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2 Integrative analysis of transcriptomic and clinical data 3 uncovers the tumor suppressive activity of MITF in 4 prostate cancer

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25 # Abstract

26 The dysregulation of gene expression is an enabling hallmark of cancer. Computational analysis of
27 transcriptomics data from human cancer specimens, complemented with exhaustive clinical annotation,
28 provides an opportunity to identify core regulators of the tumorigenic process. Here we exploit well-
29 annotated clinical datasets of prostate cancer for the discovery of transcriptional regulators relevant to
30 prostate cancer. Following this rationale, we identify Microphthalmia-associated transcription factor
31 (MITF) as a prostate tumor suppressor among a subset of transcription factors. Importantly, we further
32 interrogate transcriptomics and clinical data to refine MITF perturbation-based empirical assays and
33 unveil Crystallin Alpha B (CRYAB) as an unprecedented direct target of the transcription factor that is,
34 at least in part, responsible for its tumor suppressive activity in prostate cancer. This evidence was
35 supported by the enhanced prognostic potential of a signature based on the concomitant alteration of
36 MITF and CRYAB in prostate cancer patients. In sum, our study provides proof-of-concept evidence of
37 the potential of the bioinformatics screen of publicly available cancer patient databases as discovery
38 platforms, and demonstrates that the MITF-CRYAB axis controls prostate cancer biology.

39

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40 # Introduction

41 Balanced integration of intracellular circuits operates within a normal cell to sustain physiological
42 homeostasis. Alterations in some, if not all, of these circuits converge in changes on gene expression,
43 which will eventually enable the acquisition and sustenance of the hallmarks of cancer cells (1). This
44 event emphasizes the importance of maintaining the transcriptional homeostasis in normal cells and
45 places gene expression deregulation at the core of cancer research interests.

46 In the last decades, transcriptomics data derived from cancer specimens has become an important
47 resource for the classification, stratification and molecular driver identification in tumors. We and others
48 have demonstrated that deregulation of gene expression is a key node for cancer pathogenesis and
49 progression (2-6). Prostate cancer (PCa) research exemplifies the effort in deciphering the genomics
50 and transcriptomics landscape of tumors, and extremely valuable data has been generated (7-13). In
51 spite of the public availability of these relevant data, they are still underexploited by the scientific
52 community to understand PCa biology. In this regard, the computational tools and dataset selection
53 strategies to carry out these studies are a bottleneck for the cancer research field.

54 By combining integrated-bioinformatics screening of clinically relevant PCa datasets with *in vivo* and *in*
55 *vitro* molecular biology assays, we have recently described the metastasis suppressor activity of
56 Peroxisome proliferator-activated receptor γ (PPARγ) coactivator alpha (PGC1α) (14, 15). This
57 transcriptional coactivator is a major regulator of mitochondrial biogenesis and function, and has an
58 inherent capacity to integrate environmental signals and cellular energetic demands. This ability
59 empowers PGC1α to be a driver in shaping responses to metabolic stress during different physiologic
60 and tumorigenic processes (16). As might be expected due to its fundamental role in normal and cancer
61 scenarios, the regulation of PGC1α expression, from the genomic to the protein level, is complex and
62 dynamic (17). At the level of mRNA expression, one of the well-defined direct regulators of PGC1αis
63 the Microphthalmia-associated transcription factor (MITF) (18).

64 MITF is a basic helix-loop-helix leucine zipper (bHLHZIP) transcription factor that regulates the
65 expression of lineage commitment programs that are essential for propagation of the melanocyte

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66 lineage (19). The existence of different MITF transcript variants is the result of both alternative splicing
67 and promoter activation that results in the cell-type-specific expression of the different MITF isoforms
68 (A, CX, MC, C, E, H, D, B, M, J) (20). The melanoma specific isoform M-MITF is the best studied isoform
69 and, despite some controversy, its expression is generally deregulated in melanoma. Although MITF
70 alone cannot act as a classical oncogene, it has been called a 'lineage survival oncogene' for melanoma
71 (19, 21). Importantly, the presence or absence of the M-MITF- PGC1α regulatory axis has stratification
72 potential in melanoma and informs on the efficacy of BRAF inhibitor treatments (18, 22). Although the
73 expression of MITF has been detected in other types of tumors different from melanoma (23, 24), its
74 active role in the progression of these diseases, including PCa, remains unexplored.

75 Crystallin Alpha-B (CRYAB) is a ubiquitous small heat shock protein that is expressed in response to a
76 wide range of physiological and non-physiological conditions preventing aggregations of denatured
77 proteins. In a wide variety of tumor types CRYAB has been found to be overexpressed and associated
78 with disease progression (25-29) and poor prognosis (30, 31). However, in PCa and nasopharyngeal
79 cancers CRYAB expression is decreased (32, 33), pointing at possible tumor suppressive activity of
80 CRYAB in these cancer scenarios.

81 In the present study, by combining an exhaustive interrogation of seven publically available prostate
82 cancer databases with refined empirical assays, we have identified MITF as a prostate tumor
83 suppressor. In addition, we have unveiled CRYAB as a novel direct target of the transcription factor that
84 is, at least in part, responsible for its tumor suppressive activity in prostate cancer. Importantly, the
85 tumor suppressive role for this novel MITF-CRYAB axis is supported by the enhanced prognostic
86 potential of a signature based on the concomitant alteration of both genes in PCa patients.

