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A novel MYCN-specific antigene oligonucleotide deregulates mitochondria and inhibits tumor growth in MYCN-amplified neuroblastoma

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

Published Version:

A novel MYCN-specific antigene oligonucleotide deregulates mitochondria and inhibits tumor growth in MYCN-amplified neuroblastoma / Montemurro L.; Raieli S.; Angelucci S.; Bartolucci D.; Amadesi C.; Lampis S.; Scardovi A.L.; Venturelli L.; Nieddu G.; Cerisoli L.; Fischer M.; Teti G.; Falconi M.; Pession A.; Hrelia P.; Tonelli R.. - In: CANCER RESEARCH. - ISSN 0008-5472. - STAMPA. - 79:24(2019), pp. 6166-6177. [10.1158/0008-5472.CAN-19-0008]

Availability:

This version is available at: <https://hdl.handle.net/11585/714781> since: 2020-01-17

Published:

DOI: <http://doi.org/10.1158/0008-5472.CAN-19-0008>

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(Article begins on next page)

1 **A novel MYCN-specific antigene oligonucleotide deregulates mitochondria and inhibits tumor**
2 **growth in MYCN-amplified Neuroblastoma**

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13

14 **Running title:** New MYCN antigene causes mitochondrial deregulation in MNA-NB

15

16 **Keywords:** neuroblastoma, MYCN, antigene oligonucleotide, mitochondria, mitophagy.

17

18 **Additional information**

19 **Financial support:** R. Tonelli, A. Pession, P. Hrelia are funded by University of Bologna. G. Teti and M.
20 Falconi are funded by University of Bologna (Ricerca fondamentale orientata - RFO 2017, 2018) and by
21 Fondazione del Monte di Bologna e Ravenna. L. Montemurro, S. Angelucci, and D. Bartolucci are funded by
22 AGEOP. S. Raieli, C. Amadesi, S. Lampis, A. Scardovi, G. Nieddu, and L. Cerisoli are funded by Biogenera SpA.

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25 **Disclosure of Potential Conflicts of Interest:** R. Tonelli and A. Pession are BIOGENERA shareholders and
26 members of the BIOGENERA board. S. Raieli, C. Amadesi, S. Lampis, A. Scardovi, G. Nieddu, and L. Cerisoli
27 are working at BIOGENERA. L. Venturelli is a former BIOGENERA employer. No potential conflicts of interest
28 were disclosed by the other authors.

29 **Others:**

30 **Word count:** 5082

31 **Figures:** 6 (plenary figures), 13 (supplementary figures), Tables (8 supplementary data tables)

32

33 **Abstract**

34 Approximately half of high-risk neuroblastoma (NB) is characterized by MYCN-amplification. N-Myc
35 promotes tumor progression by inducing cell growth and inhibiting differentiation. MYCN has also been
36 shown to play an active role in mitochondrial metabolism, but this relationship is not well understood.
37 While N-myc is a known driver of the disease, it remains a target for which no therapeutic drug exists. Here,
38 we evaluated a novel MYCN-specific antigene PNA oligonucleotide (BGA002) in MYCN-amplified (MNA) or
39 MYCN-expressing NB, and investigated the mechanism of its anti-tumor activity. MYCN mRNA and cell
40 viability were reduced in a broad set of NB cell lines following BGA002 treatment. Furthermore, BGA002
41 decreased N-myc protein levels and apoptosis in MNA-NB. Analysis of gene expression data from
42 neuroblastoma patients revealed that MYCN was associated with increased reactive oxygen species (ROS),
43 downregulated mitophagy and poor prognosis. Inhibition of MYCN caused profound mitochondrial damage
44 in MNA-NB cells through downregulation of the mitochondrial molecular chaperone TRAP1, which
45 subsequently increased ROS. Correspondingly, inhibition of MYCN reactivated mitophagy. Systemic
46 administration of BGA002 downregulated N-myc and TRAP1 with a concomitant decrease in MNA-NB
47 xenograft tumor weight. In conclusion, this study highlights the role of N-myc in blocking mitophagy in NB
48 and in conferring protection to ROS in mitochondria through upregulation of TRAP1. BGA002 is a potentially
49 improved MYCN-specific antigene oligonucleotide that reverts N-myc dysregulated mitochondrial
50 pathways, leading to loss of the protective effect of N-myc against mitochondrial ROS.

51

52 **Significance:**

53 A second generation antigene peptide oligonucleotide targeting MYCN induces mitochondrial damage and
54 inhibits growth of MYCN-amplified neuroblastoma cells.

55

56 **Introduction**

57

58 Neuroblastoma (NB) is the deadliest pediatric tumor. While patients with a low or intermediate risk have a
59 favorable outcome, the high-risk group has a survival rate below 50% (1). The latter group often presents
60 with MYCN amplification (50% of the high risk group) (2). N-Myc is a well-known driver of the disease (3)
61 and is strongly associated with poor survival prognosis (4,5). N-Myc promotes cell growth, inhibits cell
62 differentiation while maintaining a stem-like phenotype; its levels correlate with metastasis and the
63 induction of angiogenesis (6,7). Furthermore, MYCN over-expression affects metabolism to support the
64 higher energy demand of the tumor cells (8–10). Beyond increasing glycolysis and glutaminolysis, N-Myc is
65 involved in mitochondrial functional alteration, however, the mechanism of this effect is not fully
66 understood (11).

67 Interestingly, MYCN is expressed during embryogenesis and is virtually absent during adulthood (12). All
68 these factors make N-Myc a promising target for neuroblastoma therapy. However, in order for an inhibitor
69 to be effective, it should either interfere with the N-Myc/MAX heterodimers, or with N-Myc interaction
70 with DNA, without inhibiting the highly homologous myc. These requirements have led to N-Myc being
71 currently considered an unlikely target for therapeutic intervention (13).

72 Although indirect therapeutic approaches in combating neuroblastoma by inhibiting N-Myc have been
73 proposed, considering the broad role of MYCN in neuroblastoma and the lack of a complete understanding
74 of its mechanism, the challenge still remains. Given the difficulties encountered in developing a small
75 molecule inhibitor, other approaches including the use of oligonucleotides, have been tested to inhibit the
76 MYC family (14,15). Differing from the use of antisense oligonucleotides, which inhibit mRNA translation,
77 the antigene approach involves binding to chromosomal DNA, resulting in the inhibition of transcription.
78 By persistently blocking transcription, the antigene oligonucleotides showed higher efficacy compared to
79 antisense oligonucleotides (14–17). Furthermore, PNAs demonstrated potent and specific antigene activity
80 (14–17) and higher therapeutic potential due to their resistance to nuclease degradation (18).

81 In the present work we show for the first time that BGA002, a new and highly improved antigene PNA
82 oligonucleotide, can specifically target a unique sequence on the MYCN gene. We also demonstrate a new
83 mechanism for the inhibition of MYCN, and ultimately confirm the efficacy of BGA002 *in vivo*.

84

85

86 **Materials and Methods**

87

88 **Cell lines.** All the cell lines used in this study were obtained during the 2018. Cell lines were obtained from
89 DSMZ (KELLY, LAN5, CHP-134, SiMa, MHH-NB11, NGP, LS, NMB, LAN-1, LAN-6, LAN-2, NBL-S), ECACC
90 (SK-N-DZ, SK-N-F1, NB69), ATCC (HEK293) and kindly gifted by Gaslini Institute, Genova (GI-LI-N, SMS-
91 KAN), by Professor Paolucci G. (SJ-N-KP), Professor Della Valle G (IMR32, SK-N-BE(2)-C, TET-21N) and by
92 professor Spampinato SM (SH-SY5Y). All cell lines are stored in liquid nitrogen and kept in culture for a
93 maximum of 30 days and less than 7 passages from the time they are obtained. The average number of
94 passages for each cell line used in this study is 3. Cell line authentication was not conducted. Cell lines were
95 verified to be negative for the presence of *Mycoplasma* every 3 months by a PCR-based method with the
96 kit LookOut Mycoplasma PCR Detection Kit (Sigma Aldrich) using the manufacturer's instructions. The list of
97 cell lines used in this study with additional details is available as part of the supplementary materials
98 (Supplementary Table 1).

99

100 **Cell line treatment.** BGA001 and BGA002 were produced by Biogenera. PNA-peptide was either available,
101 stored at -20°C, and ready for use, or freshly produced by the Chemistry department and delivered to the
102 Biology department after purification and dilution. PNA was designed and prepared according to
103 previously published studies (16,17). Cell lines were expanded in RPMI 1640, with 10% fetal bovine serum
104 (FBS). Adherent cells were detached with PBS-EDTA, collected, washed, and centrifuged. Cells were
105 counted and resuspended in OPTIMEM. For PNA-peptide treatment (BGA001 and BGA002), 50000 cells
106 were plated in a 24-well flat-bottom plate for RNA extraction, 5000 cells were plated in a 96-well flat-
107 bottom plate for cell viability assays. Cell lines were treated with increasing concentrations (range: 0.08 µM
108 to a maximum of 20 µM) of PNA-peptide. Small interfering RNA (siRNA) for MYCN (sense:
109 UGAUGAAGAGGAAGAUGAAtt, antisense: UUCAUCUCCUCUCAUCAtt), TRAP1 (S179, Thermo Fisher
110 Scientific) were mixed with Lipofectamine (Invitrogen) and then diluted in OPTIMEM. Fifty thousand cells
111 were plated in a 24 well plate and incubated with siRNA (100 nM MYCN siRNA, 50 nM TRAP1 siRNA). After
112 6 hours of treatment, 4 % FBS was added to the cells.

