increase in
51 proliferation (Extended Data Fig. 3). These data replicate the degree of differential proliferation
52 observed when Nlgn3 was sequestered from active slice conditioned medium, with residual
53 proliferation-inducing capacity of the CM accounted for by activity-regulated Bdnf¹. Taken
54 together, these findings indicate that glioma growth is more dependent on Nlgn3 *in vivo* than
55 would have been predicted from these *in situ/in vitro* experiments, at least during early phases of
56 glioma growth. The role that other activity-regulated glioma mitogens such as Bdnf may play at
57 later stages of growth or in the observed ability of some tumors to circumvent dependency on
58 Nlgn3 remains to be determined.

59 The nearly normal neurological function of *Nlgn3* knockout mice is thought to be due to
60 compensatory expression of other neuroligins^{7,11} such as Nlgn1, a molecule that is also cleaved
61 and secreted in activity-dependent fashion^{5,6}. Questioning the apparent lack of compensatory
62 effects of Nlgn1 on glioma growth in the *Nlgn3* knockout mouse, we tested the effects of
63 recombinant Nlgn1 exposure on glioma cell proliferation. Unlike Nlgn3¹, we did not find a
64 proliferation-inducing effect of Nlgn1 on HGG proliferation *in vitro* (Extended Data Fig. 4).
65 Similarly, we previously demonstrated that neuroligin-2 (Nlgn2) does not promote glioma
66 proliferation¹. Thus, compensatory expression of other neuroligins would not be expected to
67 influence glioma growth, supporting a unique role for Nlgn3 in glioma pathobiology.

68 To determine if Nlgn3 plays a similarly important role in the growth of additional types
69 of malignant glioma, patient-derived xenografts of DIPG and adult glioblastoma were tested in

70 the Nlgn3-deficient brain. A patient-derived culture of DIPG (SU-DIPGVI-GFP-luc) was
71 xenografted to the pons at p35 and monitored using *in vivo* bioluminescent imaging, and a
72 similar stagnation of growth was seen in this pontine DIPG xenograft (Fig. 2a-c). At 6 weeks,
73 tumor burden had increased by ~15 fold in *Nlgn3^{y/+}*;NSG mice yet remained unchanged in
74 *Nlgn3^{y/-}*;NSG mice as determined by *in vivo* bioluminescent imaging (Fig. 2c) and histological
75 analysis (Fig. 2b). A second patient-derived culture of DIPG (SU-DIPGXIII-GFP-luc) isolated
76 from a DIPG frontal lobe metastasis was xenografted to the frontal cortex and similarly
77 displayed growth inhibition in the Nlgn3-deficient brain (Fig. 2d), indicating that DIPG exhibits
78 a dependency on microenvironmental Nlgn3 in both cortical and pontine locations. Similarly, a
79 patient-derived adult glioblastoma culture (SU-GBM035-GFP-Luc) xenografted to the frontal
80 cortex of *Nlgn3^{y/-}*;NSG mice exhibit a striking stagnation of growth compared to GBM cells
81 xenografted to the *Nlgn3^{y/+}*;NSG mouse brain (Fig. 2e). In contrast, frontal cortex xenografts of a
82 patient-derived HER2⁺ breast cancer brain metastasis (DF-BM354-Luc)¹² did not exhibit any
83 difference in growth in the presence or absence of Nlgn3 in the brain microenvironment (Fig. 2f,
84 g). These results indicate a conserved dependency on Nlgn3 across molecularly and clinically
85 distinct types of HGG and underscore the importance of microenvironmental Nlgn3 to glioma
86 growth.

87 **Neuroigin-3 signaling in glioma cells**

88 The stagnation of growth observed in the Nlgn3-deficient brain is more robust than can
89 be explained by the known effects of Nlgn3 on glioma cell PI3K-mTOR signaling¹ alone. We
90 therefore sought to better delineate the signaling consequences of Nlgn3 exposure in glioma.
91 First, we utilized phosphoproteomic analyses (phosphoantibody array) of pediatric glioblastoma
92 (SU-pcGBM2) cells at 5 and 30 minutes following the addition of recombinant NLGN3.

93 Phosphorylation events upregulated by ~1.3 fold or more following NLGN3 exposure are
94 summarized in Figure 3a-b. These analyses demonstrated early phosphorylation of focal
95 adhesion kinase (FAK) and numerous phosphorylation events classically downstream of FAK,
96 including activation of the SRC kinase cascade, activation of the PI3K-mTOR cascade, and
97 activation of the SHC-RAS-RAF-MEK-ERK cascade (Fig. 3a, b). Additional oncogenic proteins
98 exhibiting increased phosphorylation following NLGN3 exposure include integrin β 3, growth
99 factor receptors EGFR, FGFR and VEGFR, as well as others (Fig. 3a, b). Central to many of
100 these signaling consequences of NLGN3 exposure is FAK activity. Phospho-tyrosine pull-down
101 analysis at 5 minute following NLGN3 exposure similarly demonstrated FAK phosphorylation.
102 FAK inhibition blocks the effects of NLGN3 exposure on glioma cell proliferation (Fig. 3c, d).
103 A time course analysis of FAK phosphorylation indicated peak phosphorylation at 5-10 minutes
104 following NLGN3 exposure, indicating this is an early signaling event (Fig. 3e). We had
105 previously shown that PI3K-mTOR signaling is necessary for NLGN3-induced glioma
106 proliferation¹ and FAK can be upstream of PI3K pathway. Accordingly, we found that FAK
107 activity is necessary for PI3K stimulation by NLGN3 as FAK inhibition abrogates NLGN3-
108 induced phosphorylation of AKT (Fig. 3f).

109 To further understand the mechanisms of NLGN3-induced glioma growth, we next
110 performed RNA sequencing (RNA-seq) of pediatric cortical glioblastoma (SU-pcGBM2) cells
111 16 hours following NLGN3 exposure (Extended Data Fig. 5). As expected, a host of genes
112 involved in cell proliferation were upregulated (Extended Data Fig. 5). We also found significant
113 upregulation of genes known to promote malignancy in glioma, including increased expression
114 of platelet-derived growth factor alpha (PDGFA), a growth factor strongly implicated in glioma
115 growth and progression¹³⁻¹⁵, and several potassium channel genes, recently shown to play a role

116 in DIPG cell viability¹⁶. In addition to the expected upregulation of NLGN3 expression itself¹, a
117 number of genes involved in synapse function were also upregulated following NLGN3 exposure
118 (Extended Data Fig. 5). While the meaning of this intriguing observation is not yet clear, it
119 suggests that the biology of NLGN3 in glioma may be more complex than simply promoting
120 proliferation.

121 **Cellular sources of secreted neuroligin-3**

122 The dramatic glioma growth inhibition observed in the Nlgn3-deficient brain highlights
123 its potential as a therapeutic target. One therapeutic strategy would be to block its release into the
124 tumor microenvironment, and therefore we sought to determine how, and from which cell types,
125 Nlgn3 is cleaved and secreted. As previously shown and re-demonstrated here, full length Nlgn3
126 is cleaved and secreted in an activity-regulated fashion by enzymatic cleavage and shedding of
127 the N-terminal ectodomain¹ (Fig. 4a, b). Optogenetic stimulation of acute cortical slices from
128 *Thy1::ChR2* mice illustrates increased Nlgn3 shedding detected in the CM using western blot
129 analysis as compared to the CM generated from slices exhibiting baseline spontaneous neuronal
130 activity. Conversely, the addition of tetrodotoxin (TTX), a specific voltage-gated sodium channel
131 blocker that abolishes neuronal action potentials, inhibits Nlgn3 secretion in the CM (Fig. 4c).

132 Nlgn3 is expressed at high levels in both neurons and in oligodendrocyte precursor cells
133 (OPCs; Fig. 4d)⁴, known to serve as a post-synaptic cell and form bona fide glutamatergic and
134 GABAergic synapses with presynaptic neurons¹⁷⁻¹⁹. To test the relative contribution of neurons
135 and OPCs to Nlgn3 secretion, mice expressing either the neuron-specific inducible Cre driver
136 *CamKIIa::Cre*^{ER} or the OPC-specific inducible Cre driver *PDGFRa::Cre*^{ER} were bred to Nlgn3^{fl/fl}
137 mice. Tamoxifen was administered for 5 days beginning at P28, resulting in recombination in
138 ~40% of cortical neurons in the *CamKIIa::Cre*^{ER} driver mouse and in ~80% of OPCs in the

139 *PDGFRa::Cre*^{ER} driver mouse (Extended Data Fig. 6). Using the acute slice paradigm together
140 with inducible, conditional deletion of *Nlgn3* from either neurons or from OPCs, we find that
141 both cells types contribute to activity-regulated *Nlgn3* secretion, and notably OPCs are a major
142 source of secreted *Nlgn3* (Fig.4e, f).

143 We previously demonstrated that NLGN3 exposure results in feed-forward glioma cell
144 expression of NLGN3 mRNA and protein¹, and thus asked what contribution glioma cells make
145 to the pool of secreted NLGN3 in the microenvironment. We find elevated levels of secreted
146 neuroligin-3 in CM from glioma xenograft-bearing *Nlgn3* WT brain slices compared to non-
147 xenograft bearing slices (Extended Data Fig S7a-b), either due to NLGN3 secreted from the
148 glioma cells or because glioma cells can increase cortical excitability²⁰. To confirm that glioma
149 cells are themselves secreting NLGN3, we collected CM from glioma cells that had been
150 exposed to recombinant NLGN3 or to control vehicle and then washed prior to CM collection.
151 We find up-regulation of NLGN3 secretion from glioma cells following NLGN3 exposure
152 (Extended Data Fig. 7c). Consistent with this finding and underscoring the importance of
153 microenvironmental NLGN3 to stimulate feed-forward glioma cell expression and secretion,
154 xenograft-bearing brain slices from *Nlgn3* KO mice secrete no detectable protein (Extended Data
155 Fig. S7b). Taken together, these data indicate that glioma cells contribute to the secreted NLGN3
156 in the tumor microenvironment in a manner regulated by neuroligin-3 exposure from normal
157 stromal cells.