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87 # Results

88 ### Bioinformatics screening identifies MITF as a transcription factor altered in prostate cancer

89 We have recently demonstrated that the reduced expression of the transcriptional co-activator PGC1α
90 is a causal event for metastatic prostate cancer (PCa) (14). We sought to identify transcriptional
91 regulators related to the alteration in PGC1α expression. We designed a bioinformatics strategy based
92 on the analysis of 16 genes directly linked to the regulation of *PGC1A* gene (17, 22, 34-38), in order to
93 identify transcription factors that could be relevant to PCa biology. For the candidate screen we applied
94 selection criteria based on the consistency of, first, the correlation with *PGC1A* expression and second,
95 the expression of each individual candidate in seven publicly available PCa datasets (7, 9-13) (Figure
96 1 A). We selected those candidates whose expression in primary tumors correlated with *PGC1A* ($R \geq$
97 0.2 and p-value ≤ 0.05 in more than 50% of the datasets) (Supplementary Figure 1 A) and was altered
98 when compared to normal specimens. For genes exhibiting various transcript variants, the correlation
99 analysis was initially performed using the average signal (Supplementary Figure 1 A) and, when
100 available (only Taylor dataset (11)), the correlation was confirmed in all the individual isoforms
101 (Supplementary table 1). The transcription factor MITF was the sole candidate that complied with the
102 established criteria. We observed a consistent correlation between *PGC1A* and *MITF* in four out of the
103 seven datasets analyzed (Figure 1 B and Supplementary Figure 1 A). In addition, not only the mean
104 expression but also the expression of the individual *MITF* isoforms were reduced in primary and
105 metastatic PCa specimens when compared with the normal prostate tissue samples (Figure 1 C and
106 Supplementary Figure 1 B). Taken together, our data reveals MITF as a PGC1A-associated
107 transcription factor that is consistently downregulated in PCa.

108

109 ### MITF exhibits tumor suppressive activity in PCa

110 The expression profile of *MITF* in PCa, together with its direct correlation with *PGC1A*, was suggestive
111 of a tumor suppressive activity of the transcription factor. We first examined the differential expression
112 of the distinct mRNA isoforms of *MITF* in normal, PCa primary tumors and PCa cell lines

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113 (Supplementary Fig. 2 A-C). *MITFA* was the isoform predominantly expressed in the three scenarios
114 analyzed, and we pursued the studies further with this isoform. Next, we aimed to analyze the biological
115 consequences of ectopic expression of MITFA in PC3 PCa cells. We transduced PC3 cells with a
116 lentiviral vector containing a doxycycline-inducible cassette for the expression of MITFA resulting in the
117 generation of the PC3 TRIPZ-MITFA cell line. The induction of MITFA expression (Figure 2 A and B)
118 as well as the regulation of known target genes, including *PGC1A* (14, 15) (Supplementary Figure 2 D-
119 E) was confirmed. We next evaluated the biological outcome of MITFA ectopic expression in PC3 cells
120 and observed that its upregulation significantly reduced two-dimensional and anchorage-independent
121 growth (Figure 2 C and D), with no effect of doxycycline treatment by itself (14). In line with its known
122 function as an inhibitor of cell cycle progression (39), the increased expression of MITFA in PC3 cells
123 resulted in a decrease in BrdU incorporation, a surrogate readout of proliferation (Figure 2 E).
124 In order to ascertain whether the regulation of endogenous *PGC1A* (Supplementary Figure 2 E) was
125 required for the anti-proliferative effect of MITFA in PC3 cells, we aim at silencing *PGC1A* by using
126 constitutive (pLKO) expression of short hairpins against it (Supplementary Figure 2 F). Transduction
127 with the shRNA prevented the upregulation of *PGC1A* upon MITFA induction (Supplementary Figure 2
128 G) but the anti-proliferative effect of the transcription factor remained unaffected (Supplementary Figure
129 2 H). These data suggested that the reduced proliferation induced by MITFA was not dependent on the
130 regulation of endogenous *PGC1A* in PC3 cells.
131 Importantly, the overall reduction in cell proliferation induced by MITFA was confirmed *in vivo*. Using
132 subcutaneous xenografts assays we observed that MITFA over-expression in PC3 cells
133 (Supplementary Fig. 2 I) led to a marked reduction in the tumor volume (Supplementary Fig. 2 J) and
134 growth rate (Figure 2 F), with no changes in angiogenesis (Supplementary Figure 2 K). Altogether these
135 results demonstrate that MITFA isoform exhibits tumor suppressive activity in PCa.
136

137 **Candidate screening of genes mediating the tumor suppressive activity of MITF**

138 In order to decipher the molecular mechanism driving the tumor suppressive role of MITFA we
139 performed gene expression profiling of both doxycycline treated and control PC3 TRIPZ-MITFA cells

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140 and identified 101 probes that showed statistically differential signal between both conditions
141 (Supplementary table 2). We first performed a gene enrichment analysis with those genes which
142 displayed upregulated expression (76 genes) upon MITFA over-expression (Figure 3 A and
143 Supplementary table 3), as the number of downregulated genes (25) was no sufficient to obtain any
144 gen enrichment. Next, we aimed at identifying potential MITFA effectors of relevance in human PCa.
145 To this end, we established a threshold of 1.5 fold change over MITFA non-induced cells, which resulted
146 in 8 probes (corresponding to 6 annotated genes) upregulated upon the induction of the transcription
147 factor (Supplementary table 2; yellow bold highlighted). We next performed correlation analysis
148 between *MITF* and each of the 6 differentially expressed genes obtained from the microarray (Figure 3
149 A and Supplementary Figure 3 A). The correlation analysis in PCa primary tumor specimens showed
150 that a single gene, *Crystallin Alpha B (CRYAB)*, had a consistent correlation (in more than 50% of
151 datasets) with *MITF*, both the mean of isoforms (Figure 3 B and Supplementary Figure 3 A) and the
152 individual isoform A (Supplementary table 4). The *MITF-CRYAB* correlation was confirmed using an
153 independent cohort of PCa patients from a local hospital (Basurto cohort, Supplementary Figure 3 A).
154 Moreover, the expression of CRYAB either at the level of mRNA (from public datasets and Basurto
155 cohort) and protein (from Basurto cohort) was consistently downregulated through the progression of
156 the disease (Figure 3 C and D and Supplementary Figure 3 B-D), supporting the association of MITF
157 and CRYAB expression in PCa.

158 The regulation of CRYAB expression by MITFA was further validated *in vitro* by western blot and
159 quantitative real-time PCR (qRTPCR) in doxycycline-treated PC3 TRIPZ-MITFA cell lines and *in vivo*
160 by qRTPCR in the xenograft samples (Supplementary Figure 3 E-G). MITF is a transcription factor that
161 regulates gene expression through the DNA binding to E-boxes (Myc-binding sites) (19). In order to
162 confirm the direct regulation of *CRYAB* expression by MITFA, we screened the promoter of the
163 chaperon and performed chromatin immunoprecipitation assays in two Myc-binding sites (UCSC-
164 Genome browser; Supplementary Figure 3 H). As predicted, upon doxycycline treatment we detected
165 differential binding of MITFA in both regions of *CRYAB* promoter (Figure 3 E).