113

114 **Quantitive real-time PCR.** After 12 hours, the cell lines were detached with PBS-EDTA, centrifuged, and
115 transferred to a 1.5 mL eppendorf tube. The pellet was lysed and stored at -20 °C. RNA was extracted using
116 the RNASpin Mini RNA isolation Kit (GE Healthcare). Prior to use, each sample of RNA was quantified with
117 the Nanodrop spectrophotometer (Thermo Fisher Scientific). One hundred ng of RNA was resuspended for
118 each sample. Retrotranscription and real-time PCR was performed as previously described (17). The list of
119 primers in this study is listed in Supplementary Table 2. Crossing points (Cp) from each analyte were

120 calculated using the second derivative maximum method, and the expression level was quantified by
121 comparison to the BIRC4 gene.

122

123 **Cell viability assay.** Four technical replicates were prepared for each experiment. After 72 hours of
124 treatment, the cells were treated according to the CellTiter-Glo Luminescent Cell Viability Assay protocol
125 (Promega). Luminescence was recorded with the Infinite F200 instrument (Tecan). The percentage of the
126 effect was calculated based on mean luminescence of the control.

127

128 **Western blot.** Cell were lysed 24 hours post treatment in sample lysing buffer (RIPA buffer (150 mM NaCl,
129 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) with Halt protease
130 inhibitor cocktail (Thermo Fisher Scientific). For N-Myc staining, the pellet was resuspended in sample
131 lysing solution on ice (about 50 µL for 5x10⁵ cells), and homogenized with a probe sonicator on ice. For
132 OPTN, TRAP1, and, cytochrome c staining, mitochondria were isolated from cultured cells as previously
133 described (19). Total protein extract was quantified using the BCA method with NanoDrop ND-1000
134 spectrophotometer against a standard curve of BSA in sample lysing solution. Ten to thirty µg of protein
135 was mixed with Bolt® Sample Reducing Agent (10X), and Bolt® LDS Sample Buffer (4X) (both from Thermo
136 Fisher Scientific). The samples were then denatured and loaded with SeeBlue® Plus2 Pre-stained Protein
137 Standard and SuperSignal® Enhanced Molecular Weight Protein Ladder onto a Polyacrylamide Bolt® Bis-Tris
138 Plus Gel and run with Bolt® MES SDS Running Buffer (20X). The gel transfer was conducted with the iBlot™
139 Gel Transfer System. Five percent dry milk in PBS-Tween (1X PBS, 0.1% Tween-20) was used as the blocking
140 solution. The membrane was incubated with the following antibodies: N-Myc (SCsc-53993, Santa Cruz), B-
141 Tubulin (SC-9104, Santa Cruz), OPTN (sc-166576C2, Santa Cruz), TRAP1 (sc-13557TR1, Santa Cruz), and
142 Cytochrome C (sc-13156A8, Santa Cruz) diluted in 3.5% BSA, PBS-0.1% Tween-20. The secondary antibody
143 used was anti-mouse antibody (SCsc-2031 HRP, Santa Cruz). Super Signal® West Pico was used as the HRP
144 substrate.

145

146 **Apoptosis analysis.** Kelly cell lines were treated as described above. The Tet21N cells were cultured with or
147 without tetracycline for at least 72 hours and were then detached, washed, and stained with annexin V / PI
148 (ROCHE) according to the manufacturer's instructions. The cell samples were analyzed by the CytoFLEX
149 flow cytometer (Beckman Coulter). The data were analyzed with FlowJo software (Tree Star).

150

151 **Mitochondrial net analysis.** Kelly (60,000 cells) were seeded on 12x12mm circular glass in a 24-well plate
152 for 24 hours in Optimem medium, treated with 5 µM BGA002 or 50 nM TRAP1 siRNA with the addition of
153 4% FBS 6 hours post treatment. At the end of the treatment, MitoTracker® Deep RedFM (M22426; Thermo
154 Fisher Scientific) was added according to the manufacturer's instructions. Cells were fixed with 4% PFA and

155 were mounted on a glass slide with SlowFade™ Diamond Antifade Mountant (S36967; Thermo Fisher
156 Scientific). Images were acquired with a confocal microscope (Leica TCS LS). ImageJ was used to capture
157 the images. Briefly, the signal was reduced with a subtraction command with a value of 25 and the images
158 were convolved with a Gaussian blur ($\sigma = 1$)

159

160 **Transmission electron microscopy.** Kelly cells were seeded onto a 24x24 mm square glass support in a 6-
161 well plate overnight. PNA oligo treatment was performed as described above. Tet21N cells, cultured for at
162 least 72 hours with or without tetracycline, were seeded as described above, and treated overnight with
163 60 μ M chloroquine (vesicular blocking) (20). After 24 hours, fixative solution (2.5% glutaraldehyde in
164 cacodylate buffer 0.1M pH 7.4) was added for 2 hours. The samples were then stored in cacodylate buffer
165 at 4°C. Cells were then post fixed with a solution of 1% osmium tetroxide in 0.1 M cacodylate buffer and
166 embedded in epoxy resins after a graded-acetone serial dehydration step. Ultrathin slices of 100 nm were
167 stained by uranyl acetate solution and lead citrate, and then analyzed by a transmission electron
168 microscope, CM10 Philips (FEI Company) at an accelerating voltage of 80 kV. Images were recorded with a
169 Megaview III digital camera (FEI Company).

170

171 **Bioinformatic analysis.** Neuroblastoma arrays were downloaded (E-MTAB-1781) and normalized. Briefly,
172 mitochondrial involved genes were selected from GO and the literature and used to train a self-organizing
173 map and to cluster patient gene expression profiles. Survival and differential expression gene analyses were
174 applied on the two found clusters. A self-organizing map and random forest model were used to perform
175 feature selection to build a score. ClueGO application was used to find pathway enrichment networks
176 differentially present in the two clusters. The genes in the ROS and Mitophagy pathway lists were obtained
177 from the ClueGO analysis. A detailed description was presented for the bioinformatic data in the extended
178 materials and methods (supplementary files).

179

180 **ROS measurement.** Kelly and Tet21N cell lines were seeded as described above. After 48 hours, the glass
181 support on which the cells were cultured were stained with 2',7'-dichlorofluorescein diacetate (DCFDA)
182 (Sigma-Aldrich) and MitoSOX™ Red Mitochondrial Superoxide Indicator (M36008, ThermoFisher) according
183 to the manufacturer's instructions. Cell were fixed as described above and analyzed by confocal
184 microscopy. The acquired data were processed by the ImageJ processing program. Kelly cells were treated
185 as described above, detached, stained with DCFDA, and subjected to flow cytometry with the CytoFLEX
186 cytometer (Beckman Coulter) for ROS quantification. Data were analyzed using FlowJo software.

187

188 **Neuroblastoma luminescent cells.** Phoenix-Ampho cells were transfected with Lipofectamine 2000
189 (Invitrogen) and plasmid pMMP-Lucneo (kindly provided by Professor Andrew Kung, Harvard Medical

190 School, Boston, MA). The viral particles were collected at 48 and 72 hours post transfection. The Kelly cell
191 line was spinoculated with the viral particles and polybrene (hexadimethrine bromide, Sigma). The cells
192 were subjected to selection for 15 days with 1 mg/mL of G418 (Calbiochem). The best cell clones were
193 selected and their luminescence was measured. The resulting cell line was named Kelly-*luc*.

194

195 **Xenograft ectopic neuroblastoma mouse model.** All experiments were approved by the Scientific Ethical
196 Committee of Bologna University (protocol n. 07/73/2013 and 564/2018-PR). Four to six week old
197 NOD/SCID CB17 mice of both sex were inoculated with 10×10^6 Kelly-*luc* cells in Corning®Matrigel® Matrix.
198 Mice were sedated with isoflurane prior to the injection. The pellet was inoculated by injection in the
199 dorso-posterior-lateral position. The growth of the tumor was evaluated by luminescence acquisition. D-
200 Luciferine was administered by I.P. injection. Luminescence was acquired by the UviTec imaging system
201 (Uvitec, Cambridge, UK). Treatment was performed after the tumor reached the pre-defined starting point
202 in the bioluminescent acquisition. PNA oligo was then administered to the treatment group every day for
203 14 days. The animals were sacrificed at day fifteen. The tumors were removed, measured, weighed, and
204 fixed in 4% formalin. For the event free survival curve, mice were treated daily with a dorsal subcutaneous
205 injection of 100 μ L of vehicle or BGA002 (10 mg/kg/day) for 28 days. Animals were monitored once every
206 other day for tumor diameter measurement (using a caliper) and total tumor volume was extrapolated. An
207 endpoint of at least 10mm tumor diameter and a total tumor volume of 523mm^3 was established.

208

209 **Immunohistochemistry.** The neuroblastoma tumors were dehydrated, embedded in paraffin, and cut into
210 4 μ m sections. Paraffin removal was accomplished by incubating histological slides in toluene followed by
211 incubation in ethanol. The slides were incubated in 2% H_2O_2 -methanol for inhibition of endogenous
212 peroxidase activity. Hydration was performed by serial incubation with 96% ethanol, 70% ethanol, and
213 distilled water. Antigen retrieval was performed by heat processing in 1mM EDTA, pH 8, for N-Myc
214 antibody and in 10mM Citrate pH 6 for TRAP1. The slides were blocked with 10% BSA in PBS, stained with
215 the N-Myc (OP13, Calbiochem), Ki-67 (MIB1, Dako), and TRAP1 (TR1, Santa Cruz) antibodies and
216 subsequently treated with secondary antibody (anti-mouse, Dako). The peroxidase coloration reaction was
217 performed using the Dako DAB kit. The slides were stained with haematoxylin, dehydrated, and mounted.
218 Images were acquired with the Leitz Diaplan microscope.

