

1 **TITLE:**

2 A machine learning classifier trained on cancer transcriptomes detects NF1 inactivation signal in  
3 glioblastoma  
4

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35 **KEYWORDS:**

36 Neurofibromatosis Type I, Glioblastoma, Machine Learning, Cancer, NF1 Inactivation, Classifier  
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48 **ABSTRACT (309/350):**

49 *Background*

50 We have identified molecules that exhibit synthetic lethality in cells with loss of the neurofibromin 1  
51 (*NF1*) tumor suppressor gene. However, recognizing tumors that have inactivation of the *NF1* tumor  
52 suppressor function is challenging because the loss may occur via mechanisms that do not involve  
53 mutation of the genomic locus. Degradation of the *NF1* protein, independent of *NF1* mutation status,  
54 phenocopies inactivating mutations to drive tumors in human glioma cell lines. *NF1* inactivation may  
55 alter the transcriptional landscape of a tumor and allow a machine learning classifier to detect which  
56 tumors will benefit from synthetic lethal molecules.

57

58 *Results*

59 We developed a strategy to predict tumors with low *NF1* activity and hence tumors that may  
60 respond to treatments that target cells lacking *NF1*. Using RNAseq data from The Cancer Genome Atlas  
61 (TCGA), we trained an ensemble of 500 logistic regression classifiers that integrates mutation status with  
62 whole transcriptomes to predict *NF1* inactivation in glioblastoma (GBM). On TCGA data, the classifier  
63 detected *NF1* mutated tumors (test set area under the receiver operating characteristic curve (AUROC)  
64 mean = 0.77, 95% quantile = 0.53 – 0.95) over 50 random initializations. On RNA-Seq data transformed  
65 into the space of gene expression microarrays, this method produced a classifier with similar  
66 performance (test set AUROC mean = 0.77, 95% quantile = 0.53 – 0.96). We applied our ensemble  
67 classifier trained on the transformed TCGA data to a microarray validation set of 12 samples with  
68 matched RNA and *NF1* protein-level measurements. The classifier's *NF1* score was associated with *NF1*  
69 protein concentration in these samples.

70

71 *Conclusions*

72 We demonstrate that TCGA can be used to train accurate predictors of NF1 inactivation in GBM. The  
73 ensemble classifier performed well for samples with very high or very low NF1 protein concentrations  
74 but had mixed performance in samples with intermediate NF1 concentrations. Nevertheless, high-  
75 performing and validated predictors have the potential to be paired with targeted therapies and  
76 personalized medicine.

77

## 78 **BACKGROUND:**

79 Genomic tools allow investigators to devise therapies targeting specific molecular abnormalities in  
80 tumors. One such alteration is the loss of neurofibromin 1 (NF1), an important tumor suppressor that  
81 regulates the activity of *RAS* GTPases [1,2]. Heterozygous mutation or deletion of *NF1* causes  
82 neurofibromatosis type 1 (NF), one of the most frequently inherited genetic disorders [3]. NF patients  
83 often develop plexiform neurofibromas (PNs), benign nerve tumors for which the only therapy is  
84 surgery. However, resection is often impossible due to the tumor's intimate association with peripheral  
85 and cranial nerves [4]. PNs can transform to malignant peripheral nerve sheath tumors (MPNSTs), which  
86 are chemo- and radiation-resistant sarcomas with a dismal 20% 5-year survival [5]. In addition, patients  
87 with NF are susceptible to a broad spectrum of other tumors including low-grade/pilocytic  
88 astrocytomas, pheochromocytomas, optic nerve gliomas, and juvenile myelomonocytic leukemias [6].  
89 Many aggressive non-NF associated (sporadic) tumors have recently been shown to harbor *NF1*  
90 mutations, including glioblastoma (GBM), neuroblastoma, melanoma, thyroid, ovarian, breast, and lung  
91 cancers [7]. Therefore, somatic and inherited loss of *NF1* function is emerging as a driver of tumors from  
92 different organ sites.

93 Several groups including our own have been working to develop therapeutic approaches to target  
94 tumors with loss of NF1. Previously, our lab developed a high throughput approach using yeast and  
95 mammalian screening platforms to identify tool compounds and drug targets for cancer cells in which

96 NF1 loss drives tumor formation. Our pipeline identified small molecules that selectively kill or stop the  
97 growth of MPNST cells carrying a mutation in *NF1* or yeast lacking the *NF1* homolog *IRA2* [8]. We also  
98 developed an assay in yeast to identify the targets of our lead tool compounds and found that one of  
99 these compounds (UC-1) shares a mechanism (phosphorylation of RNA Pol II CTD Ser2/5) with  
100 experimental drugs in clinical trials [8]. UC-1 impacts CTD phosphorylation, which is regulated by the  
101 CTD kinase Ctk1, the yeast homolog of human Cdk9. We showed that deletion of *CTK1* was synthetic  
102 lethal with loss of the yeast *NF1* homolog *IRA2*. Furthermore, we have found that inhibitors of this  
103 process (dinaciclib, SNS-032) can inhibit other types of RAS-dysregulated tumor cells [9].

104 However, relying on genetic data alone to identify tumors that may be susceptible to therapies  
105 targeting NF1 loss may leave a proportion of potentially actionable tumors unrecognized. NF1 tumor  
106 suppressor activity can be lost via mutation of the genomic locus, proteasome-mediated degradation,  
107 inhibition by miRNA, *de novo* insertion of an ALU element, and C→U editing of the *NF1* mRNA [10–14].  
108 This complexity presents challenges when trying to identify tumors that will benefit from molecules that  
109 exert synthetic lethality with dysregulation of *NF1/RAS* pathways.

110 The Cancer Genome Atlas (TCGA) has released a large volume of data on several cancer tissues  
111 measured on a variety of genomic platforms. In the present study, we leverage TCGA GBM RNAseq  
112 expression data with matched mutation calls to construct a classifier capable of identifying an NF1  
113 inactivation signature. This strategy sidesteps the problem of functional characterization of mutations  
114 by evaluating a regulator's downstream gene expression activity. We applied this signature to predict  
115 NF1 inactivation in a cohort of biobanked GBMs. In general, this approach can be translatable to any  
116 gene producing measurable downstream transcriptome-wide effects.

