**Supplementary information for " Anti-tumor Effects and Potential Therapeutic Response Biomarkers in α-Emitting *meta*-$^{211}$At-Astato-Benzylguanidine Therapy for Malignant Pheochromocytoma Explored by RNA-sequencing "**

## Supplementary materials and methods

### PC12 cell culture

PC12 cells were cultured in RPMI1640 (Wako Pure Chemical Industries, Osaka, Japan) containing 10% heat-inactivated horse serum (Thermo Fisher Scientific, Inc., Waltham, MA), 5% heat-inactivated fetal bovine serum (AusGeneX, Loganholme, OLD, Australia), penicillin (100 units/ml) and streptomycin (100 μg/ml) and L-glutamine (2 mM). Incubation conditions of PC12 were 37°C, 5% $CO_2$ and 95% air.

### Cell survival assay

Cells treated with $^{211}$At-MABG or irradiated with γ-rays were washed with phosphate buffered saline (PBS), suspended in growth medium, and seeded at 400 cells/well in a 96-well plate for 2 weeks incubation. After incubation, cell survival was evaluated by the

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously reported [1].

**Absorbed dose for $^{211}$At-MABG treatment**

Absorbed dose of $^{211}$At-MABG treated cells was estimated from the cellular uptake and release experiments. For uptake, $10^5$ cells were incubated with 5.0 kBq of $^{211}$At-MABG in 5 mL growth medium (1.0 kBq/mL) for 0, 1, 3, 6 and 12 h. Just after incubation, cells were washed with ice-cold PBS, and dissolved in 0.1 N NaOH. Radioactivity of $^{211}$At in the solution was measured by γ-counter. On the other hand, the cellular release was examined as follows: (i) Cells were treated with $^{211}$At-MABG for 1 h, (ii) after $^{211}$At-MABG exposure, cells were washed by PBS and (iii) cells were incubated in growth medium for 1, 3, 6, 12 and 24 h. Cells were washed by PBS after incubation, and $^{211}$At radioactivity of cells was measured.

Absorbed dose of $^{211}$At-MABG treated cells was basically estimated by the published method [2] with some modifications. Time activity curves (TACs) of cellular uptake and release experiments were fitted by the following functions using real-coded genetic algorithm (Real-GA) [3] (**Figure S1A**):

$$\Delta (\%AD_1) = -k_{12} * \%AD_1 * \Delta t + k_{21} * \%AD_2 * \Delta t \quad \text{(a1) and}$$

$$\Delta (\%AD_2) = -k_{21} * \%AD_2 * \Delta t + k_{12} * \%AD_1 * \Delta t \quad \text{(a2).}$$

Here, $\%AD_1$ and $\%AD_2$ are the percent applied dose of growth medium (5 mL) and cells ($10^5$), respectively. $k_{12}$ and $k_{21}$ are transport coefficients in $s^{-1}$. $\Delta$ indicates the difference and $\Delta t$ is the time step of 1 s. For uptake, when the calculation time was 0, $\%AD_2$ was set to be 0, and $\%AD_1$ was 0 for release. Using the estimated values of $k_{12}$ ($1.50\times10^{-5}$) and $k_{21}$ ($4.18\times10^{-5}$), we simulated well both uptake and release experiments (**Figure S1B-C**).

These parameters made our model simulate TAC in the survival experiment. **Figure S2A-B** show the TACs during the incubation for $^{211}$At-MABG exposure (1.0 kBq/ml at the start time) and 10 times of half-life in $^{211}$At ($7.2 * 10 = 72$ h).

**Western blot analysis**

Cells were dissolved in sample buffer and incubated at 95°C for 15 min. Aliquots of samples containing 40 μg protein were analyzed by 10% SDS-PAGE and transferred onto a polyvinylidene fluoride membranes. Blots were incubated at 4°C overnight in tris-buffered saline and 0.1% polysorbate-20 (TBST) containing 5% w/v milk. Blots were then incubated with rabbit anti-TSPO antibody (1/200, Biorbyt, Cambridge, UK) or rabbit anti-$\beta$-actin

antibody (1/1000, Cell Signaling Technology, Beverly, MA) at room temperature for 2.5 h. After washing with TBST, the blots were incubated with horseradish peroxidase conjugated anti-rabbit IgG antibody for 1.5 h at room temperature. The blots were further washed with TBST, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

**MIBG-control experiment**

Since there is no stable isotope in astatine, nonradioactive MIBG which shows a similar biological kinetics to $^{211}$At-MABG was used for the control experiments [1]. The radionuclidic purity of $^{211}$At was over 99% in our study. Therefore, molar concentrations of $^{211}$At-MABG were 49.7 fM for 10% survival dose (0.8 kBq/ml) and 6.2 fM for 80% survival dose (0.1 kBq/ml) according to the following formula; $A=0.693/T\times6.02\times1023\times M$ (A: radioactivity, T: half-life (sec), M: molar concentration). PC-12 cells were treated with culture medium or 80% and 10% survival equivalent dose of MIBG for 0, 3, 6, 12 h. After harvesting cells, RNA extraction, sequencing, differential expression analysis were performed according to Materials and Methods.