219

220 **Statistical analysis.** Statistical analysis was performed with the Prism software version 6 (GraphPad) or with
221 R software version 3.5. Python software version 3.0 was used to perform t-SNE. The different analyses and
222 tests were specifically designed for each experiment.

223

224 **Results**

225

226 **BGA002 is a novel MYCN-specific antigene oligonucleotide with potently improved MYCN transcriptional**
227 **inhibition**

228

229 We have previously reported on the effect of a MYCN-specific antigene PNA (agPNA) oligonucleotide
230 (BGA001) for the selective inhibition of MYCN in NB cell lines. This inhibition led to decreased transcription,
231 reduced cell viability, and apoptosis (16). Furthermore, the MYCN agPNA was able to inhibit MYCN
232 transcription in rhabdomyosarcoma cell lines, which led to anti-tumor activity *in vivo* in mice (17). Thus, our
233 first aim was to compare the effects of the novel MYCN-specific agPNA oligonucleotide (BGA002) with the
234 previous one (BGA001). BGA002 was conjugated to a nuclear localization signal (NLS) peptide for delivery
235 (16,21,22), because it was previously found to facilitate penetration of BGA001 into cells, without requiring
236 a transfection agent, and localization to the nucleus. Indeed, BGA002 showed potently enhanced activity in
237 down-regulating MYCN mRNA expression in comparison with the agPNA BGA001 in MNA-NB cells (Fig. 1A).
238 Moreover, BGA002 was much more efficient in reducing cell viability and N-Myc protein degradation than
239 BGA001 (Fig. 1B and 1C), and in inducing apoptosis at 24 and 48 hours (Fig. 1D and S1A). Therefore, as
240 demonstrated by the EC₅₀ comparison, BGA002 shows a stronger anti-tumor effect *in vitro* in comparison
241 with the previous agPNA (Supplementary Table 3).

242 To evaluate the *in vitro* activity of BGA002 in NB, we selected a panel of twenty cell lines to cover the broad
243 landscape of NB tumors: MNA cell lines (n = 10), MNA/p53_{mut} (n = 4), not-MNA (n = 5), and not-MNA/p53_{mut}
244 (n = 1). All the selected NB cell lines, showed expression of MYCN mRNA, with consistently higher levels
245 detected in MNA cell lines (Fig. S2A). BGA002 shows a strong dose-dependent inhibitory effect on MYCN
246 transcription and on cell viability (Fig. 1E and F, S2B and D). MNA cell lines were significantly more
247 susceptible to the effects of BGA002 as demonstrated by a lower EC₅₀ compared to the MNA/p53_{mut} cell line
248 (Fig. 1F).

249 As expected, BGA002 was MYCN-specific, and did not influence cell viability in the MYCN-unexpressed
250 HEK293 cells (Fig. S3A), while a mutated version of BGA002 (BGA002_{mut}) did not have any effect on MYCN
251 transcription (Fig. S3B), on cell viability of MYCN-expressing MNA-NB cells (Fig. S3C), on N-Myc downstream
252 targets (Fig. S3D), and on inducing apoptosis (Fig. S3E-F). Moreover, BGA002 bound to the unique target
253 DNA sequence in the MYCN gene, while BGA002_{mut} showed no binding (Fig. S3G).

254

255 **Specific MYCN-inhibition by BGA002 leads to profound mitochondrial damage in MNA-NB cells**

256

257 Since MYCN mRNA inhibition persisted at 48 hours (Fig. S2C), at which time we found extensive apoptosis
258 levels (Fig. 1D), we decided to perform transmission electron microscopy to investigate the leading cause of
259 this phenomenon. Ultrastructural analysis showed that MYCN inhibition by BGA002 caused profound

260 mitochondrial changes in MNA-NB cells. After 12 hours, we observed initial mitochondrial damage (Fig.
261 S4A) without concomitant apoptosis (Fig. S4B and C), suggesting that apoptosis is a consequence of the
262 observed phenomenon, rather than the cause. After 48 hours (or 72 hours) of BGA002 treatment, the
263 mitochondria had sustained extensive damage (Fig. 2A, Fig. S5C) while at 24 hours we noticed that
264 mitochondria became smaller and the cristae patterns were much less elaborate (Fig. S5B). Indeed,
265 mitochondrial alterations were not observed after treatment with a mutated control antigene PNA
266 (BGA002_{mut}), after 48 hours or at 72 hours (Fig. S5A and C).

267 Generally the distribution and connection pattern is indicative of mitochondrial mass and function (23,24).
268 While Mito-Tracker staining reveals that in the untreated MNA-NB cells the mitochondria are highly
269 interconnected, anti-MYCN BGA002 treatment disrupted these mitochondrial nets, and the mitochondrial
270 content appears to be reduced (Fig. 2B and S5D). Moreover, BGA002 induced a change in the mitochondrial
271 pattern, resulting in a perinuclear distribution (Fig. 2B). As before, BGA002_{mut} failed to affect the
272 mitochondrial nets (Fig. S5D). Interestingly, we observed much less mitochondrial damage and pattern
273 alteration after 48 hours of BGA001 (Fig. S6A-B). Furthermore, BGA002 treatment led to a decrease in
274 mitochondrial area per cell (25) in the MNA cells (Fig. S7A), and we observed a BGA002 dose-dependent
275 mitochondrial mass reduction after 48 and 72 hours of treatment (Fig. S7B-C).

276

277 **Alterations in mitochondrial pathways can identify neuroblastoma patients with poor survival prognosis**

278

279 To verify the impact of mitochondrial gene signature on neuroblastoma prognosis we selected genes from
280 the GO mitochondrial pathways and from the available literature (1718 genes, Supplementary Table 4). For
281 this purpose we used a publicly available dataset that included patient clinical annotations (26). We used
282 the mitochondrial related signature to conduct a self-organization map to separate gene expression profiles
283 from NB in two different clusters of patients (Fig. 3A-C, S8A, Supplementary Table 5). The two clusters have
284 a statistically significant difference in the overall survival rates and event-free probability (Fig. 3D, S8B),
285 with cluster 2 strongly linked with a poor survival prognosis. Indeed, cluster 2 shows a similar worsening of
286 the overall survival probability for the MNA patient subgroup (Fig. 3E). We further investigated which genes
287 had a larger effect on event-free and overall survival. For this purpose, we used a random forest model
288 optimized for censored data to conduct feature selection. The variables extracted from the model and from
289 the self-organization map were used to construct a MitoScore. The top 200 genes qualifying for MitoScore
290 are shown in word cloud (Fig. 3F, S8C, Supplementary Table 6). The genes present in MitoScore and
291 differentially expressed in the two clusters (Supplementary Table 7) were used to identify which functional
292 pathways were linked to each cluster. Filtering the insignificant pathways, we found 20 GO pathways
293 specific for cluster 1 and 45 for cluster 2 (Fig. 3G, Supplementary Table 8 and 9). As expected, analysis
294 highlighted the presence of a substantial number of folic acid pathway genes in cluster 2, since it is known

295 that MYCN amplified neuroblastomas have an enhanced dependency on folate (27). Moreover, cluster 2
296 contains a number of genes in response to ROS genes (RROS) (Fig. 3G, Supplementary Table 9). Conversely,
297 we found that cluster 1 contained a significant number of genes related to mitophagy (Fig. 3G,
298 Supplementary Table 9). Based on pathway analysis we built an RROS score and a mitophagy score. The
299 high presence of genes related to response to ROS was significantly predictive for overall survival. It was
300 also noted that that a lower number of genes present related to mitophagy significantly worsened the
301 overall survival probability (Fig. 3H). The RROS and mitophagy scores showed significant inverse correlation
302 (Pearson coefficient = -0.73, pvalue = 2.2e-16). Interestingly, the presence of MYCN correlated well with the
303 RROS score (0.63, pvalue = 2.2e-15) and showed inverse correlation with the mitophagy score (-0.73,
304 pvalue = 2.2e-16) (Fig. S8E). Ultimately, it was noted that the results from MYCN-amplified patients showed
305 a substantial presence of mitochondrial related signature genes and a higher RROS score in comparison
306 with patients lacking MYCN-amplification (Fig S9A)

307

308 **BGA002 reverts N-Myc dysregulated mitochondrial pathways in MNA-NB**

309

310 MYCN alteration of the transcriptional program is critical in promoting tumorigenesis in MNA-NB. Given
311 this view, we investigated the effects of MYCN inhibition by BGA002 on gene expression profiles in several
312 NB cell lines. As expected, genes present in the mitochondrial signature, and with a high score, show
313 different expression levels between cluster 1 and cluster 2 (Fig. 4A). Surprisingly, we found the same
314 behavior in MYCN-amplified versus MYCN non-amplified patients (Fig. 4A). Furthermore, we noticed that
315 these genes are significantly predictive for overall survival (Fig. 4B). We also found that different genes in
316 the mitochondrial signature correlate well or show inverse correlation with MYCN in the dataset of patient
317 gene expression profiles (Fig. 4C). Based on these results, we investigated if MYCN inhibition by BGA002
318 was able to down-regulate genes from the mitochondrial signature (Fig. 4D). MYCN inhibition led to the
319 down-regulation of a wide group of mitochondrial genes in the different NB cell lines. MYCN silencing by
320 BGA002 also down-regulated other previously described MYCN-related genes (Fig. 4D), including TERT and
321 SKP2 (28,29). The use of an anti-MYCN siRNA and the Tet21N cells (in which inducible MYCN silencing is
322 achieved by tetracycline administration, but not by BGA002, since these cells lack the agPNA target
323 sequence in the inserted MYCN construct (Fig. S10A)(30) as controls, resulted in the same gene expression
324 pattern (Fig. 4D). Interestingly, by confirming MYCN correlation with a response to ROS and an inverse
325 correlation with mitophagy as previously shown, we found that MYCN-inhibition down-regulated the gene
326 expression of TRAP1 while up-regulating the expression of OPTN (Fig. 4D). These genes are involved in
327 mitochondrial ROS control and in mitophagy, respectively (31–33). We also confirmed mitochondrial
328 protein production variation in MNA-NB cells, a concomitant decrease in TRAP1, and an increase in OPTN
329 production (Fig. 4E). Moreover, TRAP1 decrease and OPTN increase are accentuated at 48 hours (Fig. 4E).