158 **ADAM10 cleaves Neuroligin-3**

159 To determine the enzyme responsible for the cleavage of *Nlgn3*, the amino acid sequence of the
160 c-terminal region of *Nlgn3* was analyzed for putative cleavage sites
161 (<https://prosper.erc.monash.edu.au/home.html>). The analysis yielded both the MMP and ADAM

162 family proteases as candidate enzymes involved in Nlgn3 secretion, a prediction that is
163 consistent with the reported enzyme(s) responsible for Nlgn1 cleavage^{5,6} and a database of neural
164 ADAM10 targets²¹. To begin to determine the identity of the enzyme responsible for activity-
165 dependent Nlgn3 cleavage, optogenetically stimulated *Thy1::ChR2* cortical slices were utilized
166 to generate conditioned media in the absence and presence of various protease inhibitors.
167 Western blot analyses of the resulting CM showed a decrease in the level of Nlgn3 in CM with a
168 pan-MMP inhibitor, an MMP2/9 inhibitor, an MMP9/13 inhibitor, and a specific ADAM10
169 inhibitor²² (Fig. 5a). An increase in full-length Nlgn3 was observed in brain slice lysate
170 concomitant with the observed decrease in cleaved Nlgn3 in CM when either MMP9 or
171 ADAM10 inhibitors were used (Fig. 5b). While these results suggest a possible role for MMP9
172 and/or ADAM10, one MMP9-specific inhibitor had no effect (Fig. 5a). To test the functional
173 effect of these protease inhibitors, HGG cells were exposed to CM generated in the presence of
174 each compound. As expected, the increased proliferation seen in response to active CM was
175 abrogated in the cells exposed to CM generated in the presence of the protease inhibitors that
176 reduce Nlgn3 secretion (Fig. 5c-d). Neither MMP nor ADAM10 inhibitors exerted direct effects
177 on glioma cell proliferation (Fig. 5c-d). Thus, decreased Nlgn3 levels in the CM results in a
178 corresponding decrease in the proliferation index of HGG cells exposed to this CM. These data
179 highlight MMP9 and ADAM10 as promising candidate enzymes mediating activity-dependent
180 NLGN3 cleavage and secretion.

181 Given the possibility of cross-inhibition with pharmacological protease inhibition, genetic
182 knockout models were employed to more definitively determine whether MMP9 or ADAM10 is
183 responsible for Nlgn3 cleavage and shedding. Acute brain slices from *Mmp9* knockout mice
184 showed no change in Nlgn3 secretion into CM as compared to those from *Mmp9* WT mice (Fig.

185 5e), suggesting that MMP9 is not responsible for Nlgn3 cleavage and that the decreased levels of
186 Nlgn3 secretion observed with pharmacologic MMP9 inhibition may be due to cross-
187 inhibition/off-target effects. Constitutive knockout of *Adam10* is embryonic lethal, thus an
188 inducible conditional *Adam10* knockout model was used to delete *Adam10* from *CamKIIa*-
189 expressing neurons using the *CamKIIa::CreER* driver mouse as above. Western blot analysis of
190 the CM generated from *CamKIIa::CreER+*; *Adam10^{fl/fl}* cortical slices demonstrated ~50%
191 decrease in secreted Nlgn3 in comparison to *CamKIIa::CreER-*; *Adam10^{fl/fl}* mice (Fig. 5f). In
192 contrast, conditional deletion of *Adam10* from OPCs using *PDGFRA::CreER*; *Adam10^{fl/fl}* mice
193 does not influence Nlgn3 secretion (Fig. 5g). ADAM10 can be released from neurons at synapses
194 where it can act on the ectodomains of synaptic proteins²³. We find that brain slice incubation
195 with TTX to silence spontaneous neuronal activity substantially abrogates ADAM10 release into
196 slice CM (Fig. 5h), demonstrating activity-regulated release of the ADAM10 sheddase. Taken
197 together these data indicate that ADAM10, released in activity-dependent fashion from neurons,
198 is the chief enzyme responsible for cleaving Nlgn3, and may be a promising target for HGG
199 therapy.

200 **ADAM10 inhibition blocks glioma growth *in vivo***

201 As ADAM10 is a key mediator of Nlgn3 secretion, and we have shown the importance of Nlgn3
202 for high-grade glioma growth *in vivo*, we investigated the therapeutic potential of ADAM10
203 inhibition for HGG. ADAM10 is expressed in gliomas as reported in the literature^{24,25} and
204 demonstrated in gene expression datasets from pediatric and adult HGG samples^{1,16,26-28}
205 (Extended Data Fig. 7d). Cell intrinsic effects of ADAM10 inhibition have been reported in adult
206 HGG^{25,29,30}, so prior to assessing possible effects of modulating Nlgn3 secretion in the glioma
207 microenvironment with ADAM10 inhibition we first sought to assess possible direct effects of

208 this inhibitor on the pHGG cells used here. Cell-intrinsic effects of ADAM10 inhibition were not
209 observed on proliferation, overall cell viability or invasion of pHGG cells, even at high
210 concentrations (Fig. 6a, b Extended Data Fig. 8a). We did find a mild effect of ADAM10
211 inhibition on pHGG self-renewal in a neurosphere formation assay (Fig. 6c and Extended Data
212 Fig. 8b), consistent with reports in adult HGG²⁵.

213 We next tested the influence of ADAM10 inhibition on HGG growth *in vivo*. Brain
214 penetration of the specific ADAM10 inhibitor GI254023X was assessed following systemic
215 administration using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Following
216 a single 100 mg/kg intraperitoneal (i.p.) dose, drug levels in brain tissue were found to be ~2-4
217 μM , indicating sufficient blood-brain-barrier penetration (Extended Data Table 1). Given the
218 possible effects of ADAM10 inhibition on glioma self-renewal and therefore tumor initiation,
219 drug treatment started well after engraftment had occurred. Pediatric glioblastoma (SU-
220 pcGBM2-GFP-luc) cells were xenografted into the cortex or DIPG (SU-DIPGVI-GFP-luc or
221 SU-DIPG-XIX) cells were xenografted into the pons of NSG mice. Four weeks after
222 xenografting, mice were treated with either the ADAM10 inhibitor GI254023X (100 mg/kg) or
223 vehicle control 5 times/week over the course of 2 weeks and monitored using *in vivo*
224 bioluminescent imaging. GI254023X was well tolerated, with no apparent adverse events. The
225 pGBM and both DIPG xenografts in mice treated with the ADAM10 inhibitor exhibited a robust
226 reduction in growth as compared to vehicle-treated controls (Fig. 6d-f), commensurate with the
227 stagnation of glioma xenografts in the Nlgn3-deficient brain described above. Histological
228 analyses revealed a reduced proliferation index in ADAM10 inhibitor-treated animals as
229 assessed by the fraction of glioma cells co-expressing the proliferation marker Ki67 (Fig. 6g-h).
230 Furthermore, we found that ADAM10 inhibition abrogates glioma cell secretion of neuroligin-3

231 from both xenograft-bearing brain slices and NLGN3-primed glioma cells *in vitro* (Extended
232 Data Fig. 7b-c), suggesting this therapeutic strategy addresses all cellular sources of neuroligin-
233 3.

234 ADAM10 inhibitors have been developed for clinical use, and the ADAM10/17
235 inhibitors INCB7839 (aderbasib) and XL-784 have been through Phase II clinical trials for
236 indications such as breast cancer^{31,32}. We used LC-MS/MS to evaluate brain penetration of these
237 compounds, and found the pharmacokinetic profile of INCB7839 was superior to XL-784 (Fig.
238 6i and Extended Data Fig. 9). INCB7839 inhibits ADAM10 enzymatic function at low
239 nanomolar concentrations^{31,32} and was found to reach a brain tissue level of 100 ng/ml (~240
240 nM) for ~4 hours following systemic administration in mice, suggesting sufficient BBB
241 penetration (Fig. 6i). We therefore tested INCB7839 at a clinically relevant dose (50 mg/kg)
242 administered 5 days per week beginning four weeks following xenografting and found that, like
243 GI254023X, INCB7839 robustly inhibits growth of pediatric glioblastoma orthotopic xenografts
244 (Fig. 6j). Targeting of the sheddase ADAM10 thus represents a strategy to modulate neuroligin-3
245 levels in the tumor microenvironment for HGG therapy.

246 **Discussion:**

247 Here, we demonstrate an unexpected and conserved dependency of HGG growth on
248 microenvironmental neuroligin-3 across multiple classes of pediatric and adult HGG. The
249 magnitude of this effect both underscores its potential as a therapeutic target and suggests that
250 the mechanisms by which NLGN3 promotes HGG growth are incompletely understood. We
251 have furthered our understanding of the mechanisms by which NLGN3 stimulates glioma
252 proliferation and now show that NLGN3 activates FAK upstream of the PI3K-mTOR pathway,
253 as well as stimulating numerous additional oncogenic signaling pathways and upregulating genes

254 associated with known mechanisms of glioma malignancy. However, loss of a single robust
255 mitogen is unlikely to account entirely for the complete stagnation of xenograft growth observed
256 in the Nlgn3-deficient brain. Future work will need to elucidate further mechanisms by which
257 NLGN3 regulates glioma progression as well as by which the cancer may circumvent this
258 dependency.

259 NLGN3 is cleaved and secreted from both neurons and OPCs. The physiological function
260 of NLGN3 cleavage remains to be determined. Whether activity-regulated cleavage of NLGN3
261 in OPCs, likely occurring at axo-glial synapses, plays a role in the adaptive responses of myelin-
262 forming precursor cells to neuronal activity³⁴ remains to be seen. However, these findings define
263 a previously unrecognized place for OPCs as a microenvironmental cell type contributing to
264 glioma growth.

265 Targeting NLGN3 holds great promise for glioma therapy as an adjuvant to traditional
266 cytotoxic treatment modalities such as radiation and chemotherapy. Possible strategies to prevent
267 the glioma growth-promoting effects of NLGN3 include blocking NLGN3 cleavage and release,
268 sequestering soluble NLGN3 in the tumor microenvironment or blocking the as-of-yet
269 unidentified NLGN3 binding partner/receptor on glioma cells. Here, we focus on the former
270 strategy, identify ADAM10 as the protease mediating NLGN3 cleavage and demonstrate robust
271 stagnation of tumor growth with ADAM10 inhibition in preclinical models of HGG. The specific
272 ADAM10 inhibitor GI254023X is presently in the preclinical phase of development, but the
273 ADAM10 inhibitor INCB7839 has been through phase II clinical testing for other indications
274 and could be re-purposed now for HGG therapy. ADAM10 mediates cleavage of numerous cell
275 surface proteins, prominently targeting synapse-associated proteins²¹, and also plays an
276 important role in amyloid protein processing³³. While ADAM10 inhibition appears well-

277 tolerated in clinical trials^{31,32}, long-term effects on synaptic plasticity, amyloid deposition and
278 neurological function should be carefully evaluated as part of any effort towards clinical
279 translation of ADAM10 inhibition for this group of lethal cancers.

280 The present study highlights a means to target the growth-promoting effects of neuronal
281 activity on high-grade gliomas that span a range of clinically and molecularly distinct glioma
282 types. Further elucidation of neural influences on other classes of brain cancers may provide
283 additional therapeutic insights. Moreover, a critical role is emerging for nervous system
284 regulation of a variety of cancers, including prostate³⁵, gastric³⁶, pancreatic³⁷ and skin³⁸ cancers.
285 Deeper understanding of the neural mechanisms driving malignancy may broadly highlight
286 avenues towards more effective cancer control.