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166 Taken together, these data presented *CRYAB* as a direct target of MITFA and the best candidate to
167 mediate its tumor suppressive activity in PCa.

168

169 **CRYAB mediates the tumor suppressive activity of MITF in PCa**

170 We next studied the functional relevance of CRYAB for the tumor suppressive activity of MITFA in PCa.
171 Towards this aim, we constitutively silenced the expression of *CRYAB* by RNAi using two independent
172 short hairpin RNA (sh#1 and sh#2) in PC3 TRIPZ-MITFA cells. After validation that RNAi was achieved
173 (Figure 4 A and Supplementary Figure 4 A-C) the tumor suppressive activity of MITFA was monitored
174 in control and *CRYAB*-silenced conditions (PC3 TRIPZ-MITFA scr, sh#1 or sh#2 cell lines). *CRYAB*
175 silencing blunted the anti-proliferative effects of MITFA *in vitro* in two-dimensional and anchorage
176 independent growth when compared with scramble shRNA (Figure 4 B and C). Moreover, the reduction
177 in BrdU induced by MITFA was prevented when *CRYAB* was silenced (Figure 4 D). Importantly, the
178 requirement of CRYAB for the tumor suppressive activity of MITFA was corroborated *in vivo* (Figure 4
179 E and Supplementary Figure 4 D-F). The *in vitro* and *in vivo* data demonstrate that the induction of
180 CRYAB is a major effector involved in the tumor suppressive activity of the transcription factor MITF in
181 PCa.

182 We next asked whether the functional association between MITF and CRYAB could be employed to
183 identify PCa patients with high disease aggressiveness. We thus ascertained the stratification potential
184 of the MITF-CRYAB axis in PCa by means of consistency and robustness. We download the mRNA
185 expression raw data together with the clinical data (recurrence or not recurrence) from Taylor (11),
186 Glinsky (8) and TCGA (7) datasets. The individual or average expression signal of *CRYAB* and *MITF*
187 genes was calculated for each patient in each dataset. Patients were separated by quartiles according
188 to the individual or average signal of *CRYAB* and *MITF* genes and then Kaplan-Meyer survival curves
189 were plotted comparing patients with low expression (Quartile 1 – (Q1)) of the individual genes or the
190 gene combination (*CRYAB* and *MITF) versus* the rest of the cohort (Q2+Q3+Q4). Strikingly, the
191 signature formed by the average signal of *MITF* and *CRYAB* outperformed the prognostic potential of

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192 each individual gene, strongly suggesting that the pathway described herein is strongly associated to
193 PCa aggressiveness (Figure 4 F and Supplementary Figure 5).

194 Our data provide solid evidence of an unprecedented MITFA-CRYAB transcriptional axis that exerts
195 tumor suppressive activity in PCa and could positively contribute to disease prognosis.

196

197 DISCUSSION

198 Technological advances in the molecular understanding of cancer have led to a paradigmatic change
199 in the way that we combat the disease. We are now able to deconstruct a tumor at a molecular level
200 using genomics, transcriptomics, proteomics and metabolomics. This, in turn, enables us to foresee,
201 identify and demonstrate the potential of patient stratification. Specifically, the transcriptomics
202 characterization of tumors is an invaluable strategy to identify clinically relevant genes that play key
203 roles in the progression of cancer, especially for those types with poorer prognosis (14). Thus, the
204 comprehensive and integrative analysis of gene expression changes and clinical parameters in cancer
205 has become a mainstream in cancer research. Mining cancer-associated transcriptome datasets is an
206 emerging approach used by top cancer research groups, but better tools are needed to increase its
207 power and user-friendliness. In order to face this challenge, new interfaces to exploit OMICs data, such
208 as cBioportal (40, 41) are being designed to help scientists interrogate, integrate and visualize large
209 amount of information contained on multiple credible and qualified cancer datasets.

210 In the present study, we exploited publicly available and well-annotated (transcriptomics and clinical
211 data) prostate cancer databases together with experimental assays to describe a novel tumor
212 suppressive activity of the transcription factor MITF in PCa, which is executed, at least in part, through
213 the direct regulation of the CRYAB expression.

214 The functional implication of MITF in cancer has been best defined in melanoma, in which the
215 expression of the transcription factor is heterogeneous. Although some controversy exists regarding its
216 oncogenic role in melanoma, MITF has been defined as a “lineage survival oncogene” with no data
217 pointing out at a tumor suppressive function (19, 21, 39, 42-45). Even though the expression of MITF

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218 has been detected in other cancer types (23, 24, 46), no data supporting a functional role of MITF
219 deregulation has been reported yet in a cancer scenario different from melanoma.

220 Here we show that MITF is downregulated in PCa when compared with normal specimens, in contrast
221 to the elevated expression reported in hepatocellular carcinoma (HCC) and chronic myeloid leukemia
222 (CML) (23, 46). Moreover, the novelty of our study relies on the observation and definition of the tumor
223 suppressive activity of MITF in PCa. In this context, MITFA upregulation was associated with a reduction
224 in cell proliferation and DNA replication. As occurs in melanoma, the modulation of MITF expression in
225 PCa cells induces the expression of the cell cycle inhibitor p21 but no changes in the cell cycle inhibitor
226 p16 were observed (data not shown). Thus, our results in PCa are in line with the canonical function of
227 MITF in cell cycle progression and proliferation in melanoma (39, 44, 45).

228 It's important to highlight that the tissue specific differences in MITF expression among different cancer
229 types suggest that in order to fully comprehend MITF's role in cancer, its expression and function has
230 to be analyzed in context of each particular cell and tissue type.