330 Based on our finding that MYCN shows inverse correlation with the mitophagy score in NB and on the well
331 described important positive role of OPTN in this pathway (32), we investigated the inhibitory role of MYCN
332 on mitophagy activation. In Tet21N cells after MYCN-silencing (72 hours of tetracycline administration)
333 while not observing apoptosis, (Fig. S10B and C), we registered a dramatic decrease in mitochondrial
334 number (Fig. S10D). Concomitantly, we observed the appearance of a high number of myelin figures (Fig.
335 4F) and co-localization of mitochondria with lysosomes (Fig.S10E). Collectively, these findings are indicative
336 of mitophagy activity after MYCN inhibition. Finally, an siRNA anti-OPTN significantly reduced mitophagy
337 activity after MYCN silencing in Tet21N cells (Fig. S11A-D)

338

339

340 **BGA002 leads to loss of protective N-Myc effect against mitochondrial ROS through TRAP1** 341 **downregulation in MNA-NB**

342

343 Based on our finding of a positive correlation between MYCN expression and the RROS score in the poor
344 survival prognosis cluster 2 from the NB patient dataset (Fig. S8E), we investigated if MYCN inhibition by
345 BGA002 could induce augmentation of mitochondrial ROS production. Indeed, BGA002 treatment induced
346 up-regulation of the ROS and an increase in superoxide production (Fig. 5A, S12A). Since TRAP1 plays a
347 determining role in mitochondrial ROS control and considering that BGA002 treatment down-regulated
348 *TRAP1* expression, we investigated if TRAP1 inhibition led to an increment in ROS production in MNA-NB.
349 Indeed, siRNA against TRAP1 (siTRAP1) led to an increment in production of ROS in mitochondria (Fig. 5A).
350 TRAP1 downregulation by siTRAP1 also inhibited the mitochondrial net structure (Fig. 5B), similar to MYCN
351 inhibition after BGA002 treatment. Moreover, siTRAP1 consistently reduced cell viability (Fig. 5C). As a
352 control, we verified that siTRAP1 did not affect MYCN mRNA expression (Fig. 5D). Interestingly, in Tet21n
353 cells, which showed mitophagy reactivation after MYCN silencing (by tetracycline administration) and did
354 not undergo apoptosis, we did not find an appreciable increase in ROS production (Fig. S12B). Moreover,
355 BGA002_{mut} failed to induce ROS production, while BGA001 showed a modest effect (Fig. S12C) and the
356 latter did not consistently reduce TRAP1 mRNA expression (Fig. S12D). This mechanism is graphically
357 represented in Fig. S13A.

358

359 **BGA002 causes elimination of MNA-NB in mice**

360

361 Finally, we evaluated the *in vivo* anti-tumor activity of BGA002 in a xenograft murine model of MNA-NB.
362 We inoculated MNA-NB Kelly-luminescent cells, which were monitored until tumor luminescence was
363 detectable. Treatment with BGA002 resulted in a statistically significant augmentation of survival in
364 comparison with the vehicle (Fig. 6A, S13B).

365 Moreover, subcutaneous administration (daily for 15 days) of BGA002 resulted in a potent and dose-
366 dependent anti-tumor activity. BGA002 at 2.5 mg/kg/day caused a tumor weight decrease of 25%, while
367 the treatment with 5 mg/kg/day resulted in a significant decrease of more than 70%, and administration at
368 10 mg/kg/day led to tumor elimination (Fig. 6B). We concluded that treatment with BGA002 showed a
369 dose-response tumor growth inhibition.

370 After treatment with BGA002 at the intermediate dose (5 mg/kg/day), histological analysis revealed a
371 consistent reduction in tumor vascularization as compared to the vehicle group (Fig. 6C), while
372 immunohistochemical analysis, showed a consistent reduction in N-Myc protein staining, a decrease in Ki-
373 67, leading to reduced TRAP1 protein expression (Fig. 6C).

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378 Discussion

379

380 The critical role of N-Myc in cancer development and its association with poor survival prognosis is not
381 restricted to neuroblastoma, with a broad range of tumors available in which MYCN-amplification and over-
382 expression play a crucial role (34). Due to the highly restricted pattern of expression of MYCN in normal
383 cells, N-Myc represents an optimal target for tumor-specific therapy for MYCN-expressing tumors.

384 While the direct targeting of the N-Myc transcription factor protein is still challenging, antigene therapy by
385 targeting MYCN transcription has great potential in treating MYCN-expressing tumors, as we previously
386 demonstrated in the preclinical treatment of neuroblastoma and rhabdomyosarcoma by a MYCN-specific
387 antigene PNA (16,17).

388 Here we report for the first time, the preclinical results for BGA002, a novel MYCN-specific agPNA with
389 potentially improved ability to block MYCN transcription. The BGA002 sequence is complementary to a
390 unique target sequence in the human (and mouse) MYCN gene. BGA002 showed dose-dependent inhibition
391 of MYCN transcription and cell viability in a panel of twenty MYCN-expressing NB cell lines with or without
392 MNA. In comparison to BGA001, it showed a potentially enhanced ability to specifically decrease MYCN
393 mRNA and protein expression while decreasing the viability of NB cell lines.

394 Interestingly, BGA002 is more effective in NB cell lines with MNA versus p53-mutated or MYCN single copy
395 cell lines. Since, MYCN inhibition led to apoptosis, the higher EC₅₀ found in p53-mutated NB cells could be
396 explained by their higher resistance to apoptosis (35). MYCN inhibition led to the down- or up-regulation of
397 highly relevant genes involved in metabolism, cell cycle control, apoptosis, metastasis, and DNA repair.

398 Surprisingly, the main ultrastructural alteration that we found in MNA-NB cells after MYCN inhibition, was
399 alteration of mitochondrial structure and organization. Interestingly, BGA001, which showed much less
400 ability to induce mitochondrial alteration, exerted a lesser effect in the promotion of apoptosis. We also
401 showed that a gene expression signature related to mitochondria allows for the identification of
402 neuroblastoma patients with poor survival prognosis. Furthermore, we found that BGA002 treatment led to
403 the down- or up-regulation of different genes involved in this signature.

404 It is known that metabolic stress generally leads to autophagy (36), but its role in cancer is still controversial
405 (37,38). The impact of autophagy in neuroblastoma is also controversial (39) and depends on the p53
406 status of the cells (40). Moreover, mitophagy (a particular type of autophagy) is a fundamental process for
407 mitochondrial turnover and health, and its deregulation can result in neurodegenerative disease and cancer
408 insurgence (41,42). Interestingly, we found that low enrichment in mitophagy related genes is significantly
409 predictive for a poor survival prognosis in a large dataset of gene expression profiles from NB patients.
410 Furthermore, we noticed that MYCN inhibition led to the up-regulation of OPTN, which plays an important
411 role in mitophagy induction (32,33). Moreover, blocking MYCN expression in Tet21N cells led to the
412 disappearance of mitochondria with concomitant presence of myelin figures, and co-localization of

413 lysosomes with mitochondria, indicating mitophagy activation. This phenotype is significantly reduced after
414 siRNA anti-OPTN administration, indicating that N-Myc blocks mitophagy through OPTN silencing.

415 ROS generation in mitochondria plays a role in cancer initiation, with many mitochondrial processes leading
416 to ROS generation in tumor cells (43). Notwithstanding their role in tumorigenesis, ROS excess in cells leads
417 to damage and ultimately to apoptosis (44). TRAP1 is a mitochondrial chaperone protein that plays a crucial
418 role in mitochondrial homeostasis (31), and its down-regulation corresponds to an increase in ROS
419 presence, and to a higher susceptibility to oxidative stress (31). We found that inhibition of MYCN led to
420 TRAP1 down-regulation, an increase ROS generation, and induction of apoptosis in MNA-NB cells.
421 Furthermore, BGA001 modestly reduced TRAP1 expression and showed a less effective ability to induce
422 ROS and promote apoptosis, indicating that N-Myc mitochondrial protection plays a relevant role in
423 neuroblastoma.

424 There is a growing corpus of evidence that mitochondrial fate is connected to tumor formation and
425 progression (45,46). Neuroblastoma insurgence leads to profound metabolic changes, where high risk
426 neuroblastoma shows a higher uptake of glucose and a reduction in oxidative phosphorylation in
427 mitochondria (47). Although it has been claimed that N-Myc was involved in mitochondrial lipid metabolism
428 in NB (11), its role in mitochondrial regulation in NB was largely unknown.