287

288 **Methods:**

289 *Mice and housing conditions:* All *in vivo* experiments were approved by the Stanford University
290 Institutional Animal Care and Use Committee and performed in accordance with institutional
291 guidelines. Animals were housed according to standard guidelines with free access to food and
292 water in a 12-hour light/dark cycle.

293 For constitutive knockout studies, *Nlgn3* KO mice (The Jackson Laboratory) were intercrossed
294 with NSG mice (NOD-SCID-IL2R gamma chain-deficient, The Jackson Laboratory) to produce
295 the *Nlgn3* KO;NSG genotype. All experiments were performed on male mice either hemizygous
296 WT *Nlgn3* or hemizygous null *Nlgn3* littermates.

297 For conditional knockout experiments, *Nlgn3*^{fl/fl} mice (a kind gift from Dr. Thomas Sudhof) or
298 *ADAM10*^{fl/fl} mice (The Jackson Laboratory) were crossed to *CamKIIa*:CreER (The Jackson
299 Laboratory) or *PDGFRa*::CreER (The Jackson Laboratory). Cre+ or control Cre- floxed mice

300 were treated with 100mg/kg tamoxifen i.p. for 5 days and experiments were performed 7 days
301 after the end of treatment. Tamoxifen was given from postnatal day 28 (P28) to P33 and slice
302 experiments performed at P40.

303 *Bioluminescent imaging:* For *in vivo* monitoring of tumor growth, bioluminescence imaging was
304 performed using an IVIS imaging system (Xenogen). Mice orthotopically xenografted with
305 luciferase-expressing glioma cells were placed under isoflurane anesthesia and injected with
306 luciferin substrate. Animals were imaged at baseline and randomized based on tumor size by a
307 blinded investigator so that experimental groups contained an equivalent range of tumor sizes.
308 All total flux values were then normalized to baseline values to determine fold change of tumor
309 growth. Statistical analysis between tumor burden in each group was assessed using Student's
310 two-tailed t-test (parametric data) or Mann Whitney test (non-parametric data). Based on the
311 variance of xenograft growth in control mice, we used at least 3 mice per genotype to give 80%
312 power to detect an effect size of 20% with at a significance level of 0.05.

313
314 *Orthotopic xenografting:* A single-cell suspension from cultured neurospheres was prepared in
315 sterile PBS immediately prior to the xenograft procedure. Animals at p34-36 were anesthetized
316 with 1-4% isoflurane and placed in a stereotactic apparatus. The cranium was exposed via
317 midline incision under aseptic conditions. 600,000 glioma cells in 3 μ L sterile PBS were
318 stereotactically implanted in either the premotor cortex (M2) of the right hemisphere (SU-
319 pcGBM2) or the pons (SU-DIPGVI and SU-DIPGXIX) through a 31-gauge burr hole, using a
320 digital pump at infusion rate of 0.4 μ L/minute and 31-gauge Hamilton syringe. For breast cancer
321 brain metastasis studies, 100,000 DF-BM354 cells were similarly xenografted in the premotor
322 cortex. Stereotactic coordinates used were as follows: for premotor cortex, 0.5 mm lateral to

323 midline, 1.0 mm anterior to bregma, -1.75 mm deep to cranial surface; for pons, 1.0 mm lateral
324 to midline, -0.8 mm posterior to lamda, -5.0 mm deep to cranial surface. At the completion of
325 infusion, syringe needle was allowed to remain in place for a minimum of 2 minutes, then
326 manually withdrawn at a rate of 0.875 mm/minute to minimize backflow of the injected cell
327 suspension.

328 *Perfusion and immunohistochemistry:* Animals were anesthetized with intraperitoneal Avertin
329 (tribromoethanol), then transcardially perfused with 20 mL of PBS. Brains were fixed in 4%
330 paraformaldehyde overnight at 4°C, then transferred to 30% sucrose for cryoprotection. Brains
331 were embedded in Tissue-Tek O.C.T. (Sakura) and sectioned in the coronal plane at 40 µm using
332 a sliding microtome (Microm HM450; Thermo Scientific). For immunohistochemistry, coronal
333 sections were incubated in blocking solution (3% normal donkey serum, 0.3% Triton X-100 in
334 TBS) at room temperature for 30 minutes. Chicken anti-GFP (1:500, Abcam), Rat anti-MBP
335 (1:300, Abcam), Mouse anti-human nuclei clone 235-1 (1:100; Millipore), or rabbit anti-Ki67
336 (1:500; Abcam), were diluted in 1% blocking solution (1% normal donkey serum in 0.3% Triton
337 X-100 in TBS) and incubated overnight at 4°C. Sections were then rinsed three times in 1X TBS
338 and incubated in secondary antibody solution (Alexa 488 donkey anti-chicken IgG, 1:500
339 (Jackson Immuno Research); Alexa 594 donkey anti-mouse IgG, 1:500 (Life Technologies);
340 Alexa 647 donkey anti-rabbit IgG, 1:500 (Life Technologies); Alexa 594 donkey anti-rat IgG,
341 1:1000 (Life Technologies)) in 1% blocking solution at 4°C overnight. Sections were rinsed 3
342 times in TBS and mounted with ProLong Gold Mounting medium (Life Technologies).

343 *Cell culture:* For all human tissue studies, informed consent was obtained and Institutional
344 Review Board (IRB) approval was granted. For all cultures, short tandem repeat (STR) DNA

345 fingerprinting was performed every three months to verify authenticity. The STR fingerprints
346 and clinical characteristics for the patient-derived cultures used have been previously reported¹
347 with the exception of SU-DIPG-XIX which is a H3.3K27M mutant tumor that was derived at the
348 time of autopsy from a male who was 2 years of age at diagnosis, was treated with radiotherapy
349 and cabazitaxel and who survived 18 months. STR fingerprint for SU-DIPG-XIX is: X/Y
350 (AMEL), 10/11 (CSF1PO1), 13/14 (D13S317), 9/13 (D16S539), 30/30 (D21S11), 11/12
351 (D5S818), 10/10 (D7S820), 9.3/9.3 (TH01), 8/11 (TPOX), 17/18 (vWA). All cell cultures were
352 routinely tested for mycoplasma.

353 All high-grade glioma cultures were generated as previously described¹. In brief, tissue was
354 obtained from high-grade glioma (WHO grade III or IV) tumors at the time of biopsy or from
355 early post-mortem donations. Tissue was dissociated both mechanically and enzymatically and
356 grown in a defined, serum- free medium designated “Tumor Stem Media (TSM)”, consisting of
357 Neurobasal(-A) (Invitrogen), B27(-A) (Invitrogen), human- bFGF (20 ng/mL) (Shenandoah
358 Biotech), human-EGF (20 ng/mL) (Shenandoah), human PDGF-AA (10 ng/mL) and PDGF-BB
359 (10 ng/mL) (Shenandoah) and heparin (2 ng/mL) (Stem Cell Technologies).

360 Breast cancer brain metastasis line, PDX DF-BM354, was provided by the Zhao lab and
361 developed as previously described¹².

362 *Generation of conditioned media from acute cortical slices:* Conditioned media was generated as
363 previously described¹. Mice (genotype varied based on experiment) between the ages of 4-7
364 weeks were briefly exposed to 4% isoflurane and immediately cervically dislocated and
365 decapitated. Extracted brains were placed in oxygenated high-sucrose solution and sliced in 350-
366 μm sections. Slices were then placed in buffering solution (aCSF) and allowed to recover for at

367 least one hour. After recovery, slices were then moved into fresh aCSF in a 24-well plate and
368 slices optogenetically stimulated using a blue-light LED to observe the effects of elevated
369 neuronal activity (in the case of *Thy1::ChR2* brain slices) or unstimulated to observe the effects
370 of baseline neuronal activity. Following recovery, media was conditioned for 30 minutes. For
371 various experiments, conditioned media was prepared in the presence of various protease
372 inhibitors (described below) or tetrodotoxin at 1 μ M (Tocris). Surrounding medium was then
373 collected for immediate use or frozen at -80°C for future experiments. All slice experiments were
374 performed in three biological replicates unless otherwise indicated.

375 *EdU incorporation assay*: 8-well chamber slides were coated with poly-L-lysine. Cells were then
376 seeded at 40,000 cells per well and exposed to aCSF, aCSF the relevant inhibitor (see below), or
377 active conditioned media (conditioning methods vary by assay). 10 μ M EdU was added to each
378 well. Cells were fixed after 24 hours using 4% paraformaldehyde in PBS and stained using the
379 Click-iT EdU kit and protocol (Invitrogen). Proliferation index was then determined by
380 quantifying percentage of EdU labeled cells using confocal microscopy at 200x magnification.
381 Group mean differences were otherwise assessed using one-way analysis of variance (one-way
382 ANOVA) with Tukey post-hoc tests to further examine pairwise differences. All experiments
383 were performed in three biological replicates.

384 *CellTiter-Glo assay of cell viability*: To assess overall cell number, 5000 glioma cells were
385 seeded in minimal growth media in a 96-well plate with varying concentrations of ADAM10
386 inhibitor. After 24, 48, or 72 hours, CellTiter-Glo reagent (Promega) was added at a 1:1 ratio.
387 Luminescence was measured after 10- minute incubation at room temperature to stabilize signal.
388 All experiments were performed in three biological replicates.

389 *Inhibitors used:* Batimastat at 20nM (Pan MMP inhibitor; BB-94; Selleck Chemicals); MMP-
390 2/MMP-9 Inhibitor II at 50nM (sc-311430; Santa Cruz biotechnology); ARP 100 at 20nM
391 (MMP2 inhibitor) (R&D Systems); MMP-13 Inhibitor at 10nM (sc-205756; Santa Cruz
392 biotechnology); MMP-9 Inhibitor I at 100nM (sc-311437; Santa Cruz biotechnology); MMP-
393 9/MMP-13 Inhibitor II at 10nM (sc-311439; Santa Cruz biotechnology); MMP2/MMP-3
394 Inhibitor I at 20uM (sc-295483; Santa Cruz biotechnology); TAPI-1 at 20uM (ADAM17
395 inhibitor; S7434; Selleck Chemicals); GI 254023X at 200nM (ADAM10 inhibitor; Sigma
396 Aldrich); PF-00562271 at 5nM (FAK inhibitor; S2672; Selleck Chemicals).