231 CRYAB is a member of the small heat shock protein family that functions as stress-induced molecular
232 chaperone. It inhibits the aggregation of denatured proteins, promotes cell survival and inhibits
233 apoptosis in the context of cancer (47). Paradoxically, CRYAB is highly expressed in some cancer types
234 but decreased in others and in both scenarios an association with cancer progression and prognosis
235 has been reported (25, 26, 28-32, 48-52). In spite of the amount of information regarding the changes
236 in CRYAB expression in cancer, the transcriptional regulation of this chaperone has been poorly
237 explored (48). In this study, we described a novel direct transcriptional regulation of CRYAB by MITF.
238 Although there is no direct nor mechanistic evidence of the MITF-CRYAB transcriptional axis in other
239 cancer types, in melanoma both MITF and CRYAB expression are upregulated by BRAF/MEK-inhibitor
240 treatments (49, 52), suggesting that this regulation can go beyond both prostate cancer scenario.
241 Indeed, we observed that the correlation between MITF and CRYAB is also present in colorectal cancer,
242 but not in breast nor lung cancer (data not shown).

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243 In our study, the MITF-CRYAB transcriptional axis is reduced and exerts tumor suppressive activity in
244 PCa. This is in agreement with the reduced expression of CRYAB observed in PCa patients and its
245 previous consideration as a protective gene against PCa (32). Yet, the exact molecular mechanism
246 underlying the tumor suppressive activity of CRYAB remains to be elucidated.

247 Importantly, the extensive interrogation of PCa transcriptomes and associated clinical data has led us
248 to propose the transcriptional axis MITF-CRYAB as a potential prognostic biomarker in PCa. The
249 individual expression of CRYAB and MITF has been previously associated with poor prognosis in
250 various tumor types (26, 29-31, 50, 51) and to therapy response in melanoma (53-55). However, our
251 data showing enhanced prognostic potential of the combined signature provides a new and exciting
252 perspective of the functional interaction of these genes in PCa.

253 Our study endorses the potential of transcriptional deregulation analysis, as either a cause or a
254 consequence of cancer, and its impact to support the discovery of novel cancer related genes and long-
255 term development of novel cancer treatment strategies.

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273

274 MATERIALS AND METHODS

275 Cell culture and Reagents

276 Human prostate carcinoma cell lines (PC3) were purchased from Leibniz-Institut DSMZ - Deutsche
277 Sammlung von Mikroorganismen und Zellkulturen GmbH, who provided authentication certificate. The
278 cell line used in this study was not found in the database of commonly misidentified cell lines maintained
279 by ICLAC and NCBI Biosample. Cells were transduced with a modified TRIPZ (Dharmacon) doxycycline
280 inducible lentiviral construct in which the RFP and miR30 region was substituted by *HA-Flag-MITF*.
281 Lentiviral shRNA constructs targeting *PGC1A* (#1-TRCN0000001165 and #2-TRCN0000001166) and
282 *CRYAB* (#1-TRCN0000010822 and #2-TRCN0000010823) were purchased in Sigma and a scramble
283 shRNA (hairpin sequence:
284 CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTG) was used as
285 control. For *PGC1A* and *CRYAB* shRNAs, Puromycin resistance cassette was replaced by Hygromycin
286 cassette from pLKO.1 Hygro (Addgene Ref. 24150) using *Bam*HI and *Kpn*I sites. Cell lines were
287 routinely monitored for mycoplasma contamination and quarantined while treated if positive.
288 Doxycycline hyclate (Dox) and Puromycin were purchased from Sigma, and Hygromycin from
289 Invitrogen.

290 Xenotransplant assays

291 All mouse experiments were carried out following the ethical guidelines established by the Biosafety
292 and Welfare Committee at CIC bioGUNE. The procedures employed were carried out following the
293 recommendations from AAALAC. Xenograft experiments were performed as previously described (14),

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294 injecting 10^6 cells per condition in two flanks per mouse (Nu/Nu immunodeficient males; 6-12 weeks of
295 age). PC3 TRIPZ-HA-MITFA cells alone or under *CRYAB* silencing were injected in each flank of nude
296 mice and 24 h post-injections mice were fed with chow or doxycycline diet (Research diets,
297 D12100402).

298 **Patient samples**

299 All samples were obtained from the Basque Biobank for research (BIOEF, Basurto University hospital)
300 upon informed consent and with evaluation and approval from the corresponding ethics committee
301 (CEIC code OHEUN11-12 and OHEUN14-14).

302 **Molecular assays**

303 Western blot was performed as previously described (14). Antibodies used: HA-Tag (Cell Signalling
304 #3724; dilution 1:10000) ; MITF (Thermo Fisher Scientific MA5-14146; dilution 1:1000); β-Actin (Cell
305 Signalling #3700; dilution 1:2000); GAPDH (clone 14C10; Cell Signalling #2218; dilution 1:1000);
306 CRYAB (Cell Signalling #45844s; dilution 1:1000).

307 RNA was extracted using NucleoSpin® RNA isolation kit from Macherey-Nagel (ref: 740955.240C). For
308 patients and animal tissues a Trizol-based implementation of the NucleoSpin® RNA isolation kit
309 protocol was used as reported (14). 1µg of total RNA was used for cDNA synthesis using Maxima™ H
310 Minus cDNA Synthesis Master Mix (Invitrogen M1682). Quantitative Real Time PCR (qRTPCR) was
311 performed as previously described (14). Universal Probe Library (Roche) primers and probes employed
312 are detailed in Supplementary Table 5. *GAPDH* (Hs02758991_g1) housekeeping assay from Applied
313 Biosystems was used for data normalization.

314 For transcriptomic analysis in PC3 TRIPZ-HA-Flag-MITFA cells, Illumina whole genome -HumanHT-
315 12_V4.0 (DirHyb, nt) method was used as reported (14). A hypergeometric test was used to detect
316 enriched dataset categories.

317 **Cellular assays**

318 Cell number quantification with crystal violet was performed as referenced (14).

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319 For starvation experiments 100,000 cells per well were seeded in a 6-well plate. Cells were initially
320 plated in 10% FBS media for 24 hours and then the media was changed to FBS free media and left
321 overnight.

322 Soft agar assays were performed as previously described (14), seeding 5,000 cells per well in 6-well
323 plates.

324 For BrDu incorporation, cells were seeded on glass cover slips in 12-well plates and after 4 days, cells
325 were incubated with 3 µg mL^{-1} BrDu (Sigma B5002). Cells were fixed with 4% para-formaldehyde,
326 permeabilized with 1% Triton X-100 and incubated with a monoclonal anti-BrDu (MoBU-1) antibody
327 (Invitrogen B35128) at a 1:100 dilution. Images were obtained with an AxioImager D1 microscope
328 (Zeiss). At least three different areas per cover slip were quantified.