429 Here, we describe for the first time that N-Myc is relevant in mitochondrial structural maintenance and
430 turnover. Our work highlights the prognostic value of mitochondrial dysregulation in NB patients and
431 provides a mechanism on how N-Myc controls previously unknown aspects of mitochondrial function, by
432 inhibiting mitophagy, and controlling ROS generation.

433 Considering the role of N-Myc in a wide range of tumors, further studies on its close relationship with
434 mitochondria will provide other valuable insights in cancer biology. Furthermore, considering the role of
435 MYCN in mitochondrial ROS protection, it will be interesting to analyze the potential use of BGA002 in
436 conjunction with other therapies that induce ROS in cancer cells.

437 BGA002 has received orphan drug designation from the Food and Drug Administration (orphan registry:
438 DRU-2017-6085) and from the European Medicines Agency (orphan registry: EU/3/12/1016). Based upon
439 its well tolerated regulatory safety profile package, BGA002 is now moving to phase I clinical trials in
440 Neuroblastoma patients.

441

442 **Acknowledgments**

443 We would like to thank Wissem Eljeder and Simone Maestri for suggestions and comments. We would like
444 also to thank Andres E. Zucchetti from the Institut Curie for insightful suggestions on confocal microscopy
445 analysis.

446

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- 567

568 **Figure legends**

569

570 **Figure 1.**

571 **BGA002 is a new specific anti-MYCN antigene oligonucleotide with potently improved MYCN**
572 **transcription inhibition properties leading to cell-growth inhibition and apoptosis in Neuroblastoma cells.**

573 **A – E**, Comparison of the *in vitro* efficacy between neuroblastoma cells (Kelly and SK-N-BE(2)c) treated with
574 different doses of BGA001 and BGA002. Bars represent the mean, whiskers indicate standard deviation. **A**,
575 MYCN mRNA expression inhibition through RT-PCR after 12 hours of treatment, BGA001 (gray) and BGA002
576 (red) (n = 3, biological replicates for each cell line). **B**, Cell viability assay showing a decrease after 72 hours
577 of treatment, BGA001 (gray) and BGA002 (red) (n = 3, biological replicates for each cell-line). **C**,
578 Representative western blot analysis of N-Myc after 24 hours. Representative staining for N-Myc (above)
579 and whole-lane coomassie staining are presented (bottom). Quantification of N-Myc expression
580 (normalized with coomassie staining) is presented in the top panel. Bars represent the mean, whiskers
581 indicate standard deviation (n = 2, biological replicates for each cell line). BGA001 is shown on the left, and
582 BGA002 on the right, Kelly cell line is shown in the first line, SK-N-BE(2)c is shown in the second line. **D**, the
583 bar represents percentage of cells stained by Annexin V⁺ / PI⁺ for the cell line treated for 24 (left) or 48
584 hours (right) (n = 3, biological replicates for each cell-line). **E**, Heatmap representing different
585 neuroblastoma cell lines treated with different doses of BGA002. MYCN mRNA expression inhibition
586 through RT-PCR (left) after 12 hours of treatment and a decrease in cell viability after 72 hours of treatment
587 (right). The red scale represents the average percentage of inhibition of different biological replicates for
588 each cell line (n = 3) normalized to the control. **F**, mRNA MYCN inhibition (left) and vitality decrement
589 (right) EC₅₀ for each cell line grouped according to MYCN amplification and/or p53 mutation. Each dot
590 represents a singular experiment (n = 3 for each cell-line). Each point represents an individual sample,
591 middle line indicates the median, box limits indicate the first and third quartiles, the whiskers indicate
592 samples within 1.5 times the interquartile range (test = Wilcoxon matched pair test. pValue: *, p < 0.05, **,
593 p < 0.01, ***, p < 0.001).

594

595 **Figure 2.**

596 **BGA002 leads to MYCN-specific structural and functional alterations in mitochondria in MNA-NB cells.**

597 **A**, Transmission electron micrographs of the Kelly cell line treated for 48 hours with 5 μM BGA002.
598 Untreated cells are shown in the first row, BGA002 treated cells are shown in the second row.
599 Abbreviations used: M: mitochondrion, N: nucleus. **B**, Evaluation of mitochondrial nets in the Kelly cell line
600 treated for 48 hours with 5 μM BGA002.

601

602 **Figure 3.**

603 **Alterations in mitochondrial pathways identify neuroblastoma patients with poor survival prognosis.**

604 **A**, Schematic representation of the bioinformatic pipeline analysis conducted. A dataset of neuroblastoma
605 gene expression profiles (GEP) has been downloaded. Genes selected from the literature or listed in GO as
606 part of the mitochondrial associated terms were used to generate a mitochondrial related signature. A
607 self-organization map (SOM) was utilized to associate patient GEPs to two different clusters with a different
608 survival probability. The genes in the mitochondrial related signature were ranked by their contribution to
609 the separation of patient GEPs in two different clusters. Their contributions to predicting overall survival
610 and event-free survival were also taken into account to build a MitoScore for all the genes present in the
611 mitochondrial related signature. We used genes in the MitoScore that were differentially expressed to build
612 a functional grouped network of pathways. **B**, Heatmap showing the two different clusters (cluster1 in
613 green, cluster2 in red) derived from the self-organization map. Each square represents a neuron; the size of
614 the inner square is proportional to the number of patient GEPs associated with that neuron. **C**, T-
615 distributed stochastic neighbor embedding showing the clustering of the transcriptional profiles
616 (considering the genes present in the mitochondrial related signature) of the patient GEPs. Each dot
617 represents a patient transcriptional profile, the dots are colored according to which cluster they belong to
618 (cluster1 (n = 541) in green, cluster2 (n= 122) in red). **D**, Kaplan–Meier plots for the probability of overall
619 survival over time for patients associated with cluster 1 (green, n = 541) and cluster 2 (red, n = 161).
620 Associated P value (log-rank test) is shown in the middle. Hazard ratio and associated P value (log-rank test)
621 are shown in the bottom left of the plot. **E**, Kaplan–Meier plots for the probability of overall survival over
622 time for patients associated with cluster 1 (green) and cluster 2 (red), MNA patient (light red, n = 122) and
623 non-MNA patient (light green, n = 580). **F**, Word cloud of the top 200 genes ranked by MitoScore, the size is
624 proportional to the associated MitoScore. **G**, Functional grouped network of the pathways up-regulated in
625 cluster 1 (in green) and cluster 2 (in red). Circle size is proportional to the P value (FDR < 0.05). **H**, Kaplan–
626 Meier plots for the probability of overall survival over time for patients associated with Response to ROS
627 score (low enriched, n = 614, high enriched, n = 88, top panel) and Mitophagy score (low enriched, n = 73,
628 high enriched, n = 629, bottom panel). P value associated with the curve is shown in the middle of the plot
629 (log-rank test). Hazard ratio and associated P value (log-rank test) are shown in the bottom left of the plot.
630 (*, p < 0.05, **, p < 0.01, ***, p < 0.001)

631

632 **Figure 4.**

633 **Blocking of MYCN leads to MYCN-specific gene expression signature inhibition in NB cells and to**
634 **mitophagy reactivation.**

635 **A**, Expression of different genes presented in the mitochondrial related signature in cluster 2, cluster 1,
636 MNA and non-MNA patient gene expression profiles and presented as z-scores. Each point represents an
637 individual sample, middle line indicates the median, the whiskers indicate samples within 1.5 times the

638 interquartile range (statistical test = Wilcoxon). **B**, Kaplan–Meier plots for the probability of overall survival
639 over time for patients associated with different genes present in the mitochondrial related signature. For
640 NME4, TRAP1, MRPL11, PPRC1, MRPS2 the dark gray line represents a z-score > 1, for OPTN a z-score < -1.
641 Hazard ratio, p value (log-rank test) are shown in the bottom left of the plot. **C-D**, the gene names in the
642 middle refer to both panels. **C**, the color scale represents Pearson’s correlation coefficient of genes present
643 in the mitochondrial related signature (top) and other N-Myc targets (bottom) with MYCN in the patient
644 GEP dataset. **D**, Heatmap of the gene expression variation in neuroblastoma cell lines (MNA, MNA/p53-
645 mut, non-MNA, non-MNA/ p53-mut) after 12 hours of BGA002 treatment (5 μ M). On the right side of the
646 heatmap, Kelly cells treated with anti-MYCN siRNA, and Tet21N cells treated with tetracycline (72 hours)
647 are shown. The color scale represents the \log_2 fold change in comparison to the untreated cell line (n = 2
648 for each cell line), gray (gene not expressed). Upper part: genes present in the mitochondrial related
649 signature, bottom part: other N-Myc targets. **E**, Western blot analysis for TRAP1 (left, top row) and OPTN
650 (right, top row), cytochrome C (middle row), and coomassie staining (bottom row) of Kelly untreated cells
651 and BGA002 (5 μ M) treated cells for 24 (first line) and 48 hours (second line). **F**, Transmission electron
652 micrographs of Tet21N cultured cells treated without (first line) and with (second line) tetracycline and
653 chloroquine for 72 hours. From left to right, increasing magnification. Abbreviations used: M:
654 mitochondrion, MF: myelin figure, L: lysosome, N: nucleus. (*, p < 0.05, **, p < 0.01, ***, p < 0.001).