397
398 *Analysis of NLGN3 secretion from glioma:* For *in vivo* studies demonstrating NLGN3 secretion
399 from xenograft-bearing slices, mice were xenografted as above with SU-DIPGXIII or
400 SUGBM035 cells in premotor cortex. Brains were extracted and used for conditioned media
401 experiments in comparison to non-xenografted littermate controls at 5 months (SU-DIPG-XIII)
402 or 6 weeks (SU-GBM035). These experiments were performed in duplicate for SU-DIPG-XIII
403 xenograft-bearing cortical slices and in triplicate for SU-GBM035 xenograft-bearing cortical
404 slices (five biological replicates in total). For *in vivo* studies demonstrating neuroligin secretion
405 from xenograft-bearing slices can be blocked by ADAM10 inhibition, mice were xenografted as
406 above with SU-GBM035 cells in premotor cortex and brains were extracted at 6 weeks post
407 xenograft. Cortical slices were made and incubated in the presence or absence of 200nM
408 ADAM10 inhibitor, GI 254023X. CM was then analyzed using western blot analyses, comparing
409 non-xenograft bearing slices to xenograft-bearing slices from WT or Nlgn3 KO mice in the
410 presence of absence of ADAM inhibitor. These experiments were performed in triplicate.

411 For *in vitro* studies of NLGN3 secretion from glioma cells, SU-pcGBM2 cells were seeded at 5

412 million cells/well in the presence of either vehicle, 100nM recombinant NLGN3, 200nM GI
413 254023X, or 100nM NLGN3 + 200nM GI 254023X for 48 hours. After thorough rinsing of the
414 cells, cells were left in either fresh media or 200nM GI 254023X for another 48 hours in the
415 presence or absence of ADAM10 inhibition. After 48 hours, media was collected and analyzed
416 for presence of cleaved NLGN3 using western blot analyses as described below. Experiments
417 were performed in three biological replicates.

418

419 *Spheroid invasion assay* was performed as previously described¹⁶.

420

421 *Neurosphere formation assay*: ELDA (extreme limiting dilution analysis) was performed to
422 evaluate self-renewal capacity³⁹. SU-pcGBM2 cells were dissociated in TrypLE (+ DNase and
423 HEPES) for 5min at 37C. Cells were triturated into a single cell solution. The solution was
424 incubated with Hoechst (Thermo, cat. 33342) for 30min at 37C. Live cells were identified using
425 a LIVE/DEAD staining kit (Thermo, cat. L10119). Live cells were sorted into 96 well plates.
426 Spheres were counted at 14 days. Cell density per well ranged from: 1, 10, 25, 50, 100, 250, 500,
427 1000. Each condition was tested in 10 independent wells. Volume of media per well was 200 ul
428 media with growth factors spike-ins every 3-4 days. The ADAM10 inhibitor was reconstituted in
429 DMSO. Each well was adjusted to have 0.1% DMSO, except for the no-DMSO control wells.
430 Neurosphere-forming capability was determined using the Extreme Limiting Dilution Analysis
431 (ELDA) web-based tool (<http://bioinf.wehi.edu.au/software/elda/>).

432

433 *Western Blot analyses*: Western blot analyses were used to probe for protein levels present in
434 either brain slice homogenate or secreted into slice conditioned media. For slice homogenates,

435 brain slices were lysed using RIPA buffer and protease inhibitors. Lysates were incubated on ice
436 for 10 minutes and then centrifuged for 10 minutes at 4°C. All samples were normalized to
437 protein concentration, mixed with Laemmli loading buffer (1:4), boiled for 5 minutes, and loaded
438 onto BioRad Mini-Protean TGX precast gels. Protein was transferred to PVDF membranes and
439 blocked with 5% bovine serum albumin (BSA) in TBST for one hour. Anti-Neuroigin-3
440 (NovusBio; 1:300), Anti-phospho FAK pTyr861 (Thermo Fisher Scientific; 1:500), and anti-
441 FAK (Cell Signaling Technologies; 1:500), or anti-ADAM10 (Abcam; 1:500) were diluted in
442 1% BSA/TBST and incubated with the membrane overnight. Secondary anti-rabbit conjugated to
443 HRP (BioRad) was then added for one hour (1:1000). Proteins were visualized using Clarity
444 ECL Western Substrate (BioRad) and quantified and analyzed using ImageJ.

445

446 *Phospho-antibody array:* The Phospho Explorer antibody array assay was performed by Full
447 Moon Biosystems on patient-derived pediatric glioblastoma (SU-pcGBM2) cells. Cell lysates
448 were prepared using Protein Extraction Kit (Full Moon BioSystems). Clear supernatant of the
449 lysates was separated, biotinylated, and incubated with Phospho Explorer Antibody Arrays (Full
450 Moon BioSystems) for two hours at room temperature. The array slides were washed with Wash
451 Buffer (Full Moon BioSystems) and rinsed with DI water. The slides were then incubated with
452 Cy3-Streptavidin for 45 minutes at room temperature, then washed, rinsed and dried. Arrays
453 were scanned on GenePix Array Scanner (Molecular Devices). Image quantification was
454 performed on GenePix Pro (Molecular Devices). Signal intensity data for each spot on the array
455 was extracted from array images. Since there are two replicated printed for each antibody, the
456 mean signal intensity of the replicates is determined. The data is then normalized to the median
457 value (signal intensity) of all antibodies on the slide. Finally, fold change between Control

458 Sample and Treatment Sample is determined using the normalized data (Treatment Sample's
459 signal is divided by Control Sample's signal).

460

461 *Phosphotyrosine pull down assay*

462 Samples were analyzed using the Cell Signaling Technology PTMScan method as previously
463 described⁴⁰⁻⁴². Cellular extracts were prepared in urea lysis buffer, sonicated, centrifuged,
464 reduced with DTT, and alkylated with iodoacetamide. 15mg total protein for each sample was
465 digested with trypsin and purified over C18 columns for enrichment with the Phosphotyrosine
466 pY-1000 antibody (#8803) and the PTMScan Direct Tyr Kinases Reagent⁴². Enriched peptides
467 were purified over C18 STAGE tips (Rappsilber). Enriched peptides were subjected to secondary
468 digest with trypsin and second STAGE tip prior to LC-MS/MS analysis.

469

470 Replicate injections of each sample were run non-sequentially for each enrichment. Peptides
471 were eluted using a 90-minute linear gradient of acetonitrile in 0.125% formic acid delivered at
472 280 nL/min. Tandem mass spectra were collected in a data-dependent manner with an LTQ
473 Orbitrap Elite mass spectrometer running XCalibur 2.0.7 SP1 using a top-twenty MS/MS
474 method, a dynamic repeat count of one, and a repeat duration of 30 sec. Real time recalibration
475 of mass error was performed using lock mass⁴³ with a singly charged polysiloxane ion $m/z =$
476 371.101237.

477 MS/MS spectra were evaluated using SEQUEST and the Core platform from Harvard
478 University⁴⁴⁻⁴⁶. Files were searched against the NCBI *rattus norvegicus* FASTA database
479 updated on May 22, 2015. A mass accuracy of +/-5 ppm was used for precursor ions and 1.0 Da
480 for product ions. Enzyme specificity was limited to trypsin, with at least one LysC or tryptic (K-

481 or R-containing) terminus required per peptide and up to four mis-cleavages allowed. Cysteine
482 carboxamidomethylation was specified as a static modification, oxidation of methionine and
483 phosphorylation on serine, threonine, and tyrosine residues were allowed as variable
484 modifications. Reverse decoy databases were included for all searches to estimate false
485 discovery rates, and filtered using a 5% FDR in the Linear Discriminant module of Core.
486 Peptides were also manually filtered using a \pm 5ppm mass error range and reagent-specific
487 criteria. For each antibody reagent results were filtered to include only phosphopeptides
488 matching the sequence motif(s) targeted by the antibodies included. All quantitative results were
489 generated using Progenesis V4.1 (Waters Corporation) to extract the integrated peak area of the
490 corresponding peptide assignments. Accuracy of quantitative data was ensured by manual review
491 in Progenesis or in the ion chromatogram files.

492

493 *RNA Sequencing*

494 Samples were processed and analyzed as previously described¹⁶ with minor modifications as
495 indicated below:

496 RNA was extracted from Trizol-lysed cells and 1 μ g of total RNA was used for each sample.

497 Polyadenylated RNA was selected using Ambion Dynabeads mRNA Purification Kit (Life

498 Technologies 61006) and fragmented with Fragmentation Buffer (Ambion, #AM8740). First

499 strand synthesis was performed using Random Hexamer Primers (Invitrogen, #48190-011) and

500 SuperScript II (Invitrogen, #18064-014). Second strand synthesis was performed using DNA Pol

501 I (Invitrogen #18010-025) and RNA was removed using RNaseH (Invitrogen #18021-014).

502 Libraries were end-repaired, 3' A-tailed, and ligated to NEBNext Multiplex Oligo Adaptors
503 (NEB E7335S). Sequencing was performed on an Illumina NextSeq 500 by Stanford Functional
504 Genomics Facility.

505 Reads were mapped to hg19 annotation using Tophat2 (PMID: 23618408) (version 2.0.13) and
506 transcript expression was quantified against RefSeq gene annotations using featureCounts⁴⁷.
507 Differential testing and log₂ fold change calculation was performed using DESeq2⁴⁸. Gene
508 Ontology analyses were performed using DAVID^{49,50}.

509

510 *Pharmacokinetic studies:*

511 *LC-MS/MS analysis of GI254023X concentrations in tissues and serum.*

512 *Sample preparation:* A single 100 mg/kg dose was delivered i.p. in NOD-SCID-IL2R γ chain–
513 deficient mice, and tissue samples collected 30 min later for analysis using liquid
514 chromatography/tandem mass spectrometry (LC-MS/MS). Tissues samples were weighed and 1
515 volume of bullet blender beads (Next Advance) and 3 volume of Milli-Q water were added.
516 Tissues were homogenized by a bullet blender (Next Advance) at 4°C according to
517 manufacturer's instruction. The neat stock solution of GI254023X was dissolved in DMSO at 5
518 mg/ml and further diluted in 50% methanol to prepare spiking solutions. For spiked standard
519 curve, 25 μ l of GI254023X spiking solutions (0.5 - 1000 ng/ml for brain samples and 1 – 100
520 μ g/ml for serum and kidney samples) was mixed with 25 μ l of blank tissue homogenate or
521 serum. For samples, the spiking solution was replaced by 25 μ l of 50% methanol to make up the
522 volume. After vortexing all standards and samples, 150 μ l of methanol/acetonitrile 20:80 (v/v)
523 was added to the mixture and vortexed vigorously for 1 min followed by centrifugation at 3,000
524 g for 10 min. The supernatant was diluted 3 times in Milli-Q water for brain samples and 100

525 times in 25% methanol for serum and kidney samples. The LC-MS/MS system consists of a
526 QTRAP 4000 mass spectrometer (AB SCIEX) coupled to a Shimadzu UFLC system. LC
527 separation was carried out on a ZORBAX SB-Phenyl column (4.6 mm × 50 mm, 3.5 μm)
528 (Agilent) at room temperature. The analysis time was 3 min. The injection volume was 5-10 μl.
529 Isocratic elution was carried out with a mobile phase composed of 55% water and 45%
530 acetonitrile with 0.1% of formic acid and a flow rate of 0.5 ml/min. The mass spectrometer was
531 operated in the positive mode with multiple-reaction monitoring (MRM) with the transition m/z
532 392.2→361.2. Data acquisition and analysis were performed using the Analyst 1.6.1 software
533 (AB SCIEX).