117

## 118 **METHODS:**

119 *The Cancer Genome Atlas Data*

120 We downloaded RNAseq and mutation data from TCGA Pan Cancer project from the UCSC Xena  
121 data portal [15] and subset each dataset to only the GBMs [16]. The data consists of 607 GBMs; of which  
122 291 have mutation calls, 172 have RNAseq measurements, and 149 have both RNAseq and mutation  
123 calls. Of these 149 samples, 15 have inactivating *NF1* mutations (10.1%) and were used as gold standard  
124 positives in building the classifier (Supplementary Table S1). Additionally, to reduce dimensionality while  
125 avoiding unexpressed and invariant genes, we subset to the top 8,000 most variably expressed genes by  
126 median absolute deviation. We z-scored all gene expression measurements. This resulted in final matrix  
127 with dimension 149 samples by 8,000 genes. For use in platform independent predictions, we used  
128 Training Distribution Matching (TDM) to transform the TCGA RNAseq data to match a microarray  
129 expression distribution [17].

130

### 131 *Hyperparameter optimization*

132 Using the GBM RNAseq data, we trained logistic regression classifiers with an elastic net penalty  
133 using stochastic gradient descent to detect tumors with *NF1* inactivation. We identified high-performing  
134 alpha and L1 mixing parameters using 5-fold cross validation ensuring balanced membership of *NF1*  
135 mutations in each fold. Briefly, alpha controls how weight penalty and the L1 mixing parameter tunes  
136 the amount of test set regularization by controlling the sparsity of the features. An L1 mixing parameter  
137 value of zero corresponds to the L2 penalty and a value of one corresponds to the L1 penalty, with L1  
138 bringing a sparser solution. We used python 3.5.1 and Sci-kit Learn for machine learning  
139 implementations [18].

140

### 141 *Ensemble classifier construction and application to the validation set*

142 After selecting optimal hyperparameters, we constructed 500 classifiers that would compose our  
143 ensemble model. Specifically, across 100 different random initializations, we subset the full TCGA GBM  
144 data into 5 folds and trained a single classifier for each training fold.

145 We borrowed terminology from the epidemiology field to describe data partitioning. We trained our  
146 models on a “training” partition and assessed model performance on a “test” partition, which refers to  
147 the held out cross-validation fold. The independent “validation set” refers to the GBM dataset  
148 generated in a different lab (see Figure 1A).

149 Because of the small number of gold standard positive training examples, we were concerned about  
150 the stability of our model solutions. Therefore, we constructed an ensemble classifier from the 500  
151 models. Specifically, we assigned each classifier a weight using the specific randomization’s “test set”  
152 cross-validation AUROC. Lastly, for the final *NF1* inactivation prediction, we used the mean of the  
153 weighted predictions across all iterations as the *NF1* inactivation prediction. We applied this ensemble  
154 classifier to the validation set in which NF1 protein levels were directly measured.

155

#### 156 *Validation Sample Acquisition*

157 Thirteen flash-frozen, de-identified GBM samples were obtained from the Maine Medical Center  
158 Biobank. Samples were received on dry ice and stored at -80°C until isolation of DNA/RNA/protein. To  
159 isolate DNA, tumor fragments of approximately 20 mg in mass were harvested on an aluminum block  
160 pre-chilled on dry ice. Samples were then immediately transferred to a mortar and pestle containing a  
161 small volume of liquid nitrogen. The fragments were pulverized in the mortar and pestle, and the liquid  
162 nitrogen was allowed to evaporate. Next, samples were immediately processed with a  
163 DNA/RNA/Protein Purification Plus kit (Norgen Biotek) following the standard operating protocol for  
164 animal tissue. DNA concentration and quality were assessed using an ND-1000 (Nanodrop), a Qubit  
165 Fluorometer (Thermo Scientific), and a Fragment Analyzer (Advanced Analytical Technologies). To

166 isolate RNA, -80 C tumor fragments were placed in 5-10 volumes of RNAlater-ICE Frozen Tissue  
167 Transition Solution (Ambion) and placed at -20°C until RNA extraction with a mirVana miRNA isolation  
168 kit, without phenol, following the standard operating protocol (Thermo Scientific). Samples were  
169 homogenized using a manual homogenizer in the presence of mirVana lysis buffer. RNA concentration  
170 and quality were determined using a Qubit Fluorometer (Thermo Scientific) and a Fragment Analyzer  
171 (Advanced Analytical Technologies). To isolate protein, small tumor fragments were pulverized and lysed  
172 in approximately 3 volumes of ice-cold radioimmunoprecipitation assay (RIPA) buffer (150 mM sodium  
173 chloride, 1% v/v nonidet P40, 0.5% w/v sodium deoxycholate, 0.05% w/v sodium dodecyl sulfate, 50 mM  
174 Tris pH 8.0) containing 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM  
175 phenylmethylsulfonyl fluoride, and 1X protease inhibitor cocktail (0.1 µg/mL leupeptin, 100 µM  
176 benzamidine HCl, 1 µM aprotinin, 0.1 µg/mL soybean trypsin inhibitor, 0.1 µg/mL pepstatin, 0.1 µg/mL  
177 antipain). Samples were passed through a 25 ½ g needle and subsequently sonicated on ice to promote  
178 efficient lysis and DNA shearing. After a 30 minute incubation on ice, lysates were cleared by  
179 centrifuging at 16100 x g for 20 minutes. HEK293T, U87-MG, and U87-MG cells treated with 1  
180 micromolar bortezomib (Selleckchem) and 10 micromolar MG132 (Selleckchem) were also prepared in  
181 RIPA buffer. Protein samples were stored at -80°C until analysis.

182

### 183 *RNA Microarray*

184 After RNA isolation and QC, samples were labeled for the GeneChip Human Transcriptome Array 2.0  
185 (HTA 2.0, Affymetrix). Labeling was performed with Affymetrix Proprietary DNA Label (biotin-linked)  
186 using a WT Plus Kit (Affymetrix) provided with the HTA 2.0, following the standard operating protocol for  
187 HTA 2.0, including PolyA controls. Hybridization, washing, and staining were performed with the WT Plus  
188 Kit, following the standard operating protocol for HTA 2.0. Washing and staining were performed using a

189 GeneChip Fluidics 450. Scanning was performed with a GeneChip Scanner 3000. These data were  
190 deposited in the Gene Expression Omnibus under accession GSE85033.

191

### 192 *Validation Sample Processing*

193 We applied a quality control pipeline [19] to all CEL files generated by the HTA 2.0. All validation  
194 samples passed processing quality control, which included an inspection of spatial artifacts, MA plots,  
195 probe distributions, and sample comparison boxplots. We summarized transcript intensities using  
196 robust multi-array analysis (RMA) [20]. We determined batch normalization was unnecessary after a  
197 guided principal components analysis (gPCA) using sample processing date and array plate ID as  
198 potential batch effect confounders [21]. Lastly, we collapsed HTA2.0 transcripts into gene level  
199 measurements using the ``collapseRows()`` function with the “maxmean” method from the R package  
200 WGCNA [22]. We used the `pd.hta.2.0` platform design file (version 3.12.1) and the Bioconductor package  
201 “`hta20sttranscriptcluster.db`” (version 8.3.1) to map manufacturer transcript IDs to genes. We  
202 performed all preprocessing steps using R version 3.2.3.