655

656 **Figure 5.**

657 **BGA002 reverts the MYCN control of ROS generation by TRAP1 down-regulation in MNA-NB cells.**

658 **A**, Representative confocal microscopy analysis of ROS production in Kelly untreated cells (first line), Kelly
659 cells treated with 5 μ M BGA002 (second line) for 48 hours, Kelly cells treated with anti-TRAP1 siRNA (third
660 line) for 24 hours. Mitosox staining in red (left), DCFDA staining in green (middle), and merge (right). From
661 left to right, increasing magnification. **B**, Evaluation of mitochondrial nets in the Kelly cell line treated for
662 48 hours with vehicle (first line), with 5 μ M BGA002 (second line), and 50 nM anti-TRAP1 siRNA. From left
663 to right, increasing magnification. **C**, Kelly cell line treated with 50 nM siRNA anti-TRAP1. TRAP1 percentage
664 of mRNA inhibition after 24 hours in the left panel, percentage of cell viability inhibition in the right panel
665 (n = 2) after 72 hours. **D**, Kelly cell line treated with 50 nM siRNA anti-TRAP1, TRAP1, and MYCN showing
666 percentage of mRNA inhibition after 24 hours (n= 3).

667

668 **Figure 6.**

669 **BGA002 causes elimination of MNA-NB in mice through TRAP1 down-regulation.**

670 **A**, Kaplan–Meier plots for the probability of event-free survival over time for mice (Kelly-luc xenograft)
671 treated with vehicle (red, n = 6) and BGA002, 10 mg/kg/day (green, n = 8). Associated P value (log-rank test)
672 is shown in the middle. Hazard ratio and associated P value (log-rank test) are shown in the bottom left of

673 the plot. **B**, Evaluation of tumor weight in neuroblastoma xenograft mice treated with different doses of
674 BGA002 (untreated (n=21), 2.5 (n= 11), 5 (n=8), 10 (n=9) mg/kg/day). Each dot represents a mouse (test =
675 Mann Whitney test). The table below the graph indicates the mean value of tumor weight reduction for
676 each treatment dose in comparison to the control. **C**, Immunohistochemical analysis of neuroblastoma
677 xenograft mice untreated (first line) or treated with 5 mg/kg/day BGA002(second line). Images of sections
678 are shown stained with haematoxylin and eosin (first), Ki-67 antibody (second), N-Myc antibody (third), and
679 Trap1 antibody (last). Similar results were obtained from four independent mice. (*, $p < 0.05$, **, $p < 0.01$,
680 ***, $p < 0.001$)
681

Figure 1

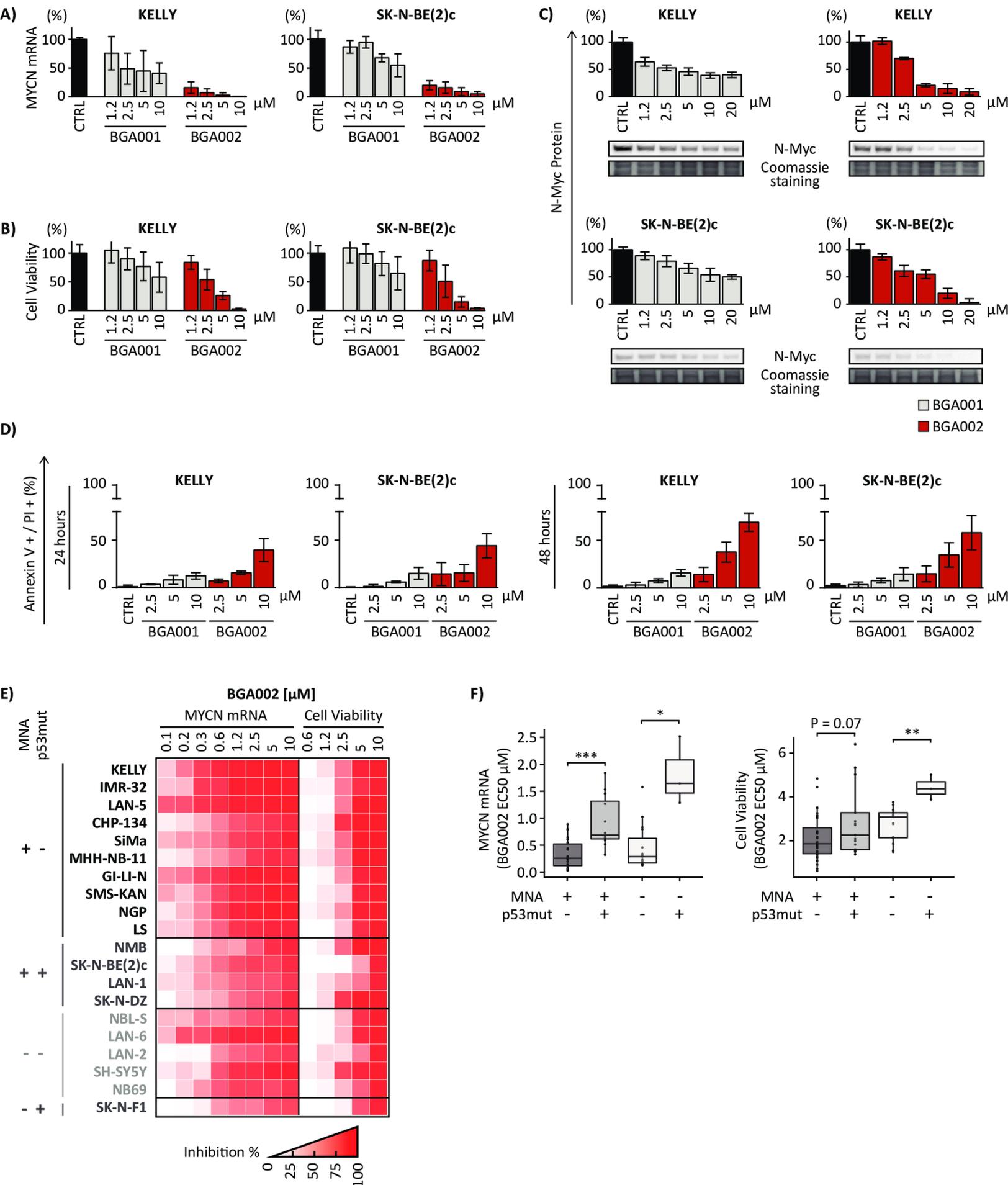
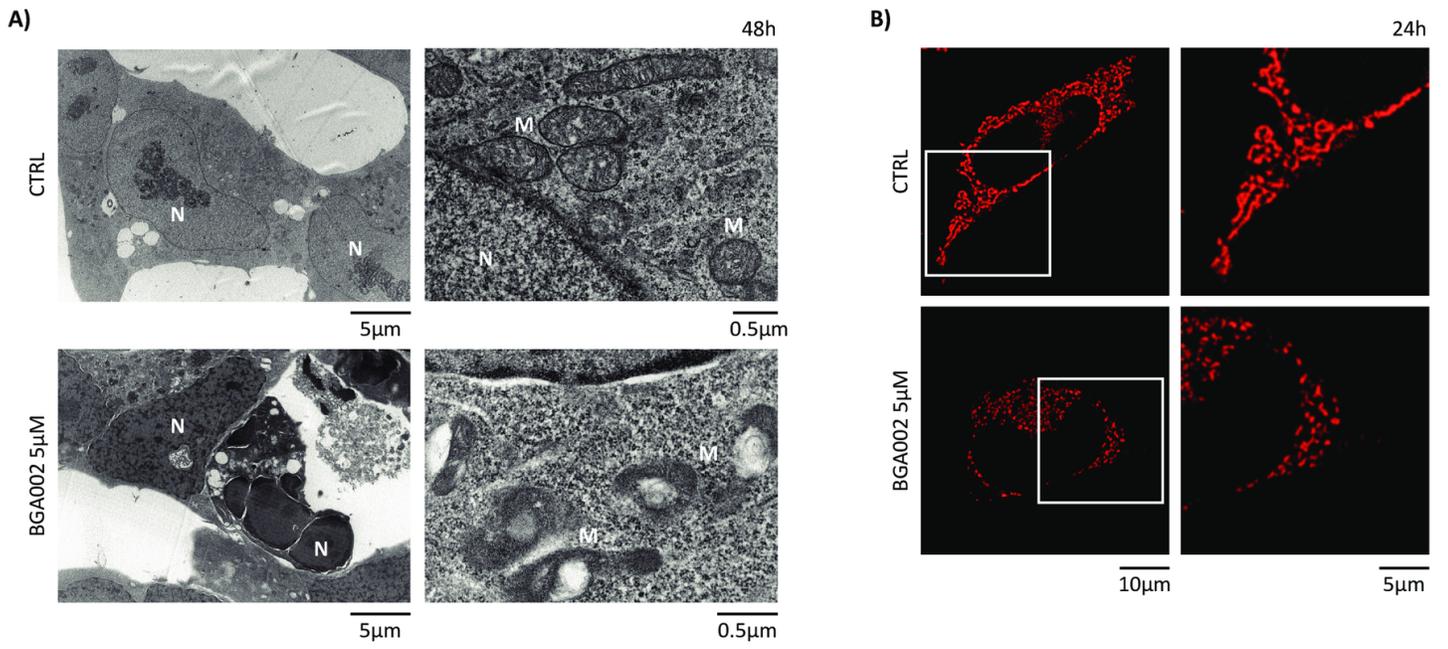
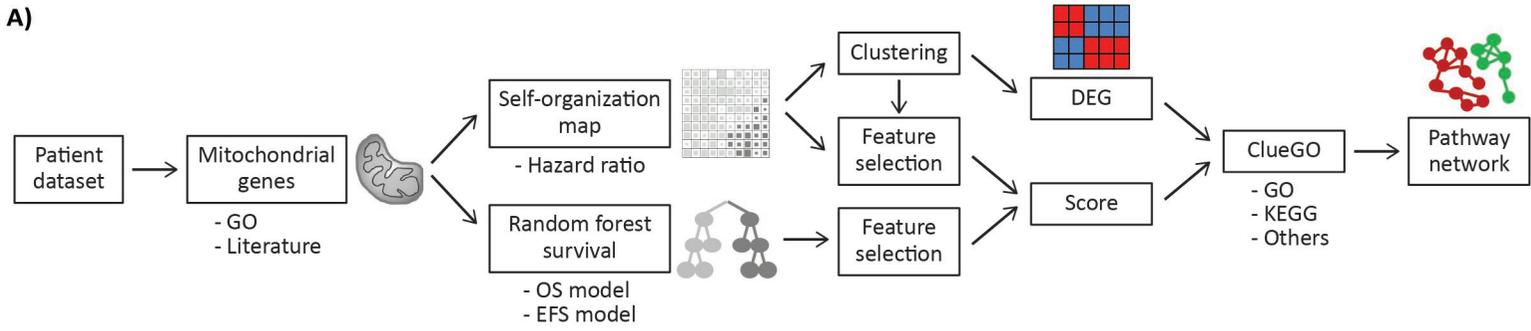