534 *LC-MS/MS analysis of INCB7839 (Aderbasib) and XL-784 in brain tissue and serum:* Aderbasib
535 was purchased from Astatech, Inc. XL-784 was provided by True Pharmachem, Inc. A single 50
536 mg/kg dose of INCB7839 (aderbasib) or XL-784 was delivered i.p. in NOD-SCID-IL2R γ
537 chain-deficient (NSG) mice, and tissue samples collected at various time points for analysis
538 using liquid chromatography/tandem mass spectrometry (LC-MS/MS). NSG mice were
539 purchased from the Model Animal Research Center of Nanjing University. Study was conducted
540 by Crown Biosciences, Inc.. Compounds were formulated in 2% DMSO, 2% Tween 80, 48%
541 PEG300, 48% water. Compounds were administered IP, with a dosing volume of 10 μL/g and
542 concentration of 5 mg/ml. Compounds were dosed at 50 mg/kg. A cohort of 24 male, NSG mice,
543 age 6-8 weeks, body weight 18-22 grams, were used for each study. Animals were housed at
544 room temperature, under 40-70% humidity, with a 12 hour light/12 hour dark schedule. Mice
545 were fed with Co⁶⁰ dry granule food, with free access to reverse osmosis water. Eight time points
546 were collected for each compound (0.25, 0.5, 1, 2, 4, 6, 8, and 24 h), with an n = 3 for each time
547 point. Blood was collected via cardiac puncture and collected into potassium-EDTA Eppendorf

548 tubes. Samples were centrifuged within 30 minutes to afford plasma samples. Brains were
549 collected at each time point, PBS (4X) was added, and the material homogenized with a Tissue
550 Lyser II to give a fine homogenate. Brain homogenate (50 μ L) was treated with 250 μ L
551 acetonitrile (containing 200 ng/mL tolbutamide), which was then vortexed and then centrifuged
552 at 4000 rpm for 20 min. The supernatant was collected and mixed with a 0.1% aqueous formic
553 acid solution. Samples were analyzed on a Waters UPLC or Agilent 1200 Liquid
554 chromatography system, with an API 4000 mass spectrometer, and a 10 μ L injection volume,
555 with tolbutamide as an internal standard. Pharmacokinetics were analyzed using WinNonlin6.3
556 (non-compartmental model).

557

558 *Mouse drug treatment studies:* For all drug studies, NSG mice were xenografted as above with
559 either SU-pcGBM2, SU-DIPGVI, or SU-DIPG-XIX. Four weeks post-xenograft, animals were
560 treated with either systemic administration of ADAM10 inhibitor, GI254023X (Sigma-Aldrich;
561 formulated in 10% DMSO in 0.1M carbonate buffer) via intraperitoneal injection 5 days per
562 week at 100mg/kg or systemic administration of ADAM10/17 inhibitor, Aderbasib (Astatech,
563 Inc; formulated in 2% DMSO, 2% Tween 80, 48% PEG300, 48% water) via intraperitoneal
564 injection 5 days per week at 50mg/kg. For both studies, controls were intraperitoneally injected
565 with an identical volume of vehicle. Drug treatment began four weeks after xenografting and
566 continued through week six. Bioluminescence imaging was performed by a blinded investigator
567 before treatment, and again 7 days and 14 days later, using an IVIS imaging system (Xenogen)
568 under isoflurane anesthesia. Similar to above, tumor burden was assessed as fold change in total
569 flux from the beginning to end of treatment. All differences were statistically verified using
570 Students two-tailed t-test.

571

572 *Confocal imaging and quantification of cell proliferation:* Cell quantification was performed by
573 a blinded investigator using live counting at 400x magnification using a Zeiss LSM700 scanning
574 confocal microscope and Zen 2011 imaging software (Carl Zeiss Inc.). The area for
575 quantification was selected as follows: of a 1 in 6 series of 40- μ m coronal sections, 3 consecutive
576 sections were selected at approximately 1.1–0.86 mm anterior to bregma (Figures 22, 23, 24;
577 Franklin & Paxinos, *The Mouse Brain in Stereotaxic Coordinates*, 3rd Ed. 2008); using our
578 stereotactic coordinates for tumor xenograft, these sections are expected to include the tissue
579 most proximal to the site of tumor cell implantation in the coronal plane. For each of the three
580 consecutive sections, the cingulum bundle was first identified as an anatomic landmark, and six
581 160x160- μ m field area for quantification were selected centered around this landmark within
582 cortical layer 6b of M2. Within each field, all human nuclear antigen (HNA)-positive tumor cells
583 were quantified to determine tumor burden within the areas quantified. HNA-positive tumor cells
584 were then assessed for double-labeling with or Ki67. To calculate proliferation index (the
585 percentage of proliferating tumor cells for each animal), the total number of HNA-positive cells
586 co-labeled with Ki67 across all areas quantified was divided by the total number of human
587 nuclei-positive cells counted across all areas quantified. Differences in proliferation indices were
588 calculated using unpaired, two-tailed Student's *t*-tests (parametric data) or Mann-Whitney test
589 (non-parametric data).

590 *Statistical Analyses:* Statistical tests were conducted using Prism (GraphPad) software unless
591 otherwise indicated. Gaussian distribution was confirmed by the Shapiro-Wilk normality test.
592 For parametric data, unpaired, two-tailed Student's *t*-tests and one-way ANOVAs with Tukey
593 post-hoc tests to further examine pairwise differences were used. For non-parametric data,

594 Mann-Whitney test was used. The limiting dilution assay to test for neurosphere forming
595 capacity was analyzed with a chi-squared test using the Extreme Limiting Dilution Analysis
596 (ELDA) web-based tool (<http://bioinf.wehi.edu.au/software/elda/>). A level of $P < 0.05$ was used
597 to designate significant differences. Based on the variance of xenograft growth in control mice,
598 we used at least 3 mice per genotype to give 80% power to detect an effect size of 20% with a
599 significance level of 0.05.

600 Statistical analyses for proteomic and RNA-seq data are described above in the respective
601 sections.

602

603 *Data Availability:* RNA-seq data are deposited in GEO, accession number GSE99045

604

605 **References:**

- 606 1. Venkatesh, H. S. *et al.* Neuronal Activity Promotes Glioma Growth through Neuroligin-3
607 Secretion. *Cell* **161**, 803–16 (2015).
- 608 2. Budreck, E. C. & Scheiffele, P. Neuroligin-3 is a neuronal adhesion protein at
609 GABAergic and glutamatergic synapses. *Eur. J. Neurosci.* **26**, 1738–1748 (2007).
- 610 3. Südhof, T. C. Neuroligins and neurexins link synaptic function to cognitive disease.
611 *Nature* **455**, 903–11 (2008).
- 612 4. Zhang, Y. *et al.* An RNA-Sequencing Transcriptome and Splicing Database of Glia,
613 Neurons, and Vascular Cells of the Cerebral Cortex. *J. Neurosci.* **34**, 11929–47 (2014).
- 614 5. Suzuki, K. *et al.* Activity-Dependent Proteolytic Cleavage of Neuroligin-1. *Neuron* **76**,
615 410–422 (2012).
- 616 6. Peixoto, R. T. *et al.* Transsynaptic Signaling by Activity-Dependent Cleavage of

- 617 Neurologin-1. *Neuron* **76**, 396–409 (2012).
- 618 7. Varoquaux, F. *et al.* Neuroligins determine synapse maturation and function. *Neuron* **51**,
619 741–54 (2006).
- 620 8. Radyushkin, K. *et al.* Neuroligin-3-deficient mice: model of a monogenic heritable form
621 of autism with an olfactory deficit. *Genes, Brain Behav.* **8**, 416–425 (2009).
- 622 9. Tabuchi, K. *et al.* A neuroligin-3 mutation implicated in autism increases inhibitory
623 synaptic transmission in mice. *Science* **318**, 71–6 (2007).
- 624 10. Etherton, M. *et al.* Autism-linked neuroligin-3 R451C mutation differentially alters
625 hippocampal and cortical synaptic function. *Proc. Natl. Acad. Sci.* **108**, 13764–13769 (2011).
- 626 11. Blundell, J. *et al.* Neuroligin-1 Deletion Results in Impaired Spatial Memory and
627 Increased Repetitive Behavior. *J. Neurosci.* **30**, (2010).
- 628 12. Ni, J. *et al.* Combination inhibition of PI3K and mTORC1 yields durable remissions in
629 mice bearing orthotopic patient-derived xenografts of HER2-positive breast cancer brain
630 metastases. *Nat. Med.* **22**, 723–726 (2016).
- 631 13. Ozawa, T. *et al.* Most human non-GCIMP glioblastoma subtypes evolve from a common
632 proneural-like precursor glioma. *Cancer Cell* **26**, 288–300 (2014).
- 633 14. Martinho, O. *et al.* Expression, mutation and copy number analysis of platelet-derived
634 growth factor receptor A (PDGFRA) and its ligand PDGFA in gliomas. *Br. J. Cancer* **101**, 973–
635 82 (2009).
- 636 15. Sakakini, N. *et al.* A Positive Feed-forward Loop Associating EGR1 and PDGFA
637 Promotes Proliferation and Self-renewal in Glioblastoma Stem Cells. *J. Biol. Chem.* **291**, 10684–
638 99 (2016).
- 639 16. Nagaraja, S. *et al.* Transcriptional Dependencies in Diffuse Intrinsic Pontine Glioma.