329 **Chromatin Immunoprecipitation**

330 Chromatin Immunoprecipitation (ChIP) was performed using the SimpleChIP® Enzymatic Chromatin IP
331 Kit (Cat: 9003, Cell Signalling Technology, Inc). Four million PC3 cells were grown in 150 mm dishes
332 either with or without 0.5 µg mL^{-1} doxycycline during 3 days. Cells from three 150 mm dishes were
333 cross-linked with 35% formaldehyde for 10 min at room temperature. Glycine was added to dishes
334 during 5 min at room temperature. Cells were then washed twice with ice-cold PBS, and scraped into
335 PBS+PMSF. Pelleted cells were lysed and nuclei were harvested following manufacturer's instructions.
336 Nuclear lysates were digested with micrococcal nuclease for 20 min at 37°C and then sonicated in
337 500µl aliquots on ice for 6 pulses of 20 s using a Branson sonicator. Cells were held on ice for at least
338 1 min between sonications. Lysates were clarified at 11,000 × g for 10 min at 4°C, and chromatin was
339 stored at -80°C. HA-Tag polyclonal antibody (Cat: C29F4, Cell Signalling Technology) and IgG antibody
340 (Cat: 2729, Cell Signalling Technology, Inc), were incubated overnight (4ºC) with rotation and protein
341 G magnetic beads were incubated 2hrs (4ºC). Washes and elution of chromatin were performed
342 following manufacturer's instructions. DNA quantification was carried out using a Viia7 Real-Time PCR
343 System (Applied Biosystems) with SybrGreen reagents and primers that amplify the predicted MITFA
344 binding region to *CRYAB* (region 1; For: ttgtttcctcgtagggcttg, Rev: tttcagagccaggagagagc- region 2;

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345 For: tctggaatggtgatgtcagg, Rev: attgggtgtggacagaaagc) and *ANGPTL4* (For: gttgacccggctcacaat, Rev:
346 ggaacagctcctggcaatc) as a negative binding control.

347 **Whole genome gene expression characterization**

348 Whole genome expression characterization was conducted using Human HT12 v4 BeadChips (Illumina
349 Inc.). In brief, cRNA synthesis was obtained with TargetAmp™ Nano-g™ Biotin-aRNA Labeling Kit for
350 the Illumina® System, Epicentre (Cat.Num. TAN07924) and subsequent amplification, labeling and
351 hybridization were performed according to Whole-Genome Gene Expression Direct Hybridization
352 Illumina Inc.'s protocol. Raw data were extracted with GenomeStudio analysis software (Illumina Inc.)
353 in the form of GenomeStudio's Final Report (sample probe profile).

354 **Bioinformatic analysis and statistics**

355 Database normalization: All the datasets used for the data mining analysis were downloaded from GEO
356 and TCGA. GEO-downloaded data was subjected to background correction, $\log_2$ transformation and
357 quartile normalization. In the case of using a pre-processed dataset, this normalization was reviewed
358 and corrected if required. TCGA data were downloaded as upper quartile normalized RSEM count,
359 which was been log2 transformed.

360 Quartile analysis in Disease Free Survival: Patients biopsies from primary tumours were organized into
361 four quartiles according to the expression of the gene of interest in three datasets. The recurrence of
362 the disease was selected as the event of interest. Kaplan-Meier estimator was used to perform the test
363 as it takes into account *right-censoring*, which occurs if a patient withdraws from a study. On the plot,
364 small vertical tick-marks indicate losses, where a patient's survival time has been right-censored. With
365 this estimator we obtained a survival curve, a graphical representation of the occurrence of the event
366 in the different groups, and a p-value that estimates the statistical power of the differences observed.

367 Correlation analysis: Spearman correlation test was applied to analyse the relationship between paired
368 genes. From this analysis, Spearman coefficient (R) indicates the existing linear correlation
369 (dependence) between two variables X and Y, giving a value between +1 and −1 (both included), where

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370 1 is total positive correlation, 0 is no correlation, and −1 is total negative correlation. The p-value
371 indicates the significance of this R coefficient.

372 Statistical analysis: No statistical method was used to predetermine sample size. The experiments were
373 not randomized. The investigators were not blinded to allocation during experiments and outcome
374 assessment. Unless otherwise stated, data analysed by parametric tests is represented by the mean ±
375 s.e.m. of pooled experiments and median ± interquartile range for experiments analysed by non-
376 parametric tests. n values represent the number of independent experiments performed, the number of
377 individual mice or patient specimens. For each independent *in vitro* experiment, at least two technical
378 replicates were used and a minimum number of three experiments were done to ensure adequate
379 statistical power. For data mining analysis, ANOVA test was used for multi-component comparisons
380 and Student T test for two component comparisons. In the *in vitro* experiments, normal distribution was
381 confirmed or assumed (for $n<5$) and Student T test was applied for two component comparisons. In the
382 statistical analyses involving fold changes, one sample t-test with a hypothetical value of 1 was
383 performed. The confidence level used for all the statistical analyses was of 95% (alpha value = 0.05).
384 Two-tail statistical analysis was applied for experimental design without predicted result, and one-tail
385 for validation or hypothesis-driven experiments.

386 Gene expression array data analysis: first, raw expression data were background-corrected, log2-
387 transformed and quantile-normalized using the lumi R package7

388 , available through the Bioconductor repository. Probes with a “detection p-value” lower than 0.01 in at
389 least one sample were considered expressed. For the detection of differentially expressed genes, a
390 linear model was fitted to the probe data and empirical Bayes moderated t-statistics were calculated
391 using the limma package from Bioconductor. Only genes with differential fold-change (FC) >1.5 or <-
392 1.5 and an adjusted p-value < 0.05 were considered as differentially expressed.

393 **Accession numbers and datasets**

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394 Primary accessions: The transcriptomic data generated in this publication have been deposited in
395 NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number
396 GSE114345 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114345).