**Supplementary result**

**Low fluence rate**

The doses for 10% survival were 10 Gy and 3.5 Gy for γ-ray and $^{211}$At-MABG, respectively. The relative biological effectiveness (RBE) at 10% survival was thus approximately 2.9, suggesting the strong anti-tumor effect of the α-particles emitted from $^{211}$At-MABG. Because nuclear DNA damage is the main cause of IR-induced cell death, the number of α-particles passing through the nucleus is an important concern [4]. The maximum number of α-particles emitted was approximately 25 per cell (Figure S2D), and 10 α-particles passing through the nucleus can induce 10% survival in mammalian cells [5]. Overall, the number was one-third to one-half of the maximum α-particles emitted by $^{211}$At-MABG. Thus, adjacent cellular $^{211}$At-MABG may contribute to nuclear-penetrating α-particles because the probability of α-particles emitted in all directions by $^{211}$At-MABG passing through the nucleus is expected to be slightly lower than one-third. Taken together, the number of α-particles derived from 0.8 kBq/mL of $^{211}$At-MABG-exposure would be sufficient for 10% survival in PC12 cells.

**Supplementary references**

1. Ohshima Y, Sudo H, Watanabe S, Nagatsu K, Tsuji AB, Sakashita T, et al. Antitumor effects of radionuclide treatment using α-emitting meta-$^{211}$At-astato-benzylguanidine in a PC12 pheochromocytoma model. Eur J Nucl Med Mol Imaging. 2018; 45(6): 999-1010.

2. Shinohara A, Hanaoka H, Sakashita T, Sato T, Yamaguchi A, Ishioka NS, et al. Rational evaluation of the therapeutic effect and dosimetry of auger electrons for radionuclide therapy in a cell culture model. Ann Nucl Med. 2018; 32(2): 114-122.

3. Herrera F, Lozano M, Verdegay JL. Tackling real-coded genetic algorithms: operators and tools for behavioural analysis. Artif Intell Rev. 1998; 12(4): 265–319.

4. Maier P, Hartmann L, Wenz F, Herskind C. Cellular Pathways in Response to Ionizing Radiation and Their Targetability for Tumor Radiosensitization. Int J Mol Sci. 2016; 17(1). pii: E102.

5. Zhou H, Randers-Pehrson G, Waldren CA, Vannais D, Hall EJ, Hei TK. Induction of a bystander mutagenic effect of alpha particles in mammalian cells. Proc Natl Acad Sci U S A. 2000; 97(5): 2099-104.