- 640 *Cancer Cell* **31**, 635–652.e6 (2017).
- 641 17. Bergles, D. E., Roberts, J. D., Somogyi, P. & Jahr, C. E. Glutamatergic synapses on
642 oligodendrocyte precursor cells in the hippocampus. *Nature* **405**, 187–91 (2000).
- 643 18. Lin, S. & Bergles, D. E. Synaptic signaling between GABAergic interneurons and
644 oligodendrocyte precursor cells in the hippocampus. *Nat. Neurosci.* **7**, 24–32 (2004).
- 645 19. Ziskin, J. L., Nishiyama, A., Rubio, M., Fukaya, M. & Bergles, D. E. Vesicular release of
646 glutamate from unmyelinated axons in white matter. *Nat. Neurosci.* **10**, 321–30 (2007).
- 647 20. Campbell, S. L., Buckingham, S. C. & Sontheimer, H. Human glioma cells induce
648 hyperexcitability in cortical networks. *Epilepsia* **53**, 1360–70 (2012).
- 649 21. Kuhn, P.-H. *et al.* Systematic substrate identification indicates a central role for the
650 metalloprotease ADAM10 in axon targeting and synapse function. *Elife* **5**, 1174–1189 (2016).
- 651 22. Ludwig, A. *et al.* Metalloproteinase Inhibitors for the Disintegrin-Like
652 Metalloproteinases ADAM10 and ADAM17 that Differentially Block Constitutive and Phorbol
653 Ester-Inducible Shedding of Cell Surface Molecules. *Comb. Chem. High Throughput Screen.* **8**,
654 161–171 (2005).
- 655 23. Lundgren, J. L. *et al.* ADAM10 and BACE1 are localized to synaptic vesicles. *J.*
656 *Neurochem.* **135**, 606–615 (2015).
- 657 24. Qu, M., Qiu, B. O., Xiong, W., Chen, D. & Wu, A. Expression of a-disintegrin and
658 metalloproteinase 10 correlates with grade of malignancy in human glioma. *Oncol. Lett.* **9**,
659 2157–2162 (2015).
- 660 25. Bulstrode, H. *et al.* A-Disintegrin and Metalloprotease (ADAM) 10 and 17 promote self-
661 renewal of brain tumor sphere forming cells. *Cancer Lett.* **326**, 79–87 (2012).
- 662 26. Grasso, C. S. *et al.* Functionally defined therapeutic targets in diffuse intrinsic pontine

- 663 glioma. *Nat. Med.* **21**, 827 (2015).
- 664 27. Verhaak, R. G. W. *et al.* Integrated genomic analysis identifies clinically relevant
665 subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1.
666 *Cancer Cell* **17**, 98–110 (2010).
- 667 28. Tirosh, I. *et al.* Single-cell RNA-seq supports a developmental hierarchy in human
668 oligodendroglioma. *Nature* **539**, 309–313 (2016).
- 669 29. Siney, E. J. *et al.* Metalloproteinases ADAM10 and ADAM17 Mediate Migration and
670 Differentiation in Glioblastoma Sphere-Forming Cells. *Mol. Neurobiol.* (2016).
671 doi:10.1007/s12035-016-0053-6
- 672 30. Kohutek, Z. A., diPierro, C. G., Redpath, G. T. & Hussaini, I. M. ADAM-10-mediated N-
673 cadherin cleavage is protein kinase C-alpha dependent and promotes glioblastoma cell migration.
674 *J. Neurosci.* **29**, 4605–15 (2009).
- 675 31. Infante J, Burris HA, L. N. *et al.* A multicenter phase Ib study of the safety,
676 pharmacokinetics, biological activity and clinical efficacy of INCB7839, a potent and selective
677 inhibitor of ADAM10 and ADAM17. *Breast Cancer Res Treat* **106(Supp1)**, S269. (2007).
- 678 32. Friedman, S. *et al.* Clinical Benefit of INCB7839, a Potent and Selective Inhibitor of
679 ADAM10 and ADAM17, in Combination with Trastuzumab in Metastatic HER2 Positive Breast
680 Cancer Patients. *Cancer Res.* **69**, (2014).
- 681 33. Postina, R. *et al.* A disintegrin-metalloproteinase prevents amyloid plaque formation and
682 hippocampal defects in an Alzheimer disease mouse model. *J. Clin. Invest.* **113**, 1456–64 (2004).
- 683 34. Gibson, E. M. *et al.* Neuronal activity promotes oligodendrogenesis and adaptive
684 myelination in the mammalian brain. *Science* **344**, 1252304 (2014).
- 685 35. Magnon, C. *et al.* Autonomic nerve development contributes to prostate cancer

- 686 progression. *Science* **341**, 1236361 (2013).
- 687 36. Zhao, C.-M. *et al.* Denervation suppresses gastric tumorigenesis. *Sci. Transl. Med.* **6**,
688 250ra115 (2014).
- 689 37. Stopczynski, R. E. *et al.* Neuroplastic changes occur early in the development of
690 pancreatic ductal adenocarcinoma. *Cancer Res.* **74**, 1718–27 (2014).
- 691 38. Peterson, S. C. *et al.* Basal cell carcinoma preferentially arises from stem cells within hair
692 follicle and mechanosensory niches. *Cell Stem Cell* **16**, 400–12 (2015).
- 693 39. Hu, Y. & Smyth, G. K. ELDA: extreme limiting dilution analysis for comparing depleted
694 and enriched populations in stem cell and other assays. *J. Immunol. Methods* **347**, 70–8 (2009).
- 695 40. Rush, J. *et al.* Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. *Nat.*
696 *Biotechnol.* **23**, 94–101 (2005).
- 697 41. Stokes, M. *et al.* Complementary PTM Profiling of Drug Response in Human Gastric
698 Carcinoma by Immunoaffinity and IMAC Methods with Total Proteome Analysis. *Proteomes* **3**,
699 160–183 (2015).
- 700 42. Stokes, M. P. *et al.* PTMScan Direct: Identification and Quantification of Peptides from
701 Critical Signaling Proteins by Immunoaffinity Enrichment Coupled with LC-MS/MS. *Mol. Cell.*
702 *Proteomics* **11**, 187–201 (2012).
- 703 43. Olsen, J. V. *et al.* Parts per Million Mass Accuracy on an Orbitrap Mass Spectrometer via
704 Lock Mass Injection into a C-trap. *Mol. Cell. Proteomics* **4**, 2010–2021 (2005).
- 705 44. Eng, J. K., McCormack, A. L. & Yates, J. R. An approach to correlate tandem mass
706 spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass*
707 *Spectrom.* **5**, 976–989 (1994).
- 708 45. Huttlin, E. L. *et al.* A Tissue-Specific Atlas of Mouse Protein Phosphorylation and

709 Expression. *Cell* **143**, 1174–1189 (2010).

710 46. Villén, J., Beausoleil, S. A., Gerber, S. A. & Gygi, S. P. Large-scale phosphorylation
711 analysis of mouse liver. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 1488–93 (2007).

712 47. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for
713 assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–30 (2014).

714 48. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion
715 for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

716 49. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Systematic and integrative analysis of
717 large gene lists using DAVID bioinformatics resources. *Nat. Protoc.* **4**, 44–57 (2009).

718 50. Huang, D. W., Sherman, B. T. & Lempicki, R. A. Bioinformatics enrichment tools: paths
719 toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* **37**, 1–13
720 (2009).

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746 application related to this work. RNA-seq data are deposited in GEO, accession number
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748
749 **Figure 1: Microenvironmental Nlgn3 is necessary for pediatric GBM growth**
750 **a**, Left: Schematic representation of GFP and luciferase-labeled patient-derived pediatric
751 glioblastoma cells (SU-pcGBM2) orthotopically xenografted into the premotor cortex of
752 immunodeficient WT or *Nlgn3* KO mice Right: Representative IVIS images in WT or *Nlgn3* KO
753 mice at 3 months. Heat map represents degree of photon emission. **b**, Representative confocal
754 micrographs illustrating tumor burden at 6 months; GFP+ tumor cells (green) and myelin basic

755 protein (MBP, red). **c-f**, Fold change in total photon flux of SU-pcGBM2 xenografts in
756 identically manipulated WT;NSG (n=11) and *Nlgn3*^{y/-};NSG (n=14) mice shown at 6, 12, 18, and
757 24 weeks post-xenograft. Experiment was replicated in five independent cohorts of mice and
758 data are shown combined. Each dot represents one mouse. P values indicated on graphs, Mann-
759 Whitney test. Data shown as mean +/- s.e.m.

760

761 **Figure 2: Microenvironmental Nlgn3 is necessary for DIPG and adult GBM growth, but**
762 **not a breast cancer brain metastasis**

763 Patient-derived DIPG cells (SU-DIPG-VI, from a pontine DIPG tumor or SU-DIPG-XIII FL,
764 from a frontal lobe DIPG metastasis), adult glioblastoma cells, (SU-GBM035 cells) or HER2+
765 breast cancer brain metastasis cells (DF-BM354 cells) expressing firefly luciferase were
766 xenografted into the orthotopic location of derivation in *Nlgn3*^{y/+};NSG and *Nlgn3*^{y/-};NSG mice. **a**,
767 Representative IVIS images of WT (left) and *Nlgn3* KO (right) mice at 6 weeks following DIPG
768 (SU-DIPG-VI) xenografting. **b**, Representative confocal images at the level of the pons in *Nlgn3*
769 WT (left) and *Nlgn3* KO (right) mouse brains (MBP, red) bearing DIPG xenografts (green) at 6
770 weeks post-xenografting. **c-e, g**, Fold change in photon emission in SU-DIPG-VI (**c**), SU-DIPG-
771 XIII FL (**d**), SU-GBM035 (**e**) and DF-BM354 (**g**) xenografts in WT and *Nlgn3* knockout (KO)
772 mice at 6 weeks (**c,d**) or 4 weeks (**e,g**) after xenografting. **f**, Representative IVIS images of WT
773 (left) and *Nlgn3* KO (right) mice at 4 weeks following breast cancer brain metastasis (DF-
774 BM354) xenografting. *n*=3-4 mice per group. Each dot represents one mouse. P values indicated
775 on graphs, Student's two-tailed t-test. Data shown as mean+/-s.e.m.

776

777 **Figure 3. Signaling consequences of NLGN3 in glioma**

778 **a**, Schematic illustration of signaling pathways activated following NLGN3 exposure; red circles
779 represent proteins exhibiting increased phosphorylation following NLGN3 exposure. **b**,
780 Phosphorylation ratio of NLGN3-exposed vs. control SU-pcGBM2 cell lysates after 5-minute or
781 30-minute exposure to NLGN3 as assessed by phospho-antibody array. **c**, Proliferation index of
782 SU-pcGBM2 cells exposed to plain media (aCSF), aCSF + 5nM FAK inhibitor (FAKi), NLGN3
783 (50nM), or NLGN3 (50nM) + 5nM FAK inhibitor (NLGN3 + FAKi), (n=3 wells per condition;
784 P and F values indicated on graph; One-way ANOVA, Data shown as mean+/-s.e.m.). **d**,
785 Representative confocal images of SU-pcGBM2 cells exposed to NLGN3 in the absence (left) or
786 presence of FAK inhibitor (right). Vimentin, green; phospho-FAK, white; DAPI, blue; EdU, red,
787 scale bar = 50 μ m. **e**, Representative Western blot demonstrating increased phosphorylation of
788 FAK (Tyr861) after 0, 5 or 15-minute exposure to NLGN3. **f**, Representative Western blots
789 demonstrating AKT phosphorylation in SU-pcGBM2 cells exposed to plain media (aCSF), aCSF
790 + 5nM FAK inhibitor (FAKi), NLGN3 (50nM), or soluble NLGN3 (50nM) + 5nM FAK
791 inhibitor, (NLGN3 + FAKi).