203

### 204 *Western Blotting*

205 Prior to sodium dodecyl sulfate polyacrylamide gel electrophoresis, protein sample concentration  
206 was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific). Protein samples were prepared  
207 with 1X Laemmli sample buffer (50 mM Tris pH 6.8, 0.02% w/v bromophenol blue, 2% w/v SDS, 10% v/v  
208 glycerol, 1% v/v beta-mercaptoethanol, 12.5 mM EDTA) and 50 µg of tumor protein. Volumes were  
209 normalized with RIPA buffer including the protease/phosphatase inhibitors described above. SDS-PAGE  
210 was performed using a 4-15% Mini-PROTEAN TGX gel (Bio-Rad) for 1 hour at 120V. The samples were  
211 then transferred to a nitrocellulose membrane for 2 hours and 45 minutes at 400 mA in cold transfer  
212 buffer (384 mM glycine, 50 mM Tris, 20% methanol, 0.005% w/v sodium dodecyl sulfate. Following this,



213 the blots were then blocked in 5% w/v BSA or 5% w/v nonfat dry milk in Tris-buffered saline (137 mM  
214 NaCl, 2.7 mM KCl, 19 mM Tris, 0.05% v/v Tween 20, pH 7.4) for 25 minutes. Immunoblotting was  
215 performed with the following antibodies and conditions (vendor, species, diluent, dilution, incubation  
216 time, incubation temperature): anti-NF1 D7R7 (Cell Signaling, rabbit, 2% BSA, 1:1000, overnight, 4°C),  
217 anti-tubulin B-1-2-5 (Santa Cruz, mouse, 2% milk, 1:10000, 1 hour, RT), anti-EGFR D38B1 (Cell Signaling,  
218 rabbit, 2% milk, 1:1000-1:2000, 1h, RT), p-ERK ½ (p44/42 MAPK) #9101 (Cell Signaling, rabbit, 2% BSA,  
219 1:2000, overnight, 4°C), SUZ12 D39F6 #3737 (Cell Signaling, rabbit, 2% milk, 1:1000, overnight, 4°C).  
220 Anti-NF1 D7R7 was a kind gift from Cell Signaling Technologies, Inc.

221 The binding of the primary antibodies was detected by incubation with secondary antibodies goat  
222 anti-rabbit HRP 1:20000 or goat anti-mouse HRP 1:10000 (Jackson Immunoresearch Laboratories Inc.) at  
223 room temperature in 2% milk in TBST and detection of HRP activity using Pierce ECL Western Blotting  
224 substrate (Thermo Scientific), or in the case of *NF1*, SuperSignal West Femto Maximum Sensitivity  
225 Substrate (Thermo Scientific). The chemiluminescent signal was captured with MED-B medical x-ray film  
226 (Med X Ray Company Inc.). Between primary antibodies, the membrane was stripped twice for 10  
227 minutes at room temperature using a mild stripping buffer containing 1.5% w/v glycine, 0.1% w/v SDS,  
228 1% v/v Tween 20 at pH 2.2 (Abcam). One sample was eliminated due to low yield, and apparent  
229 degradation as determined by western blotting (all proteins examined were undetectable with the  
230 exception of tubulin, not shown). Densitometry was performed using Li-COR Image Studio Lite 5.0.  
231 Briefly, intensity measurements for *NF1* and tubulin were taken using equally-sized regions for all bands.  
232 The background was subtracted using the local median intensity from the left and right borders (size=2)  
233 of each measurement region. *NF1* values were divided by tubulin intensity to adjust for protein loading.  
234 All measurement ratios were then normalized by dividing values by the “U87+PI” measurement for each  
235 blot, respectively.

236

## 237 *Reproducibility of Computational Analyses*

238 We provide software with a permissive open source license to reproduce all computational analyses  
239 [23]. Ensuring a stable compute environment, we performed all analyses in a Docker image [24]. This  
240 image and source code can be used to freely confirm, modify, and build upon this work.

241

## 242 **RESULTS:**

### 243 *Classifier performance*

244 Using 5-fold cross validation across a parameter sweep, we identified optimal hyperparameters at  
245  $\alpha = 0.15$  and L1 mixing = 0.1 (Supplementary Figure S1). To assess model performance, we  
246 performed 100 random initializations of five-fold cross-validation. These models had mean test area  
247 under the receiver operating characteristic curve (AUROC) of 0.77 (95% Quantiles: 0.53 – 0.95) and a  
248 mean train AUROC of 0.997 (95% Quantile: 0.98 – 1.00) (Supplementary Figure S2). We repeated this  
249 procedure after TDM transformation (Supplementary Figure S3) and achieved comparable results with  
250  $\alpha = 0.15$  and l1 mixing = 0.1 (mean test AUROC = 0.77, 95% Quantiles: 0.51 – 0.96; mean train  
251 AUROC = 0.998, 95% Quantiles: 0.99 – 1.00) (Figure 1). Because the validation set was measured by  
252 microarray, we used the classifier trained on TDM transformed data to construct our ensemble  
253 classifier.

254

### 255 *Identification and characterization of NF1 deficient glioblastoma tumor samples*

256 We characterized NF1 protein concentrations as well as other molecules involved in RAS signaling in  
257 the 12 GBM samples (Figure 2A). Two samples (CB2, 3HQ) had no apparent NF1 protein. Eight other  
258 samples had similar or less NF1 signal than the U87-MG NF1-low control (H5M, LNA, YXL, VVN, R7K,  
259 TRM, UNY, W31). Two samples (PBH, RIW) had equal or greater NF1 than the positive control, U87-MG +  
260 proteasome inhibitors (preventing *NF1* degradation). We also observed variable EGFR content in these

261 samples, with non-existent to low levels (3HQ, YXL, R7K), or medium to large EGFR signal (CB2, H5M,  
262 PBH, LNA, YXL, VVN, RIW, TRM, UNY, W31). All GBM samples had high concentrations of phospho-  
263 ERK1/2 signal relative to cell line controls. Samples with increased phospho-ERK1/2 may have greater  
264 Ras pathway activation. This can be attributed to multiple factors, including increased EGFR expression  
265 and/or NF1 inactivation.