397 Referenced accessions: TCGA https://cancergenome.nih.gov/, Grasso *et* al., GEO: GSE35988 (9);
398 Lapointe *et* al., GEO: GSE3933 (10); Taylor *et* al., GEO: GSE21032 (11); Tomlins *et* al., GEO:
399 GSE6099 (12); Varambally *et* al., GEO: GSE3325 (13); and Glinsky et al (8).

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400 **AUTHOR CONTRIBUTIONS**

401 LV-J and AM performed the majority of *in vitro* and *in vivo* experiments, unless specified
402 otherwise. NM-M contributed to the in vivo experiments, experimental design and
403 discussion. ARC carried out the bioinformatics and biostatistical analysis. AS-C, MP-V,
404 IH and IA contributed to *in vitro* analysis and provided technical support. IL-V, AS-M, AL-
405 I and MU-U provided BPH and PCa samples for gene expression analysis from Basurto
406 University Hospital. MGr carried out microvessel staining and quantifications. JS
407 contributed as supervisor of IH. JDS and RB performed or coordinated (RB) the cloning
408 of *MITFA* in lentiviral vectors. AMMA contributed to experimental design and discussion.
409 AC contributed to experimental design, data analysis and discussion. VT supervised the
410 project, contributed to the experimental design, data generation, analysis and discussion
411 and wrote the manuscript.

412

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564 # Figure legends

565 **Figure 1. MITF expression correlates with PGC1A expression and is**
566 **downregulated in PCa. A.** Schematic representation of candidate screening to mediate
567 PGC1A downregulation in PCa. Candidate selection was performed by applying two
568 different selection criteria based on the consistency within the datasets used (>50%): the
569 expression of the candidate must be consistently (i) correlated with the PGC1A´s and (ii)
570 altered in the disease. **B.** Correlation analysis between PGC1A and MITF expression in
571 primary tumor (PT) specimens of different PCa datasets ((9-11) and TCGA provisional).
572 Sample sizes: Grasso n=45; Lapointe n=13; Taylor n=131 and TCGA provisional n=495.
573 **C.** MITF expression in normal prostate and primary tumors (PT) specimens in different
574 data sets (9-11). Correlation (B) and expression (C) data from Taylor dataset
575 corresponds to the mean signal of all isoforms of the transcripts. In B and C, each dot
576 corresponds to an individual specimen. Sample sizes: Grasso et al. (Normal, n=12; PT,
577 n=45); Lapointe et al. (Normal, n=9; PT, n=13); Taylor et al. (Normal, n=29; PT, n=131).
578 Error bars represent s.e.m. Statistic test: Spearman correlation *R* (B) and Mann Whitney
579 test (C). p, p-value.

580 **Figure 2. MITF exhibits tumor suppressive activity in PC3 PCa cell line. A-B.**
581 Analysis and quantification of MITF expression by qRTPCR (A, n=8)) and western blot
582 (B, representative experiment out of 3 independent ones) in PC3 TRIPZ-MITFA cells
583 after treatment with 0.5 µg mL^{-1} doxycycline (Dox). **C.** Relative cell number quantification
584 by crystal violet in doxycycline treated and non-treated PC3 TRIPZ-MITFA cells. Data is
585 normalized to day 0. Asterisks indicate statistics of 5 independent experiments. One
586 representative experiment out of 5 is shown. Error bars represent standard deviation. **D-**
587 **E.** Effect of MITF induction on anchorage independent growth (D, soft agar; n=4
588 independent experiments) and BrdU incorporation (E, n=3 independent experiments). **F.**
589 Impact of MITF induction in tumor growth rate of PC3 TRIPZ-MITFA cells (n=7 animals
590 per group; 14 injections/tumors). No dox: MITFA non-induced conditions; Dox: MITFA

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591 induced conditions. Error bars represent s.e.m (A, D and E) or minimun and maximum
592 values (H). Statistic test: One sample t-test (A, D and E) and Student t-test (C and F. *p
593 < 0.05, **p < 0.01, ***p < 0.001.

594 **Figure 3. CRYAB is the candidate to mediate its tumor suppressive activity in PCa.**
595 **A.** Workflow of the candidate screening. **B.** Correlation analysis between MITF and
596 CRYAB expression in primary tumor (PT) specimens of different PCa datasets. Sample
597 sizes: Taylor, n=131; Grasso, n=49; Lapointe, n=13; TCGA provisional data, n=495; and
598 Glinsky, n=78. **C.** CRYAB expression in normal prostate and primary tumor (PT)
599 specimens in different PCa datasets (9-13). Sample sizes: Taylor (N, n=29; PT, n=130);
600 Grasso (N, n=12; PT, n=49); Varambally (N, n=6; PT, n=7); Lapointe et al. (N, n=9; PT,
601 n=13) and Tomlins (N, n=22; PT, n=32). Data from Taylor dataset corresponds to the
602 mean signal of all isoforms of the transcripts. In B and C, each dot corresponds to an
603 individual specimen. **D.** Western blot analysis of CRYAB expression in benign prostatic
604 hyperplasia (BPH) and PCa specimens from Basurto University Hospital cohort (BPH
605 n=7 patient specimens; PCa n=14 patient specimens). **E.** Chromatin
606 immunoprecipitation (ChIP) of exogenous MITF on CRYAB promoter in PC3 TRIPZ-
607 MITFA cells after induction with 0.5 µg mL-1 doxycycline for 3 days (n=4-5). Binding to
608 ANGPT4 was used as a negative control. Final data was normalized to IgG (negative-
609 immunoprecipitation control) and to No dox condition. No dox: MITFA non-induced
610 conditions; Dox: MITFA induced conditions. Statistic tests: Spearman correlation (B);
611 Mann Whitney test (C); one sample t test (E); Error bars represent s.e.m. *p < 0.05, **p
612 < 0.01.