792

793 **Figure 4: Activity-regulated neuroligin-3 secretion from both neurons and OPCs**

794 **a**, Schematic illustration of proteolytic cleavage of neuroligin-3 at the membrane. **b**,
795 Representative Western blot showing full length Nlgn3 in slice lysate and cleaved Nlgn3 N-
796 terminal ectodomain fragment in conditioned media (CM). **c**, Representative Western blot
797 illustrating secreted Nlgn3 levels in CM from *Thy1::ChR2* cortical slices that have been
798 optogenetically stimulated, WT slices at baseline neuronal activity, and WT slices in the
799 presence of 1 μ M tetrodotoxin (TTX). **d**, *Nlgn3* RNA expression (FPKM values) in various cell
800 types; (data from Brain-seq Barres dataset⁴) **e**, *CamKIIa::Cre^{ER};Nlgn3^{fl/fl}* mouse model; Western

801 blot analysis and quantification of CM generated from *CamKIIa::Cre^{ER};Nlgn3^{fl/fl}* or *Nlgn3^{fl/fl}* (no
802 Cre) cortical slices at baseline neuronal activity. **f**, *PDGFRa::Cre^{ER};Nlgn3^{fl/fl}* mouse model;
803 Western blot analysis and quantification of CM from *PDGFRa::Cre^{ER};Nlgn3^{fl/fl}* or *Nlgn3^{fl/fl}* (no
804 Cre) cortical slices at baseline neuronal activity. Note that the Western blots in panels e and f
805 were run on the same gel. P values indicated on graphs; Student's two-tailed t-test. Data are
806 shown as mean+/-s.e.m.

807

808 **Figure 5: ADAM10 is the primary sheddase involved in neuroligin-3 cleavage**

809 **a**, Representative Nlgn3 Western blot analyses of CM generated from optogenetically stimulated
810 *Thy1::ChR2* slices in the presence and absence of specific protease inhibitors as indicated. **b**,
811 Nlgn3 Western blot analyses of optogenetically stimulated *Thy1::ChR2* cortical slice
812 homogenates +/- ADAM10 and MMP2/9 inhibitors. **c**, Proliferation indices of SU-pcGBM2 cells
813 exposed to plain media (aCSF) or CM in the present of absence of pan-MMP inhibitor (BAT,
814 left) or ADAM10 inhibitor (right). n=3 wells per condition. **d**, Representative confocal
815 micrographs of SU-pcGBM2 cells (EdU, red; DAPI, blue) exposed to CM +/- ADAM10
816 inhibitor; Scale bar = 50µm. **e**, *MMP9^{-/-}* mouse model; Nlgn3 Western blot analysis and
817 quantification illustrating secreted Nlgn3 levels from WT and *MMP9^{-/-}* cortical slices CM at
818 baseline neuronal activity, expressed as ratio of Nlgn3 levels in *MMP9* KO to WT slice CM. **f**,
819 *CamKIIa::Cre^{ER};ADAM10^{fl/fl}* mouse model; Nlgn3 Western blot analysis and quantification of
820 CM from *CamKIIa::CreER;ADAM10^{fl/fl}* and *ADAM10^{fl/fl}* (no cre) cortical slices at baseline
821 neuronal activity expressed as ratio of Nlgn3 levels in Cre+ to Cre- slice CM. **g**, as in f but with
822 *PDGFRa::Cre* driver mouse. **h**, ADAM10 Western blot analysis and quantification of cortical

823 slice CM in the present or absence of TTX. $n = 3$ biological replicates (e-h). P values shown in
824 graphs; one-way ANOVA (c); two-tailed Student's t-test (e-h). Data shown as mean \pm s.e.m.

825

826 **Figure 6: ADAM10 inhibition blocks glioma growth**

827 **a**, Cell viability of SU-pcGBM2 cells exposed to ADAM10 inhibitor (GI254023X, 10nM-2 μ M)

828 or vehicle control at 24-, 48-, and 72-hours, ($n = 3$ wells/condition) **b**, Proliferation index of SU-

829 pcGBM2 cells exposed to GI254023X (0-2 μ M; ($n = 3$ wells/condition) **c**, Neurosphere formation

830 assay in SU-pcGBM2 cells in the presence of GI254023X (0-2 μ M; ($n = 10$ wells/condition)) **d-f**,

831 Growth (change in photon emission) of orthotopic xenografts following systemic administration

832 of GI254023X or vehicle control for SU-pcGBM2 (d), SU-DIPG-VI (e), SU-DIPG-XIX (f)

833 xenografts ($n = 4-7$ mice/group.) **g**, *In vivo* proliferation index of SU-pcGBM2 cells in vehicle

834 control and ADAM10i treated mice ($n = 4$ /group). **h**, Representative confocal images (Ki67⁺,

835 green; human nuclear antigen, HNA⁺ red) of vehicle-treated or ADAM10i-treated mice. Scale

836 bars=50 μ m. **i**, Brain and tissue penetration of INCB7839; ($n = 3$ mice/data point). **j**, Growth

837 (change in photon emission) of SU-pcGBM2 xenografts following systemic administration of

838 INCB7839 or vehicle control; ($n = 4-5$ mice/group.) P values indicated in graphs. n.s. = not

839 significant. one-way ANOVA (a,b), χ^2 test (c), Student's two-tailed t-test (d,e,j); Mann-Whitney

840 (f,g). Each dot represents one mouse. Data are shown as mean \pm s.e.m except in c) error bars

841 are \pm confidence intervals and in i) error bars are st. dev.

842

843 *Extended Data Figure Legends:*

844 **Extended Data Figure 1: Engraftment is equivalent in Nlgn3 knockout and wild type mice**

845 **a**, *In vivo* bioluminescence imaging of SU-pcGBM2 xenografts two weeks following xenograft

846 in WT;NSG (“WT”; left) or *Nlgn3* KO;NSG mice (“KO”; right). The heat map superimposed
847 over the mouse heads represents the degree of photon emission by cells expressing firefly
848 luciferase. **b**, Absolute flux of pHGG cells in identically manipulated WT;NSG (n=11) and
849 *Nlgn3* KO;NSG (n=14) mice, measured by IVIS imaging two weeks post-xenograft illustrates no
850 significant difference in tumor engraftment. Mann-Whitney test, n.s. = not significant ($P > 0.05$).
851 Data are shown as mean +/- s.e.m.

852 **Extended Data Figure 2: Microenvironmental Nlgn3 is necessary for pediatric GBM**
853 **growth.** Data from main Figure 1 shown on the same axis (**a**) and with each independent cohort
854 color coded for comparison of littermates (**b**). Data illustrate growth of pHGG (SU-pcGBM2)
855 xenografts in identically manipulated WT;NSG (black dots, n=11) and *Nlgn3*^{y/-};NSG (grey dots,
856 n=14) mice, measured by IVIS imaging (fold change in total photon flux) and shown at 6, 12, 18,
857 and 24 weeks post-xenograft. Data were replicated in five independent cohorts (litters) of mice
858 xenografted with different cell preparations on different days and the data from these five
859 biological replicates are shown combined with each cohort color-coded (i.e. littermates are
860 shown in the same color). ** $P < 0.01$, **** $P < 0.0001$, Mann-Whitney test. Data are shown as
861 mean +/- s.e.m.

862

863 **Extended Data Figure 3: Nlgn3 is necessary for the full proliferative effect of brain slice**
864 **conditioned media**

865 Schematic representation of active conditioned media generation (left). Proliferation index
866 (EdU+ and DAPI co-positive nuclei/total DAPI+ nuclei) of pHGG cells (SU-pcGBM2) exposed
867 to plain media (aCSF), optogenetically stimulated *Nlgn3* WT cortical slice CM, or

868 optogenetically stimulated *Nlgn3* KO cortical slice CM (one-way ANOVA, $F = 30.8$, $P < 0.001$).

869 * $P < 0.05$, *** $P < 0.001$

870

871 **Extended Data Figure 4: Neuroligin-1 does not stimulate glioma proliferation**

872 Proliferation index of patient-derived pediatric cortical glioblastoma (SU-pcGBM2) cells as

873 measured by EdU incorporation 24 hrs after *in vitro* exposure to recombinant human neuroligin-

874 1 (NLGN1) at concentrations ranging from 0-100 nM. $n = 3$ wells per condition. Data are

875 presented as the mean EdU+/DAPI+ fraction \pm s.e.m. n.s. = not significant ($p > 0.05$) by one-

876 way ANOVA.

877

878 **Extended Data Figure 5: Gene expression changes induced by neuroligin-3 in glioma**

879 **a**, Scatterplot showing SU-pcGBM2 ($n=2$) gene expression changes following 16 hours of

880 treatment with vehicle ($\sim 1\%$ DMSO) or NLGN3 (100 nM). The x-axis shows mean FPKM value

881 in vehicle treated cells and the y-axis shows $\log_2(\text{fold-change})$ of NLGN3 over vehicle. Points

882 shown in red represent genes showing statistically significant change (adjusted p value < 0.1 ,

883 Benjamini-Hochberg). **b**, Gene Ontology Biological Processes enriched in significantly

884 upregulated genes with NLGN3 treatment, as identified by DAVID^{49,50} with p values shown with

885 Benjamini-Hochberg adjustment. Genes associated with each GO BP term shown in (c).

886

887 **Extended Data Figure 6: Efficiency of Cre driver mice**

888 Recombination rate of inducible Cre driver models 7 days after treatment with tamoxifen

889 (100mg/kg for 5 days) in *Rosa26::tdTomato*^{lox-stop-lox} reporter mice. **a**, To assess the neuron-

890 specific *CamKIIa::CreER* Cre driver, recombination efficiency was quantified as percent of

891 NeuN⁺ neurons that co-express tdTomato⁺ in the cortex of either *CamKIIa*:CreER⁻ or
892 *CamKIIa*:CreER⁺ mice 7 days following completion of tamoxifen administration. **b**, To assess
893 the OPC-specific Cre driver *PDGFRa*:CreER, recombination efficiency was quantified as
894 number PDGFRa⁺ OPCs that co-express tdTomato in the cortex of either *PDGFRa*:CreER⁻ or
895 *PDGFRa*:CreER⁺ mice. n = 3 mice per group. Data are shown as mean +/- s.e.m.