**Table S1 Representative DEGs between $^{211}$At-MABG treatment and γ-irradiation.**

| Rank | *Gene name* | LogFC | Rank | *Gene name* | LogFC | Rank | *Gene name* | LogFC |
|---|---|---|---|---|---|---|---|---|
| **1** | *Snrpg* | 4.075 | **56** | *Ero1b* | 1.615 | **111** | *Nup93* | 1.311 |
| **2** | *Mien1* | 3.665 | **57** | *Csrp2* | 1.612 | **112** | *Slirp* | 1.306 |
| **3** | *Hnrnpa3* | 2.925 | **58** | *Shfm1* | 1.606 | **113** | *Timm8b* | 1.304 |
| **4** | *Vdac1* | 2.897 | **59** | *Dbi* | 1.587 | **114** | *Siva1* | 1.304 |
| **5** | *Otub1* | 2.697 | **60** | *Sass6* | 1.584 | **115** | *Hnrnpf* | 1.3 |
| **6** | *Ppial4d* | 2.672 | **61** | *Cdkn2aipnl* | 1.563 | **116** | *Map1s* | 1.287 |
| **7** | *Gm5471* | 2.644 | **62** | *Cox6c* | 1.562 | **117** | *Ankfy1* | 1.286 |
| **8** | *Tuba1a* | 2.623 | **63** | *Btf3* | 1.556 | **118** | *Slbp* | 1.285 |
| **9** | *Sos2* | 2.551 | **64** | *App* | 1.555 | **119** | *Hdac1l* | 1.282 |
| **10** | *Dmap1* | 2.443 | **65** | *Fkbp5* | 1.539 | **120** | *Txn1* | 1.28 |
| **11** | *Snrpf* | 2.377 | **66** | *Uap1* | 1.535 | **121** | *Rbx1* | 1.261 |
| **12** | *Usmg5* | 2.339 | **67** | *Slc16a1* | 1.51 | **122** | *Med21* | 1.261 |
| **13** | *Vegfa* | 2.328 | **68** | *Ercc4* | 1.51 | **123** | *Bre* | 1.259 |
| **14** | *Uqcrb* | 2.305 | **69** | *Epm2a* | 1.506 | **124** | *Paip2b* | 1.252 |
| **15** | *Fam222b* | 2.305 | **70** | *Snrpe* | 1.498 | **125** | *Gtf2f2* | 1.242 |
| **16** | *Cilp2* | 2.299 | **71** | *Fkbp11* | 1.491 | **126** | *Atg14* | 1.241 |
| **17** | *Adk* | 2.279 | **72** | *Sgol2* | 1.49 | **127** | *Pole4* | 1.24 |
| **18** | *Lsm5* | 2.277 | **73** | *Clybl* | 1.489 | **128** | *Adck1* | 1.231 |
| **19** | *Eif3h* | 2.24 | **74** | *Zdhhc13* | 1.48 | **129** | *Morf4l1* | 1.223 |
| **20** | *Ybx1-ps3* | 2.22 | **75** | *Fam83d* | 1.477 | **130** | *Sub1* | 1.217 |
| **21** | *Fen1* | 2.218 | **76** | *Pitpnb* | 1.476 | **131** | *Rgs7* | 1.217 |
| **22** | *Slc6a17* | 2.208 | **77** | *7-Sep* | 1.474 | **132** | *Hnrnpa2b1* | 1.214 |
| **23** | *Zbed5* | 2.195 | **78** | *Exoc6b* | 1.473 | **133** | *Kntc1* | 1.206 |
| **24** | *Atp5i* | 2.17 | **79** | *SNORA44* | 1.462 | **134** | *Chchd1* | 1.2 |
| **25** | *Cdk5r2* | 2.078 | **80** | *Trmt112* | 1.457 | **135** | *Ahcyl2* | 1.197 |
| **26** | *Atp5j2* | 2.061 | **81** | *Fam64a* | 1.447 | **136** | *Sdhaf3* | 1.194 |
| **27** | *Ska2* | 1.978 | **82** | *Cdca5* | 1.44 | **137** | *Mb21d1* | 1.191 |
| **28** | *Msh2* | 1.975 | **83** | *Nuf2* | 1.436 | **138** | *Rtcb* | 1.187 |
| **29** | *Atp5e* | 1.97 | **84** | *Galnt2* | 1.436 | **139** | *Ddx19b* | 1.187 |
| **30** | *Acp1* | 1.93 | **85** | *Ncam1* | 1.434 | **140** | *Srsf7* | 1.177 |
| **31** | *Chmp6* | 1.928 | **86** | *Abi1* | 1.427 | **141** | *Ccne2* | 1.175 |
| **32** | *Tubg1* | 1.865 | **87** | *Cox7b* | 1.417 | **142** | *Rell1* | 1.168 |
| **33** | *Cwc22* | 1.864 | **88** | *Vrk2* | 1.406 | **143** | *Slc36a1* | 1.145 |
| **34** | *Lin9* | 1.857 | **89** | *Dazap1* | 1.406 | **144** | *Ndufb3* | 1.141 |
| **35** | *Ddx6* | 1.848 | **90** | *Clspn* | 1.402 | **145** | *Parp2* | 1.135 |
| **36** | *Csde1* | 1.832 | **91** | *Ppih* | 1.399 | **146** | *Cox7c* | 1.133 |
| **37** | *Ptma* | 1.812 | **92** | *Exosc8* | 1.399 | **147** | *Actr2* | 1.129 |
| **38** | *Lims1* | 1.808 | **93** | *Gabpb1l* | 1.39 | **148** | *Atp11b* | 1.124 |
| **39** | *MGC95208* | 1.795 | **94** | *Tuba1a* | 1.388 | **149** | *Gins3* | 1.116 |
| **40** | *Phb* | 1.794 | **95** | *SNORA24* | 1.377 | **150** | *Osbpl8* | 1.112 |
| **41** | *Zwilch* | 1.774 | **96** | *Nt5c3b* | 1.377 | **151** | *Strbp* | 1.11 |
| **42** | *Matr3* | 1.766 | **97** | *Usp12* | 1.368 | **152** | *Dhx40* | 1.108 |
| **43** | *Map4* | 1.764 | **98** | *Mpv17l* | 1.365 | **153** | *Eif4h* | 1.106 |
| **44** | *Glis2* | 1.752 | **99** | *Rpa3* | 1.362 | **154** | *Dpy19l1* | 1.105 |
| **45** | *Mgst3* | 1.751 | **100** | *Cycs* | 1.362 | **155** | *Snx4* | 1.094 |
| **46** | *Tmem161b* | 1.726 | **101** | *RT1-A1* | 1.355 | **156** | *Racgap1* | 1.07 |
| **47** | *Brca1* | 1.716 | **102** | *Sbf2* | 1.349 | **157** | *Cpd* | 1.07 |
| **48** | *Pkm* | 1.705 | **103** | *Magohb* | 1.348 | **158** | *Smim20* | 1.068 |
| **49** | *Ppil2* | 1.678 | **104** | *Tgds* | 1.343 | **159** | *Cry2* | 1.066 |
| **50** | *Set* | 1.661 | **105** | *Arhgap11a* | 1.341 | **160** | *Smarcc1* | 1.064 |
| **51** | *Eif3i* | 1.647 | **106** | *Crnkl1* | 1.328 | **161** | *Cox8a* | 1.064 |
| **52** | *Mboat1* | 1.646 | **107** | *Tipinl1* | 1.327 | | | |
| **53** | *S100a6* | 1.643 | **108** | *Arhgef3* | 1.324 | | | |
| **54** | *Ndufab1* | 1.631 | **109** | *Selk* | 1.321 | | | |
| **55** | *Sap18* | 1.625 | **110** | *Suclg2* | 1.316 | | | |