613 **Figure 4. CRYAB mediates the tumor suppressor activity of MITF. A.** Analysis of
614 MITFA and CRYAB protein expression in doxycycline-treated PC3 TRIPZ-MITFA cells
615 transduced with shScramble (scr) or two independent shCRYAB (sh#1 and#2) (one
616 representative experiment with technical duplicates is shown; similar results were
617 obtained in three independent experiments). **B.** Relative cell number quantification by

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618 crystal violet in doxycycline-treated and non-treated PC3 TRIPZ-MITFA cells, in the
619 presence (scr) or absence (sh#1, 2) of CRYAB (n=5 independent experiments). Data is
620 normalized to day 0 and represented as cell number at day 6 relative to No Dox condition
621 (depicted by a dotted line). **C-D**. Effect of CRYAB silencing on anchorage independent
622 growth (C, soft agar; n=4 independent experiments) and BrdU incorporation (D, n=3
623 independent experiments) in PC3 TRIPZ-MITFA cells after treatment with 0.5 μg mL^{-1}
624 doxycycline. **E**. Impact of CRYAB silencing in tumor growth rate of MITF-induced cells
625 (n=10 animals per group-scr or sh#1; 2 injections per mice; (scr No dox, n=10 tumors;
626 sh#1 No dox, n=8 tumors; scr Dox, n=6 tumors; sh#1 Dox, n=11 tumors). **F**. Association
627 of the mean signal of MITF and CRYAB with disease-free survival (DFS) in three PCa
628 data sets (Q1: first quartile distribution; rest: second, third and fourth quartile distribution.
629 Sample sizes: Taylor, primary tumors n=131; TCGA provisional data primary tumors
630 n=490; Glinsky, primary tumors n=78. No dox: MITFA non-induced conditions; Dox:
631 MITFA induced conditions. HR: Hazard Ratio. Statistic tests: One sample t test (B, C and
632 D – No dox vs Dox conditions); Unpaired Student *t*-test (*t*) (B, C and D – Dox-treated scr
633 vs Dox-treated sh#1/2); Log-rank (Mantel-Cox) test (F). Error bars represent s.e.m. */$
634 $p < 0.05$, **/$$p < 0.01. Asterisks indicate statistic between No dox and Dox conditions
635 and dollar symbol between Dox-treated scr and Dox-treated sh#1 or 2.

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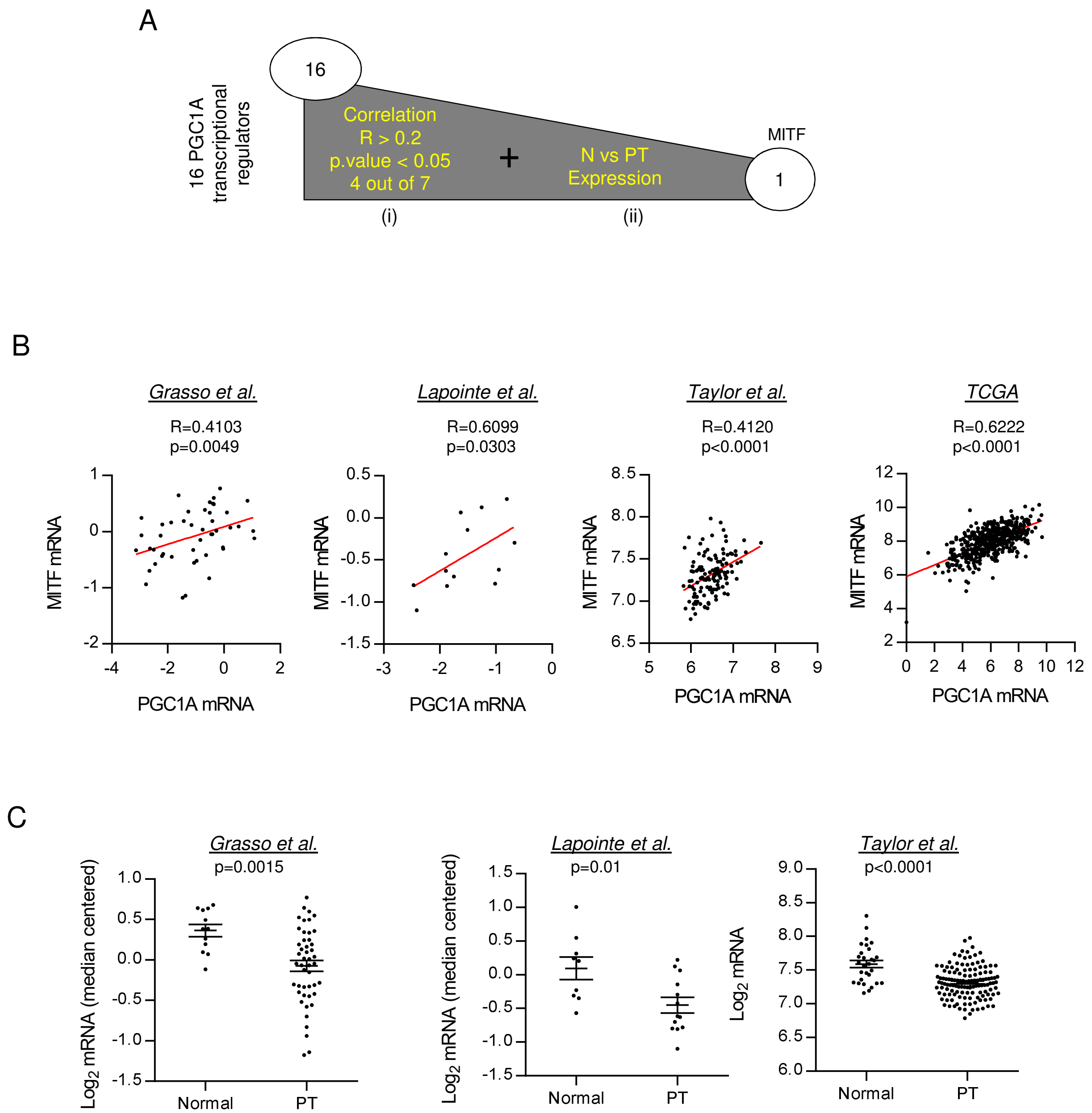