896

897 **Extended Data Figure 7: NLGN3 shedding from glioma cells is regulated by NLGN3**
898 **exposure and is mediated by ADAM10**

899 **a**, NLGN3 Western blot illustrating neuroigin-3 secreted into CM from optogenetically
900 stimulated *Thy1::ChR2*; NSG cortical slices (ChR2 stim slice) or SU-DIPGXIII xenograft-
901 bearing *Thy1::ChR2*; NSG cortical slices (ChR2 stim slice with xenograft). **b**, NLGN3 western
902 blot illustrating neuroigin-3 secreted into CM from wild type brain slices (WT), WT brain slices
903 bearing xenografts of adult GBM SU-GBM035 (WT + xeno), or from *Nlgn3* knockout brains
904 bearing SU-GBM035 xenografts (*Nlgn3* KO + xeno) in the absence (left 3 lanes) or presence
905 (right 3 lanes) of 200 nM ADAM10 inhibitor GI254023X (+ADAM10i). **c**, NLGN3 western blot
906 illustrating glioma cell secretion of NLGN3 in vitro at baseline media conditions (aCSF),
907 following exposure to recombinant NLGN3 with subsequent washing (NL3), at baseline media
908 conditions in the presence of ADAM10 inhibitor GI254023X (aCSF + AD10i) or following
909 NLNG3 exposure in the presence of ADAM10 inhibitor (NL3+AD10i). **d**, mRNA expression
910 levels of *ADAM10* in primary tumor and cultures of DIPG by RNA-seq with values reported as
911 FPKM^{16,26} (left) and in 493 individual adult glioblastoma samples from TCGA²⁷ (right). Values
912 are reported as robust multi-array averages (RMA; right).

913

914 **Extended Data Figure 8**

915 **a**, Spheroid invasion index of SU-DIPGVI cells exposed to ADAM10i (0-5 μ M) at 24-, 48- and
916 72-hours expressed as the diameter of the sphere of glioma cells relative to the initial diameter at
917 time 0-hours. **b**, Extreme limiting dilution assay (ELDA) data presented in main Figure 6c re-
918 plotted here as a log fraction plot with the slope of the solid line representing the log-active cell
919 fraction and confidence intervals shown as dotted lines. SU-pcGBM2 cells treated with
920 ADAM10 inhibitor GI254023X at 0.5 μ M (black), 1 μ M (red) or 2 μ M (green), with vehicle
921 (DMSO) control (royal blue) or no DMSO (cyan) and analyzed for neurosphere formation at two
922 weeks.

923

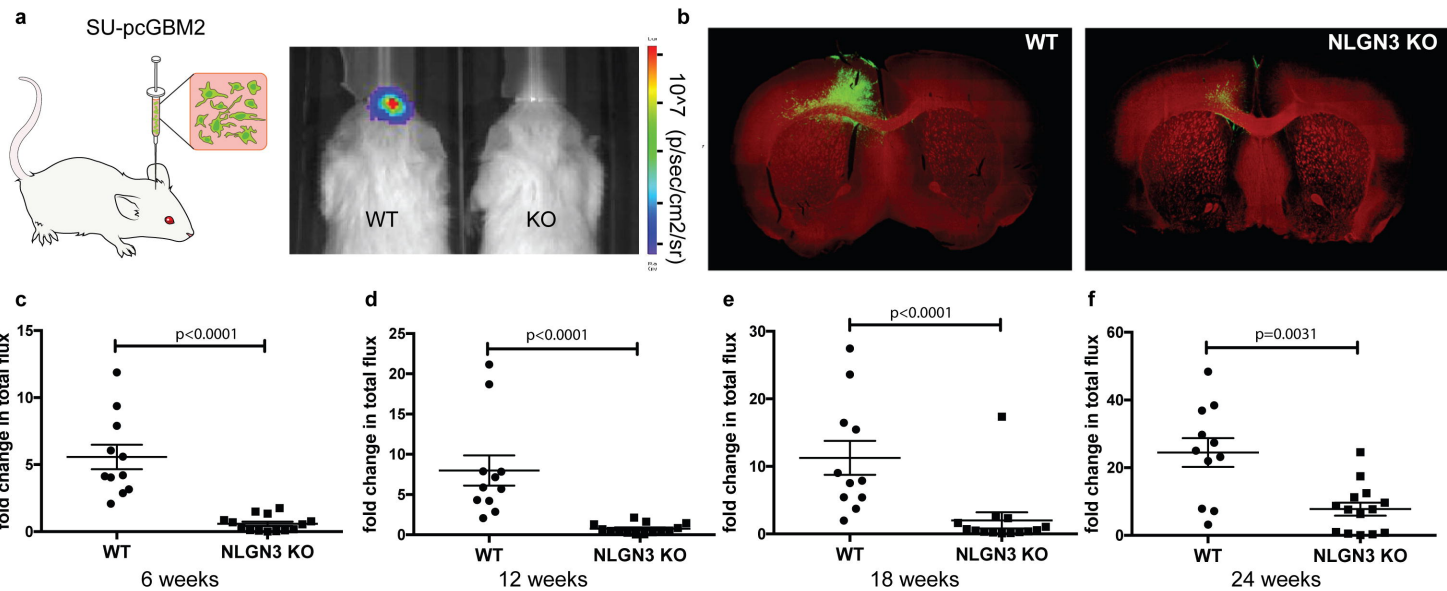
924 **Extended Data Figure 9: Brain Penetration of XL-784**

925 Brain tissue and plasma levels of XL-784 at various time points following a single 50 mg/kg i.p.
926 dose in NSG mice as assessed by liquid chromatography/tandem mass spectrometry (LC-
927 MS/MS). n = 3 mice at each data point. Data are shown as mean +/- st.dev.

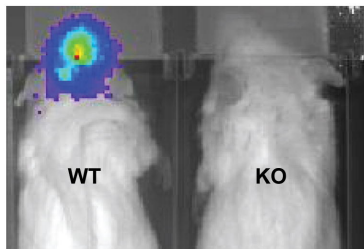
928

929 **Extended Data Table 1: Brain penetration of ADAM10 inhibitor GI254023X**

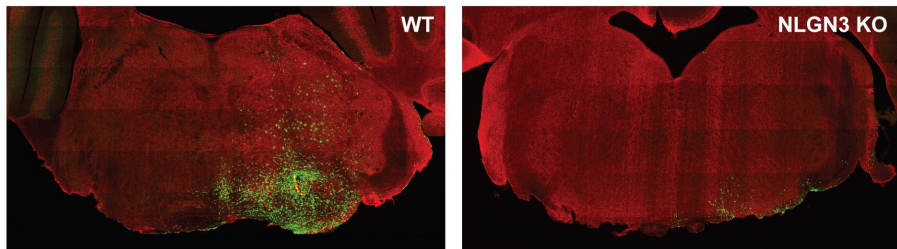
930 A single 100 mg/kg dose was delivered intra-peritoneally in NSG mice, and tissue samples
931 collected 30-minutes later for analysis using liquid chromatography/tandem mass spectrometry
932 (LC-MS/MS). Brain tissue concentrations show reasonable penetration across the blood brain
933 barrier, achieving 2-4 μ M concentration of drug at this time point.



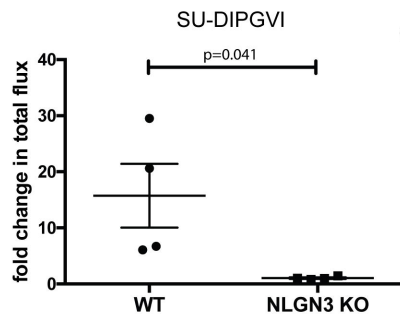
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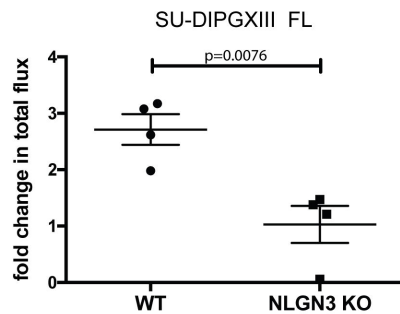
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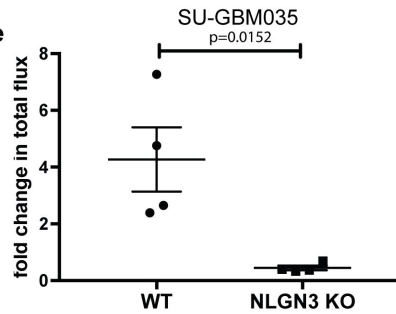
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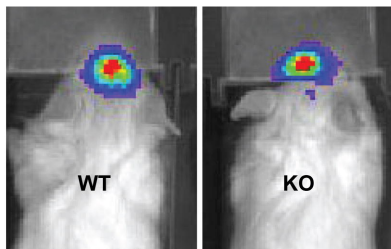
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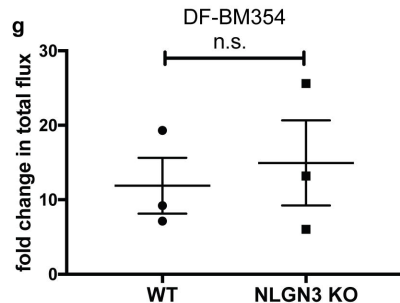
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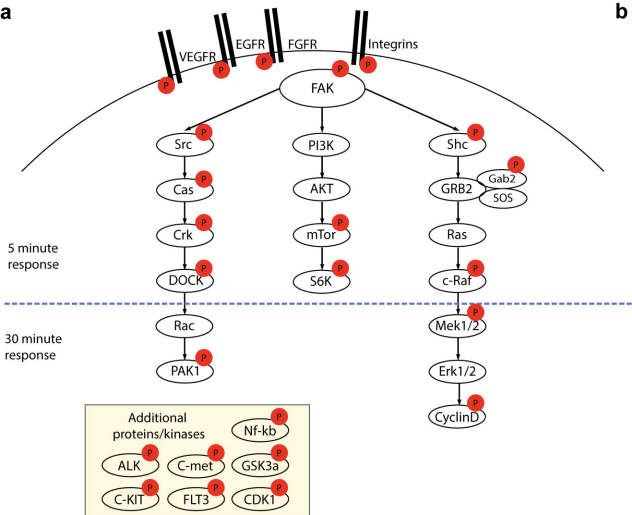


f



g





b

5 minute phosphorylation	ratio of fold change	30 minute phosphorylation	ratio of fold change
LYN	2.72	NFKB1	4.13
PLCG2	2.21	CCND1	3.87
FGFR1	1.97	FLT3	3.37
SHC1	1.88	FAK2	2.13
SYK	1.88	MEK1	2.06
NFKB1	1.86	JUN	1.91
TP53	1.79	EFNB1/B2/B3	1.87
DOK1	1.79	CDK1	1.80
AURKA	1.78	PLD2	1.65
EGFR	1.76	PTPN11	1.62
RAF1	1.67	CDH5	1.56
RPS6KB1	1.58	ELK1	1.52
PAK2	1.55	GSK3A	1.49
PTK2B	1.53	PAK1	1.45
MET	1.52	ETK	1.44
JUN	1.52	ITGB3	1.36
VEGFR2	1.51	CRK	1.30
PLD1	1.47	TP53	1.30
GAB2	1.47	PXN	1.29
TNK2	1.47	MTOR	1.28
CCNE1	1.41		
ITGB3	1.39		
LCK	1.37		
BCAR1	1.35		
PXN	1.31		