266 Our ensemble classifier predicted four samples to have NF1 inactivation (CB2, UNY, R7K, and 3HQ)  
267 and eight samples to be NF1 wildtype (W31, TRM, PBH, VVN, LNA, RIW, H5M, and YXL) (Figure 2B).  
268 Because two samples, (CB2 and H5M) were measured on both western blots (Figure 2C), we used the  
269 mean of their NF1 protein level across both experiments (Figure 2D).

270 One of the samples predicted to be NF1 inactive contains detectable NF1 protein (R7K), suggesting  
271 that this sample may have NF1 inactivation not detectable by assaying protein, have a different  
272 alteration that phenocopies NF1 loss, or is incorrectly predicted by the classifier. Conversely, there are  
273 three samples predicted to be NF1 wildtype that have low or undetectable protein (YXL, VVN, W31),  
274 which either indicates unknown elements that confound the detection of some NF1 dysregulated  
275 tumors or a classification error.

276

### 277 *Highly Contributing Genes*

278 We observed several genes that consistently contributed to the ensemble classifier performance  
279 (Figure 3). Since we applied several classifiers to the validation set as an ensemble, we took the sum of  
280 all classifier's gene weights across all 500 iterations to define these consistently contributing genes.  
281 Expression of genes such as *TXNIP*, *ARRDC4*, *ISPD*, *C10orf107*, and *DUSP18* appear to be predictive of  
282 intact NF1 signaling. Among the list of genes that appear to be expressed in tumors with loss of NF1  
283 function are *QPRT*, *ATF5*, *HUS1B*, *PEG10*, *HMGA2*, *RSL1D1*, and *NRG1*. A full list of positive and negative

284 weight genes that were two standard deviations beyond the gene weight distribution is provided in  
285 Supplementary Table S2.

286 We also performed over-representation analysis of the most influential genes in the classifier to  
287 identify gene ontology (GO) sets and pathways that may be predictive of NF1 status [25–28]  
288 (Supplemental Table S3). For high-weight genes predictive of intact NF1 signaling, we observed GO sets  
289 involved in plasma membrane-localized proteins (GO:0005886, GO:0071944, GO:0016324) and  
290 homeostasis (GO:0048871, GO:0001659, GO:0048873, GO:0031224), among others. Annotated  
291 pathways associated with genes from this dataset include hematopoietic stem cell differentiation,  
292 thyroid cancer, voltage-gated potassium channels, and RHO GTPase functional pathways.

293 For high-weight genes predictive of NF1 loss of function, we observed GO sets related to cellular  
294 adhesion (GO:0007155, GO:0098742), negative regulation of signaling (GO:0009968, GO:0023507,  
295 GO:0010648), and nervous system development (GO:0051962, GO:0007416, GO:0050808), among  
296 others. These genes were also enriched for elements of the phototransduction cascade and thyroxine  
297 production pathways.

298

## 299 **DISCUSSION:**

300 A machine learning classifier, based on gene expression data, can capture signal associated with the  
301 inactivation of a tumor suppressor. Our classifier is able to detect subtle downstream changes in gene  
302 expression as a result of the tumor responding to NF1 loss of function. This finding supports using mRNA  
303 as a summary measurement capable of capturing system-wide responses to molecular events beyond  
304 transcription factor alterations. Machine learning has been applied to gene expression in a variety of  
305 studies with various goals [29–33]. In a similar study, Guinney *et al.* trained a classifier to model RAS  
306 activity in colorectal cancer and demonstrated its clinical utility by predicting response to MEK inhibitors  
307 and anti-EGFR based treatments [34]. With a wealth of signal embedded in gene expression and a

308 rapidly growing library of datasets, the performance of machine learning models is likely to rapidly  
309 improve. An increase in performance leads to more reliable clinical applications that would potentially  
310 predict the effectiveness of pathway-specific targeted therapies.

311 While our classifier was able to predict NF1 inactivation status to an extent, its performance is far  
312 from being clinically actionable. A major difficulty in developing a reliable classifier in this case is  
313 contamination in gold standard positives and negatives. While we aim to detect NF1 inactivation events,  
314 our gold standard positives can only include samples with known *NF1* mutation status. Conversely, we  
315 expect that negative samples (about 90% of the data) are also contaminated with NF1 inactivated  
316 samples due to protein loss and other mechanisms. We cannot determine scenarios where NF1 is  
317 inactivated beyond mutation at scale in the TCGA data. Another challenge with the construction of  
318 classifiers from such data is overfitting. Even after hyperparameter optimization we observed  
319 substantial overfitting (Figure 2), which has also been observed in competitions (see, for example,  
320 supplementary figure S2 of Noren *et al.* 2016 [35] in which the best performing algorithms also overfit).  
321 Finally with a small number of positive examples the model performance is unstable, which  
322 demonstrates high variability in gold standard samples used to train the model [36]. We employed  
323 ensemble classification to mitigate this issue. In summary, our results are promising but these challenges  
324 are substantial and significant work remains to reach a robust classifier with clinical utility.

325 The performance of the classifier appears to be impacted by many *NF1* related genes. For example,  
326 genes such as *TXNIP* and *ARRDC4*, which are both indicative of lactic acidosis, correlate with better  
327 clinical outcomes, and contribute to predicting tumors with intact NF1 signaling [37]. We also observed  
328 transcripts that are more highly expressed in brain tissue than either other normal tissue (*ISPD*,  
329 *C10orf107*), or more highly expressed in normal brain tissue than glioma (*EPHA5*) [38–40]. *DUSP18*  
330 contributes to the prediction of *NF1* wildtype status and is a negative regulator of ERK phosphorylation,  
331 possibly by regulating *SHP2* phosphorylation [41]. Over-representation analysis of these data

332 highlighted changes in potassium channel expression. It was previously demonstrated that *NF1* wild-  
333 type Schwann cells have altered K<sup>+</sup> channel activity as compared to *NF1*<sup>-/-</sup> Schwann cells suggesting that  
334 this may be one factor by which *NF1* mutant and wild-type cells can be distinguished [42].