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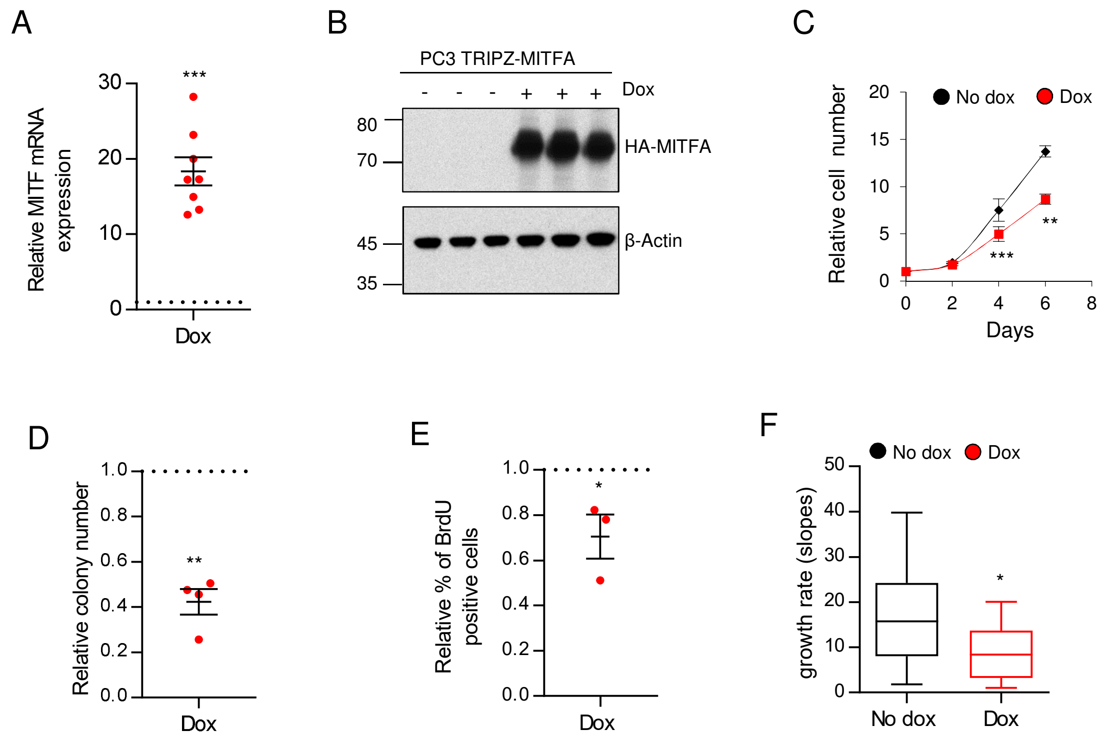
Valcarcel-Jimenez at al. Figure 2



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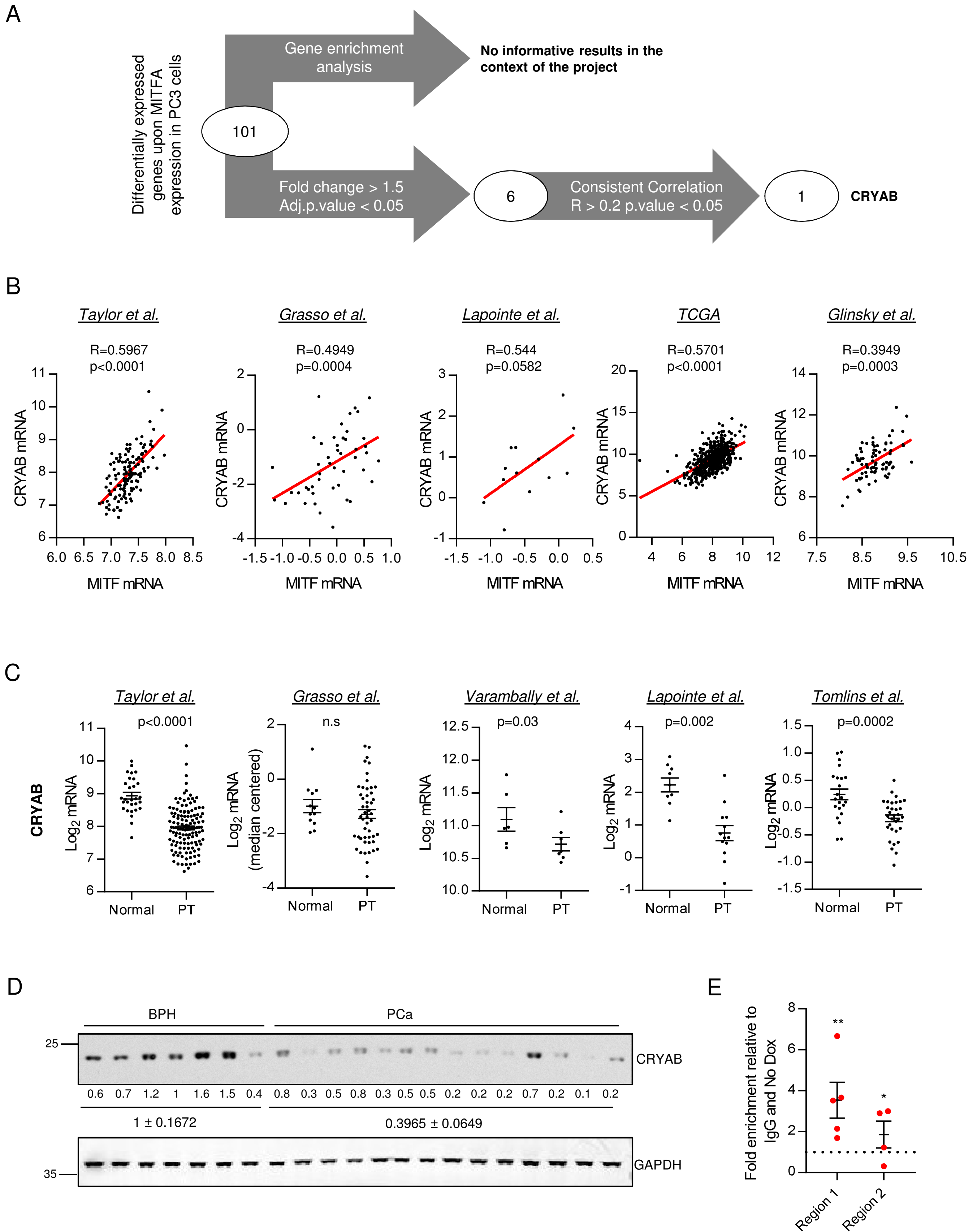
Valcarcel-Jimenez at al. Figure 3



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Valcarcel-Jimenez at al. Figure 4