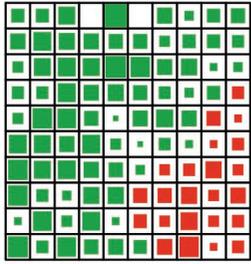
Figure 2



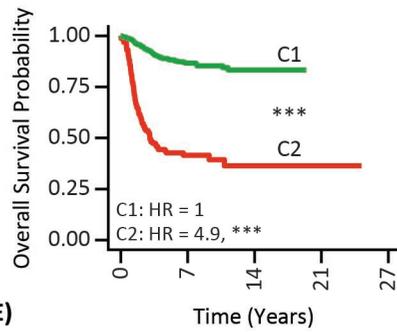
A)



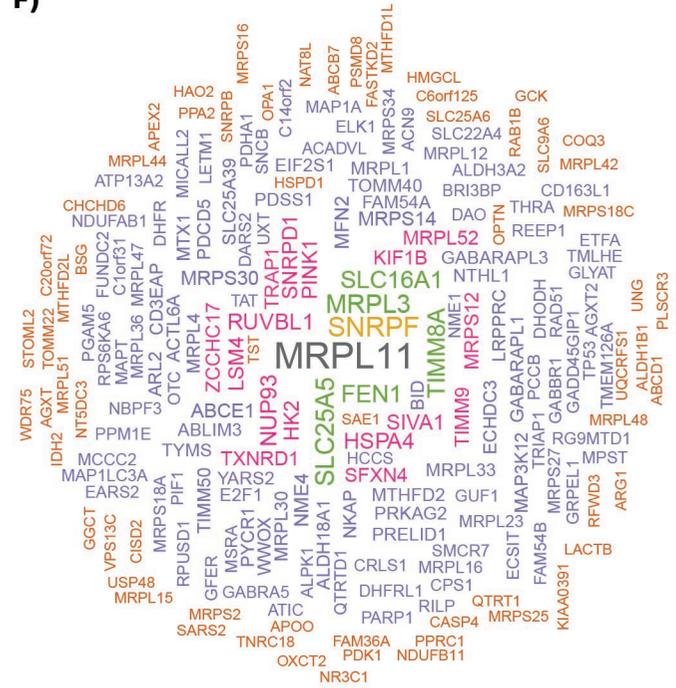
B)



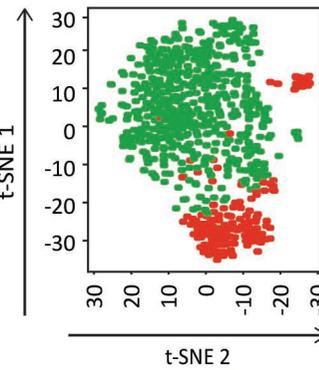
D)



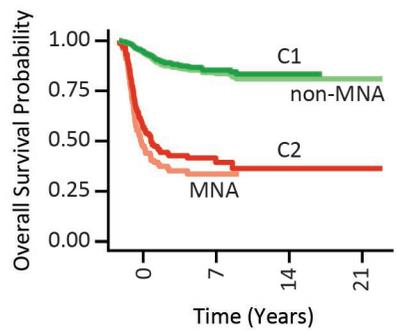
F)



C)

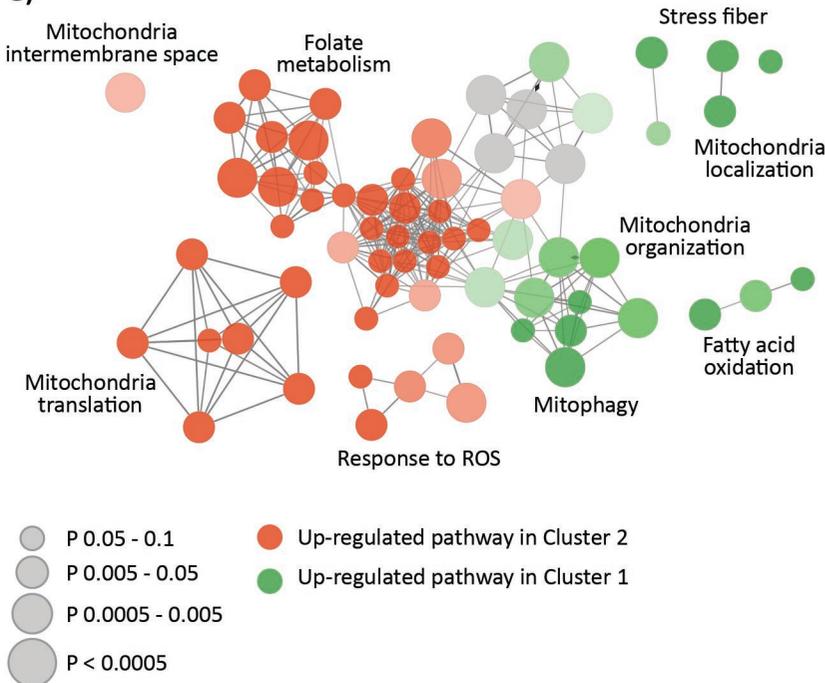


E)



■ C1 = Cluster 1 ■ MNA = MYCN-Amplified
■ C2 = Cluster 2 ■ non-MNA = MYCN Non-Amplified

G)



H)