335       Regarding prediction of *NF1* inactivated tumors, we observed several genes that have been linked to  
336 cancer such as *QPRT*, which is highly expressed in malignant pheochromocytomas as compared to  
337 benign; *RSL1D1* (CSIG), which stabilizes *c-myc* in hepatocellular carcinoma; *PPEF*, which is highly  
338 expressed in astrocytic gliomas as compared to normal brain tissue [43–45]; and *PEG10*, a poor  
339 prognostic marker and regulator of proliferation, migration, and invasion in several tumor types [46–48].  
340 We also observed *ATF5*, a gene for which expression in malignant glioma is correlated with poor survival  
341 [49]. Knockdown of *ATF5* in GBM cells causes cell death *in vitro* and *in vivo* [50]. Analysis of genes that  
342 contribute to the prediction of *NF1* inactivation yielded several GO terms related to neural  
343 development. It is well established that loss of *NF1* can result in abnormal neural development and/or  
344 tumorigenesis [14,51,52]. We also observed genes associated with the mesodermal commitment  
345 pathway, components of which are linked to the epithelial to mesenchymal transition in human cancer  
346 cells [53–55]. Analysis of this pathway may be informative in identifying tumors with *NF1* loss because  
347 mesenchymal GBMs are enriched for tumors with *NF1* loss [56].

348       Our ensemble classifier was able to robustly detect the samples with the highest and lowest *NF1*  
349 protein concentrations, but it struggled with samples of intermediate *NF1* concentrations. This could be  
350 a result of an enrichment of mechanisms causing *NF1* inactivation beyond protein abundance, an  
351 overrepresentation of mesenchymal tumors in *NF1* inactivated samples contaminating dataset splits  
352 [56], poor classifier generalizability, or incomplete data transformation between RNAseq and microarray  
353 data. Because training and testing performance were similar between transformed and non-  
354 transformed data (see Figure 1 and Supplementary Fig S2) we don't anticipate performance to be  
355 impacted much by platform differences or classifier generalizability. Nevertheless, we demonstrated the

356 ability of system-wide gene expression measurements to capture downstream consequences of a  
357 complex biological mechanism that would otherwise require several different types of data acquisition  
358 to capture.

359

#### 360 **CONCLUSIONS:**

361 A machine learning classifier for transcriptomic data was able to detect signal associated with the  
362 inactivation of *NF1*, a tumor suppressor gene. The gene is an important regulator of the oncogene *RAS*  
363 and is inactivated frequently in GBM and in other tumors. The measurement of NF1 inactivity cannot be  
364 comprehensively captured by any single genomic characterization such as targeted sequencing or  
365 fluorescence in situ hybridization. This difficulty arises from diverse and complex biological mechanisms  
366 that inactivate the tumor suppressor in a variety of ways. However, we demonstrated that measuring  
367 system-wide RNA can capture subtle downstream changes that occur in response to NF1 inactivation.  
368 Improving classification performance is required before transitioning such a model into clinical use, but  
369 our method could be used to characterize cell lines or patient-derived xenograft (PDX) models with  
370 inactive NF1. Eventually, with more data and improved classification, we expect machine-learning  
371 models constructed on system-wide transcriptomics will translate into clinically relevant predictions that  
372 will guide targeted therapy.

373

#### 374 **DECLARATIONS:**

375 *Ethics approval and consent to participate*

376 The Dartmouth-Hitchcock Medical Center declared IRB review and approval is not required since the  
377 study does not meet the regulatory definition of human subjects.

378

379 *Consent for publication*

380 Not Applicable

381

382 *Availability of data and materials*

383 All source code is available under a permissive open source license in the nf1\_inactivation GitHub

384 repository, [https://github.com/greenelab/nf1\\_inactivation](https://github.com/greenelab/nf1_inactivation). We also provide a docker image to

385 replicate the computational environment at [https://hub.docker.com/r/gregway/nf1\\_inactivation/](https://hub.docker.com/r/gregway/nf1_inactivation/).

386 Additionally, all validation data is available under Gene Expression Omnibus accession number

387 GSE85033.

388

389 *Competing interests*

390 The authors declare no competing interests.

391

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400

401 *Authors' contributions*

402 GPW built, analyzed, and interpreted the classifier, generated the figures, created all source code,

403 and wrote the manuscript. RJA performed all experiments, interpreted the classifier, and wrote the



404 manuscript. SJB interpreted the results and was a major contributor to the manuscript. CEF was a  
405 major contributor to the study design and was a major contributor to the manuscript. YS designed  
406 the study, interpreted the results, and wrote the manuscript. CSG designed the study, interpreted  
407 the results, and wrote the manuscript. All authors read and approved the final manuscript.

408

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412 Center.

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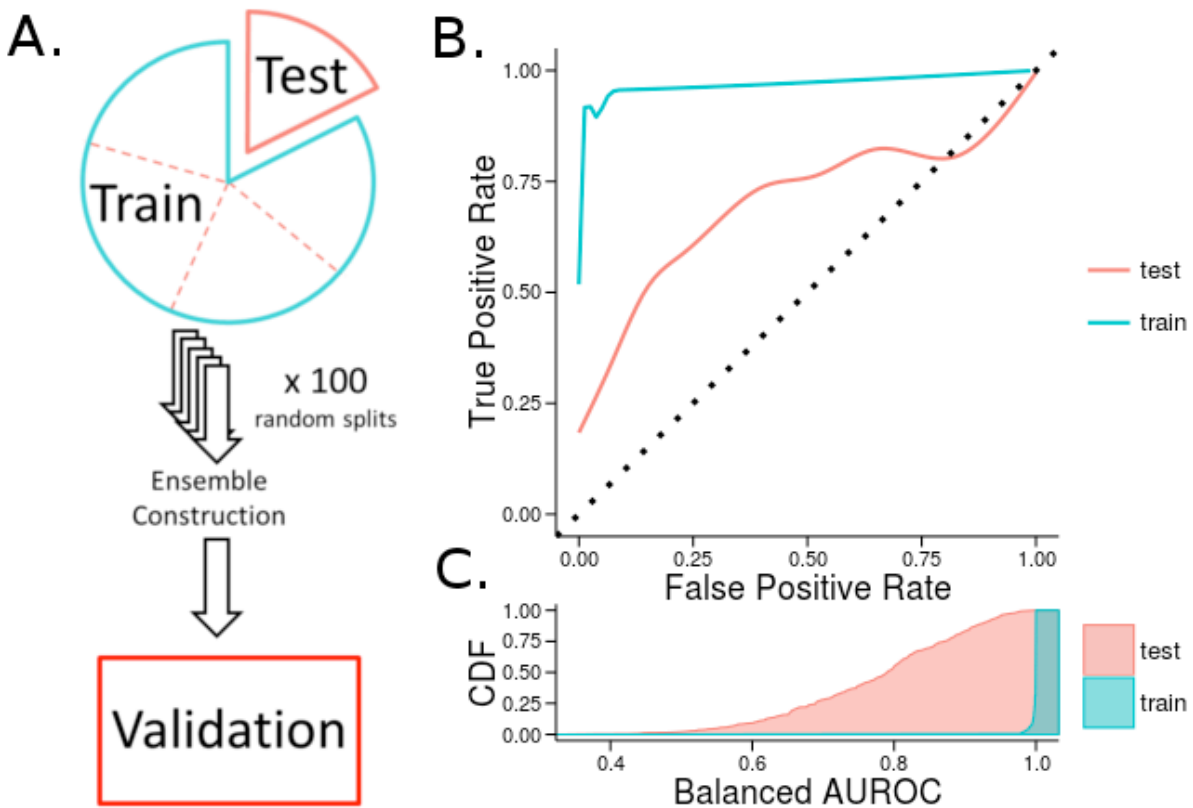
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428 FIGURES:



429

430 Figure 1: Logistic regression classifier with elastic net penalty training and testing errors over 100

431 *iterations for Training Distribution Matching (TDM) transformation of The Cancer Genome Atlas*

432 *Glioblastoma RNAseq data. (A) Schematic describing the terms used for training, testing, and validating*

433 *our model. We applied 5-fold cross validation to the full dataset which consists of training and testing*

434 *splits in each fold. The model is then applied as an ensemble classifier on a set of in-house samples*

435 *(validation set) (B) Receiver operating characteristic (ROC) curve and shows the average training and*

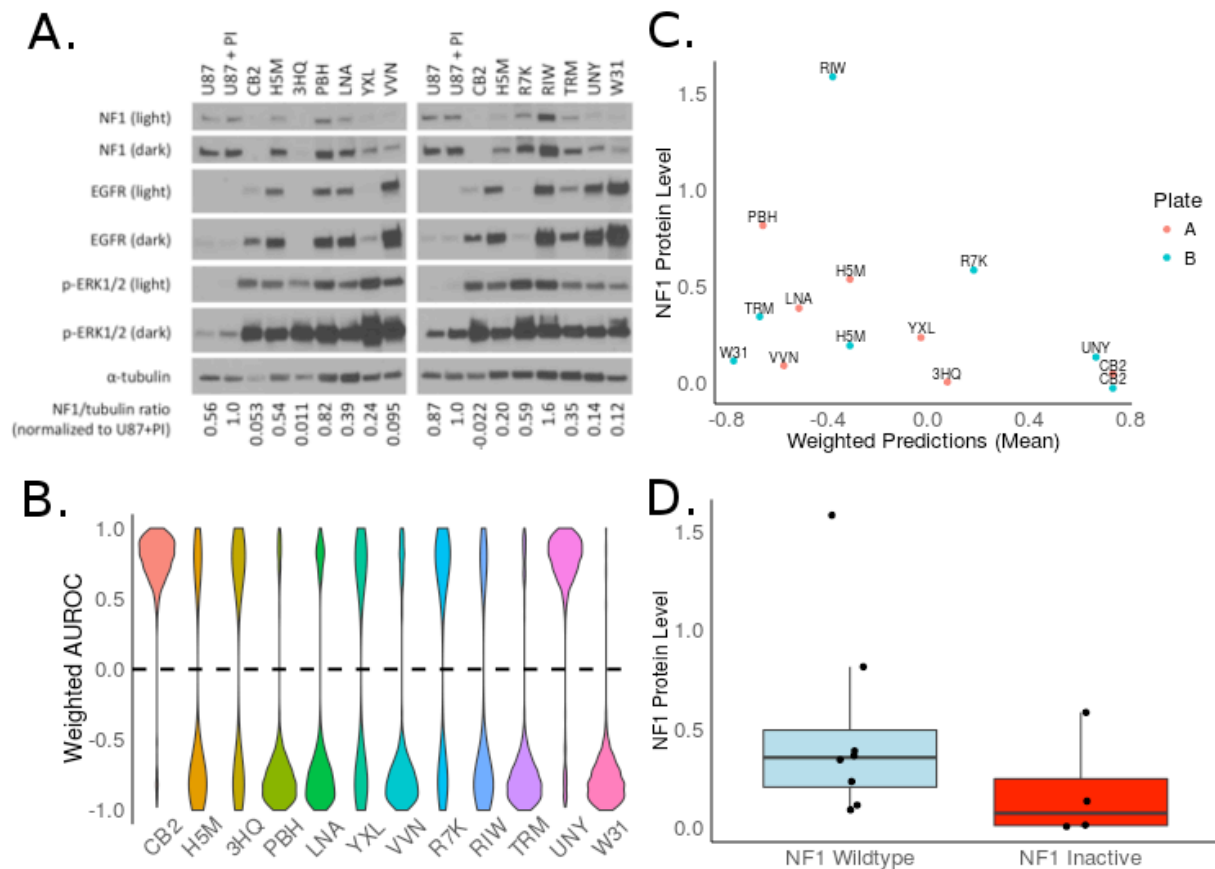
436 *testing performance of 5-fold cross validation. (C) The cumulative density of area under the ROC curve*

437 *(AUROC) balanced by 0-1 class for training and testing partitions.*

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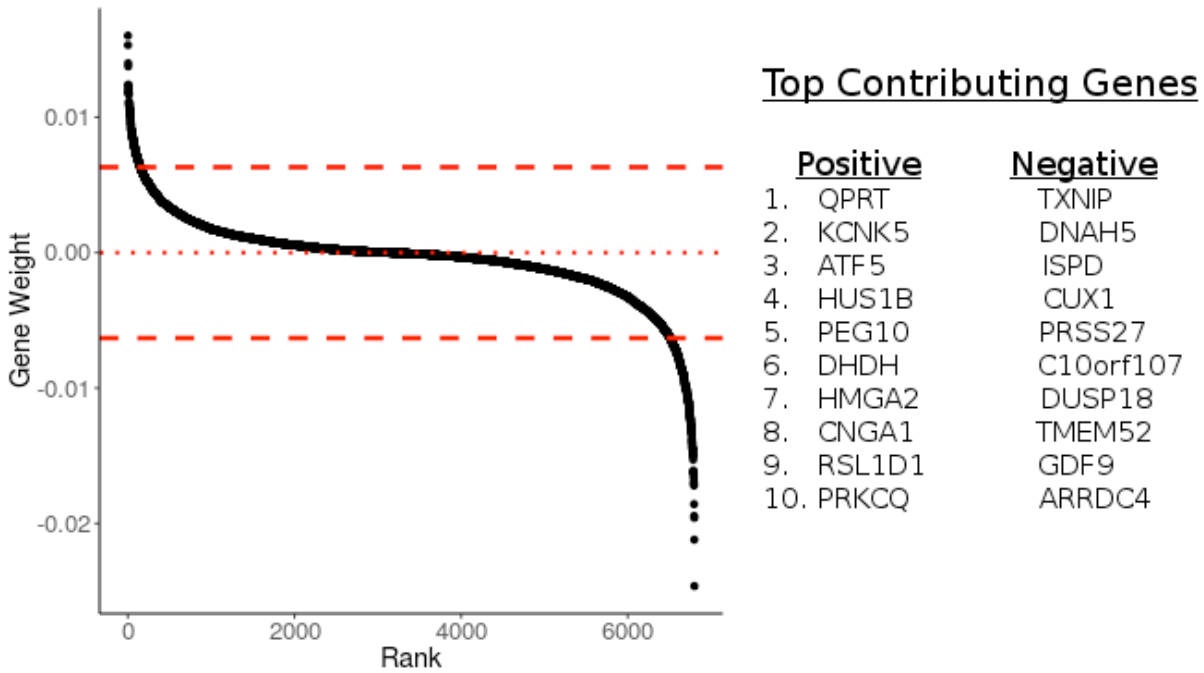
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 442 **Figure 2: Performance of our classifier on a validation set.** (A) Two distinct western blots for each of our  
 443 twelve samples. The controls are U87-MG, an *NF1* WT glioblastoma cell line that exhibits proteasomal  
 444 degradation of the *NF1* protein. U87+PI are U87-MG cells are treated with the proteasome inhibitors (PI)  
 445 MG-132 and bortezomib to block proteasome-mediated degradation of *NF1*. We used the *NF1*/tubulin  
 446 ratio normalized to U87+PI as our *NF1* protein level estimate. (B) Our ensemble classifier performance  
 447 weighted by test set AUROC where a negative number indicates *NF1* wildtype and a positive number is  
 448 predictive of *NF1* inactivation. (C) We quantify protein against U87+PI and provide the mean of the  
 449 weighted predictions. (D) Based on weighted predictions, we show the abundance of *NF1* protein  
 450 compared to U87+PI.

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454 Figure 3: Genes that contribute to the classifier performance. Genes are shown ranked by their weighted  
455 contribution to the ensemble classifier. Weights are scaled to unit norm. The top 10 positive and top 10  
456 negative contributing high weight genes are given on the right.

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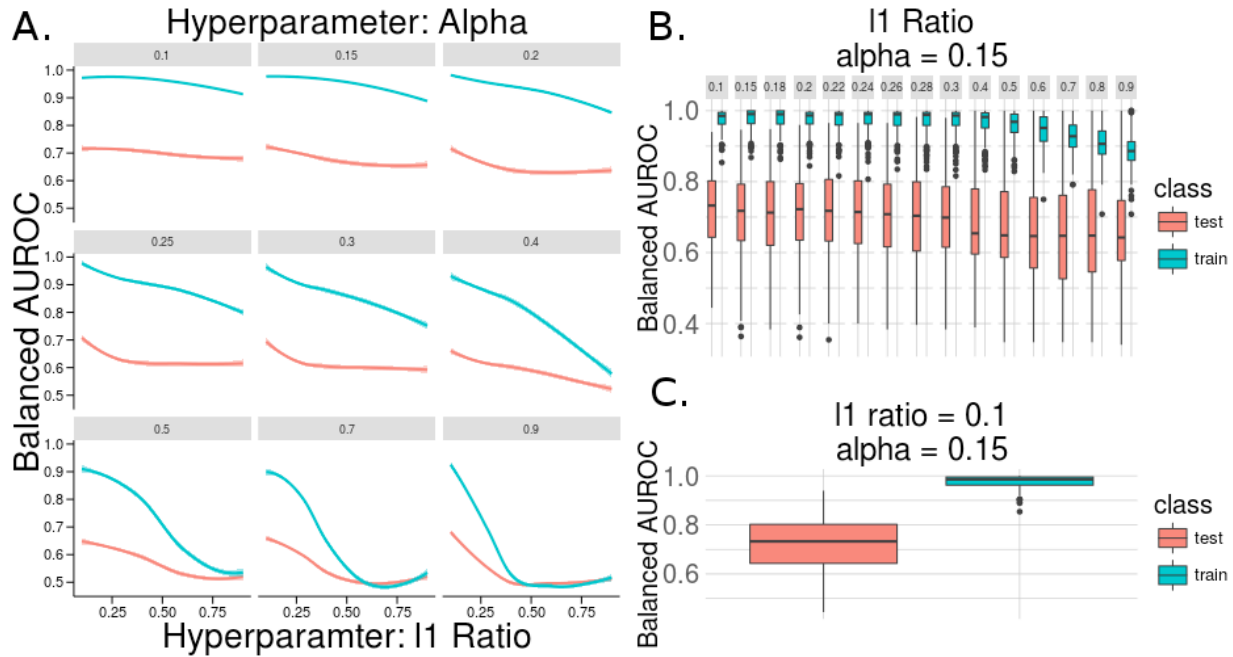
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468 Supplementary Figure S1: Non-transformed RNAseq results of The Cancer Genome Atlas Glioblastoma

469 *parameter sweep for stochastic gradient descent logistic classifiers with elastic net penalty.* (A) Training

470 and testing area under the receiver operating characteristic curve (AUROC) balanced by class is given for

471 each parameter tested. All accuracies are presented following 5-fold cross validation after 50 random

472 initializations. (B) The l1 mixing parameter with the optimal alpha and (C) the classifier performance

473 across all random starts for the best hyperparameters.