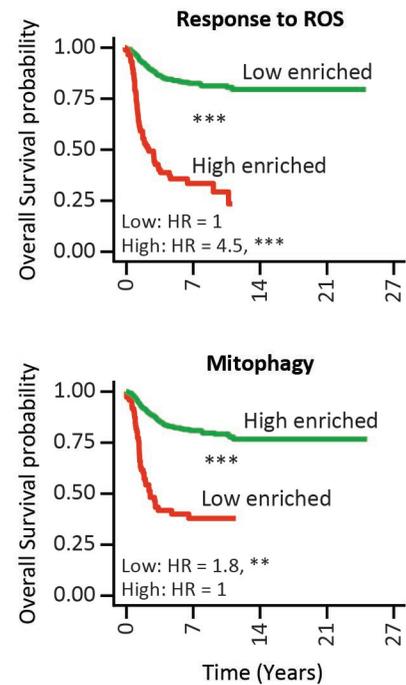


Figure 4

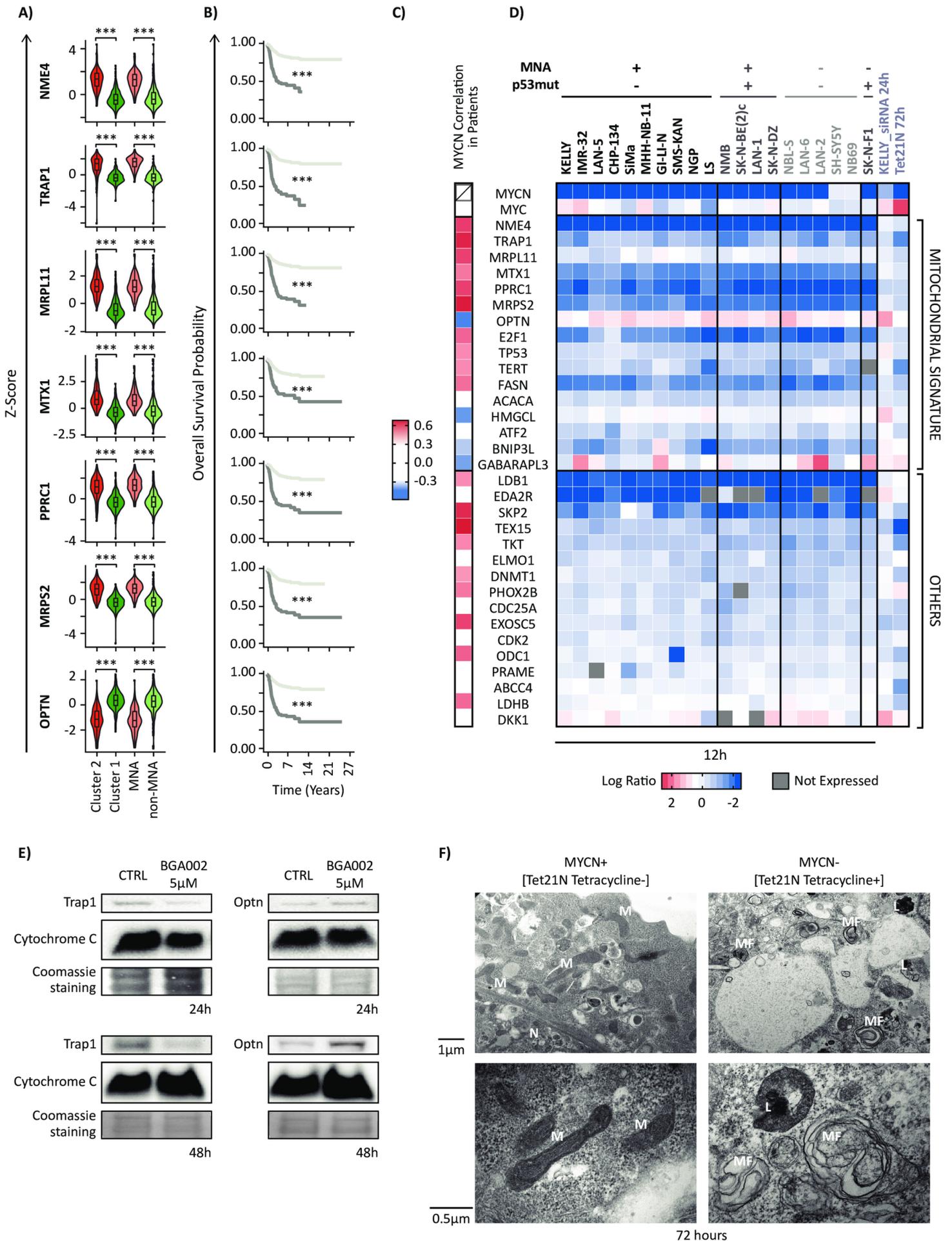


Figure 5

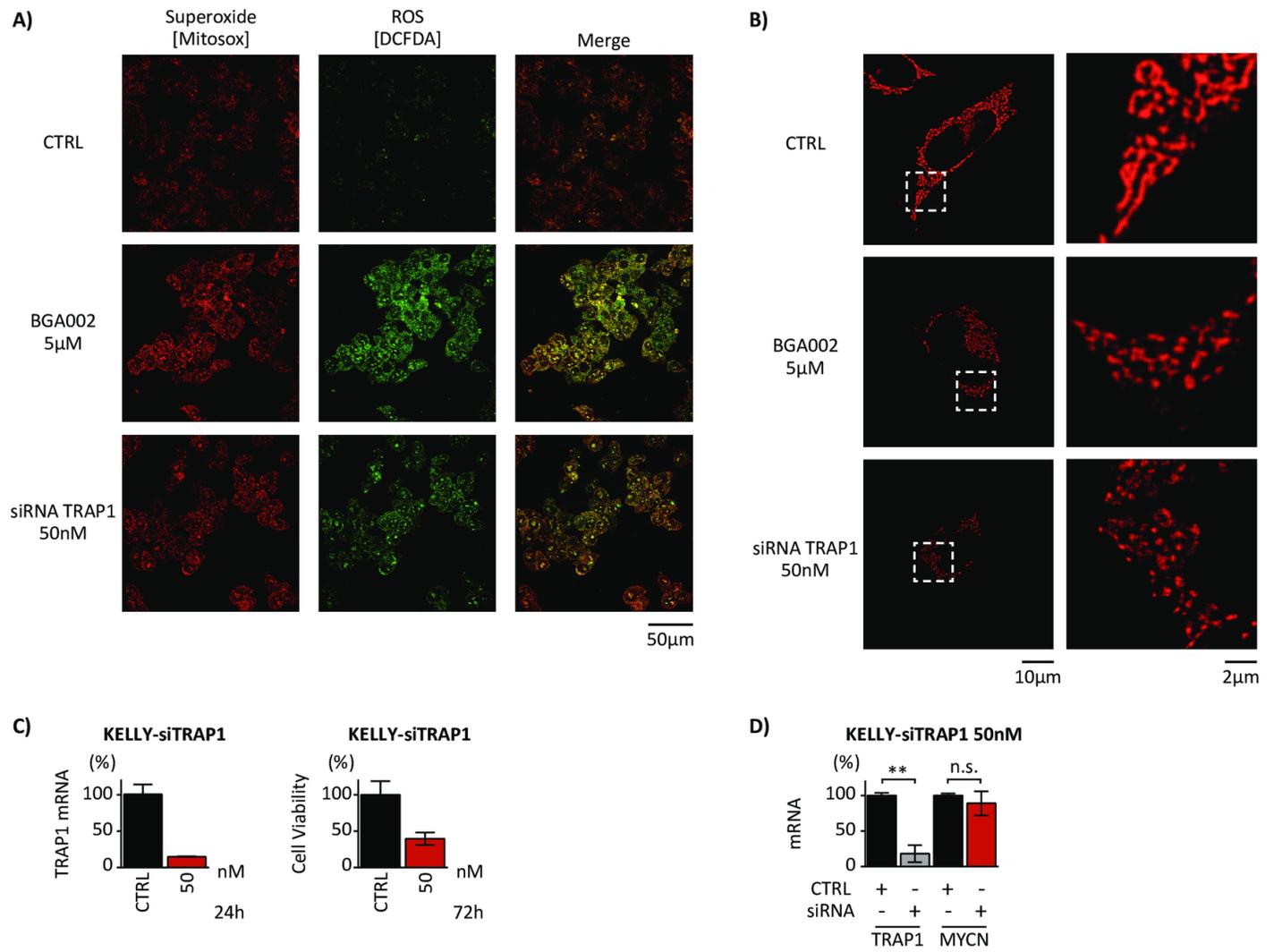
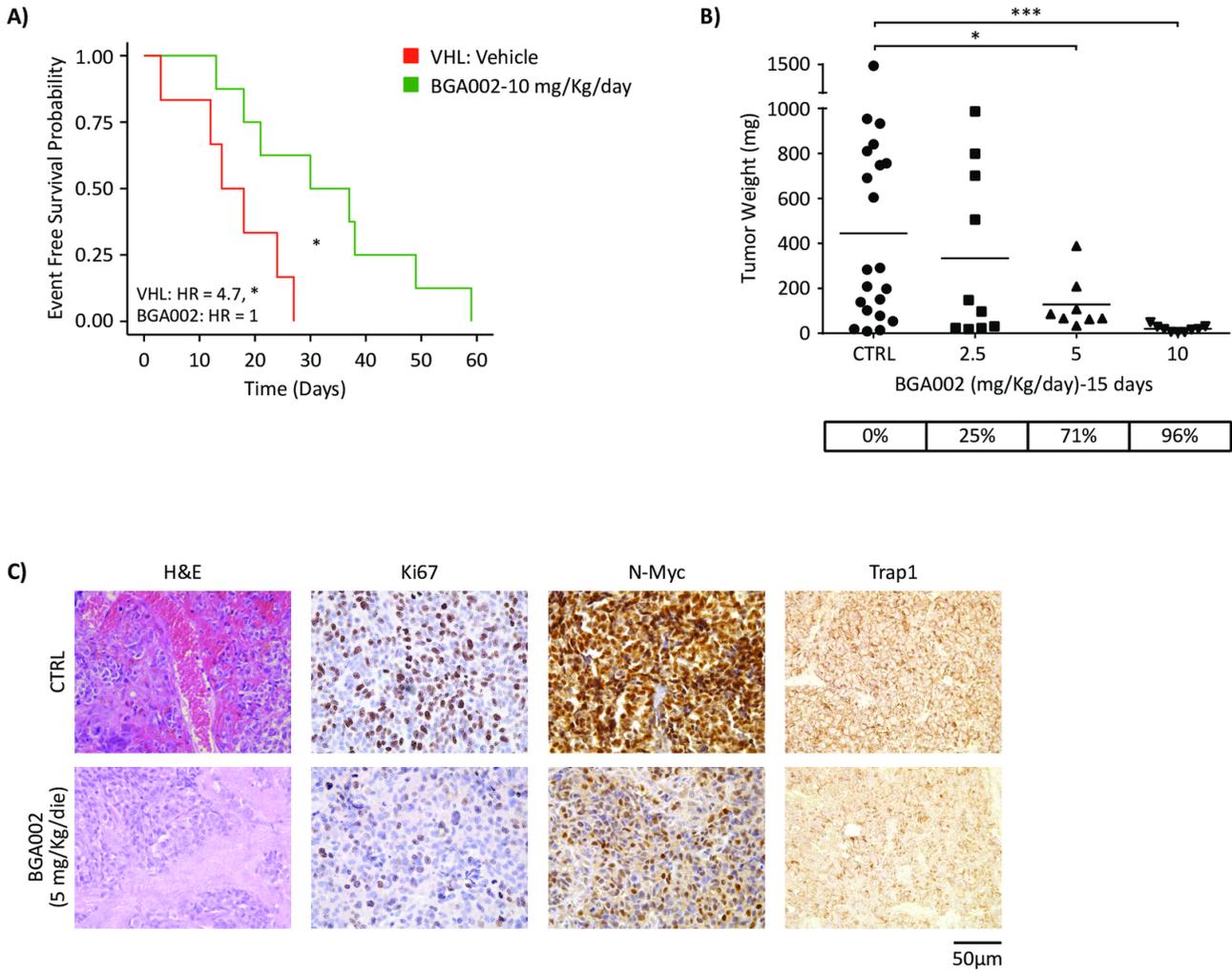


Figure 6



Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

A novel MYCN-specific antigene oligonucleotide deregulates mitochondria and inhibits tumor growth in MYCN-amplified Neuroblastoma

Luca Montemurro, Salvatore Raieli, Silvia Angelucci, et al.

Cancer Res Published OnlineFirst October 15, 2019.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-19-0008
Supplementary Material	Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2019/10/15/0008-5472.CAN-19-0008.DC1
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