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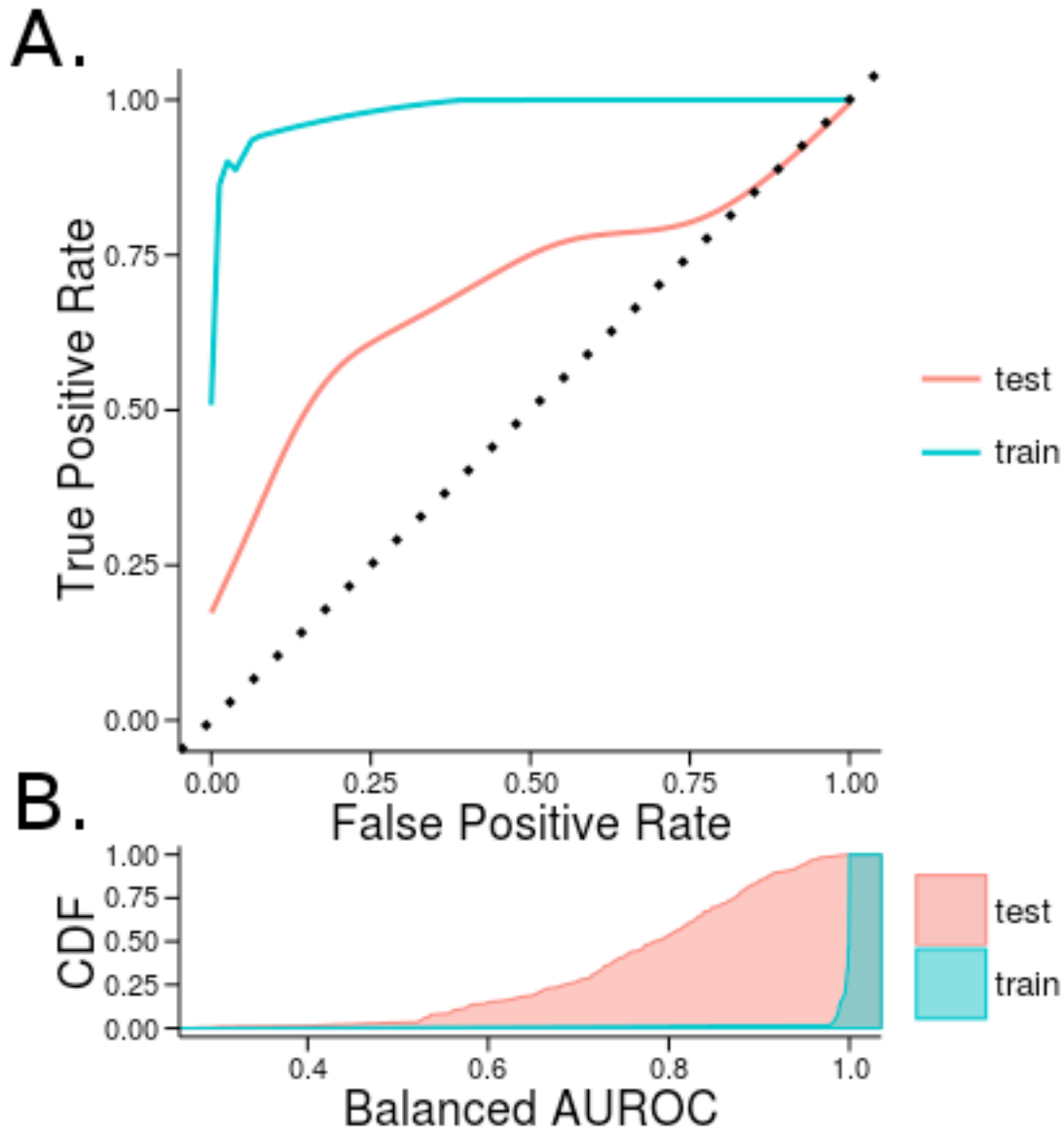
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483 Supplementary Figure S2: Logistic regression classifier with elastic net penalty training and testing errors

484 *over 100 iterations for non-transformed The Cancer Genome Atlas Glioblastoma RNAseq data. (A)*

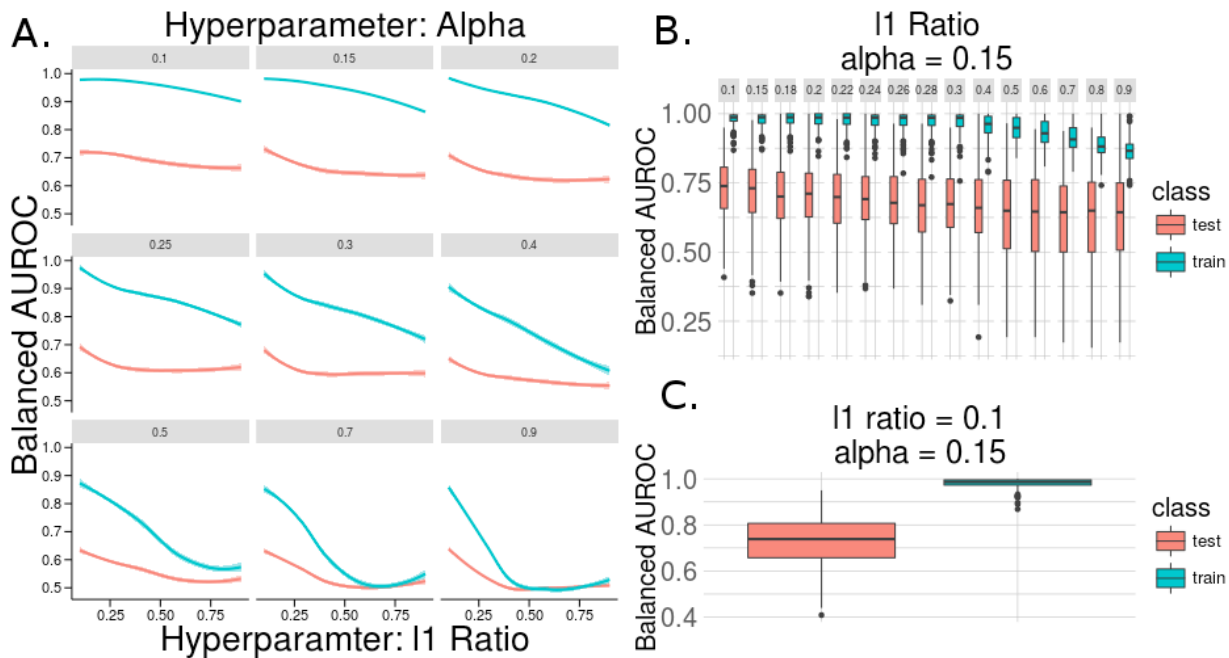
485 Receiver operating characteristic (ROC) curve and shows the average training and testing performance

486 of 5-fold cross validation over 100 random initializations. (B) The cumulative density of area under the

487 ROC curve (AUROC) balanced by 0-1 class for training and testing partitions.

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491 Supplementary Figure S3: Training Distribution Matching (TDM) transformation of RNAseq results of The  
492 *Cancer Genome Atlas Glioblastoma parameter sweep for stochastic gradient descent logistic classifier*  
493 *with elastic net penalty.* (A) Training and testing area under the receiver operating characteristic curve  
494 (AUROC) balanced by class is given for each parameter tested. All accuracies are presented following 5-  
495 fold cross validation after 100 random initializations. (B) The l1 mixing parameter with the optimal alpha  
496 and (C) the classifier performance across all random starts for the best hyperparameters.

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