**Supplementary figure legends**

**Figure S1 Pharmacokinetics of *in vitro* $^{211}$At-MABG treatment. (A)** 2 compartment model which consists of "growth medium" and "cells" compartments. $k_{12}$ and $k_{21}$ parameters are transport coefficients in h. $\%AD_1$ and $\%AD_2$ indicate the percent applied dose. **(B)** Cellular uptake simulation of $^{211}$At-MABG. **(C)** Cellular release simulation of $^{211}$At-MABG.

**Figure S2 Time activity curves (TACs) of *in vitro* $^{211}$At-MABG treatment. (A)** % Applied dose of the "cells" compartment. **(B)** TAC of the "cells" compartment in kBq treated with the applied dose of 1.0 kBq/mL. **(C)** Dose rate in $10^{-5}$ Gy/s for $^{211}$At-MABG treatment and γ-ray irradiation. **(D)** Cumulated activity in Bq s per cell for 0.8 kBq/mL $^{211}$At-MABG treatment.

**Figure S3 Vastly different transcriptional profiles.** A pair comparison of all treatment conditions was performed to test the correlation. There were similar expression levels among γ-ray irradiated samples, in which early (3 h) response of weak irradiation (80% survival) showed very weak correlation (around 0.2 in pearson correlation) to 3 h control conditions

**Figure S4 Number of DEGs of γ-ray irradiation and $^{211}$At-MABG treatment.** Number of DEGs was summarized in each condition, 80% and 10% survival rates, 3, 6 and 12 h post treatments. Yellow and purple circles with light or dark filled color indicate the conditions compared for DEGs. Here the DEGs are FDR < 0.05. Number with a gray bar demonstrates the number of DEGs significantly expressed in all selected conditions.

**Figure S5 Heatmap of clustered correlations between MIBG-control experiments.** The expression level of all genes with log-normalized TPM (Transcript Per Million, +1 to avoid taking log of zero) is used to cluster samples according to overall Pearson correlation. Regardless of the time point or survival rate, the two conditions (control, stable-iodine labeled MIBG treatment) showed similar expression pattern.

**Figure S6 Number of DEGs of stable-iodine labeled MIBG treatment.** Number of DEGs was summarized for each comparisons, where DEGs > two fold change (FC) shown in orange, and those below in blue. Actual numbers are displayed above the bars. Overall, comparison between control conditions and MIBG treatment resulted in extremely low number of DEGs exceeding FC > 2, indicating very limited effects of the compounds themselves.

**Figure S7 Gene expressions of the regulatory network for cell cycle checkpoints.**

Expressions (FPKM) of all genes configured at the pathway map of **Figure 4A** are shown at 15 panels. Error bars represent standard deviation among the three replicates, and median values are represented by the symbols.

**Figure S1**

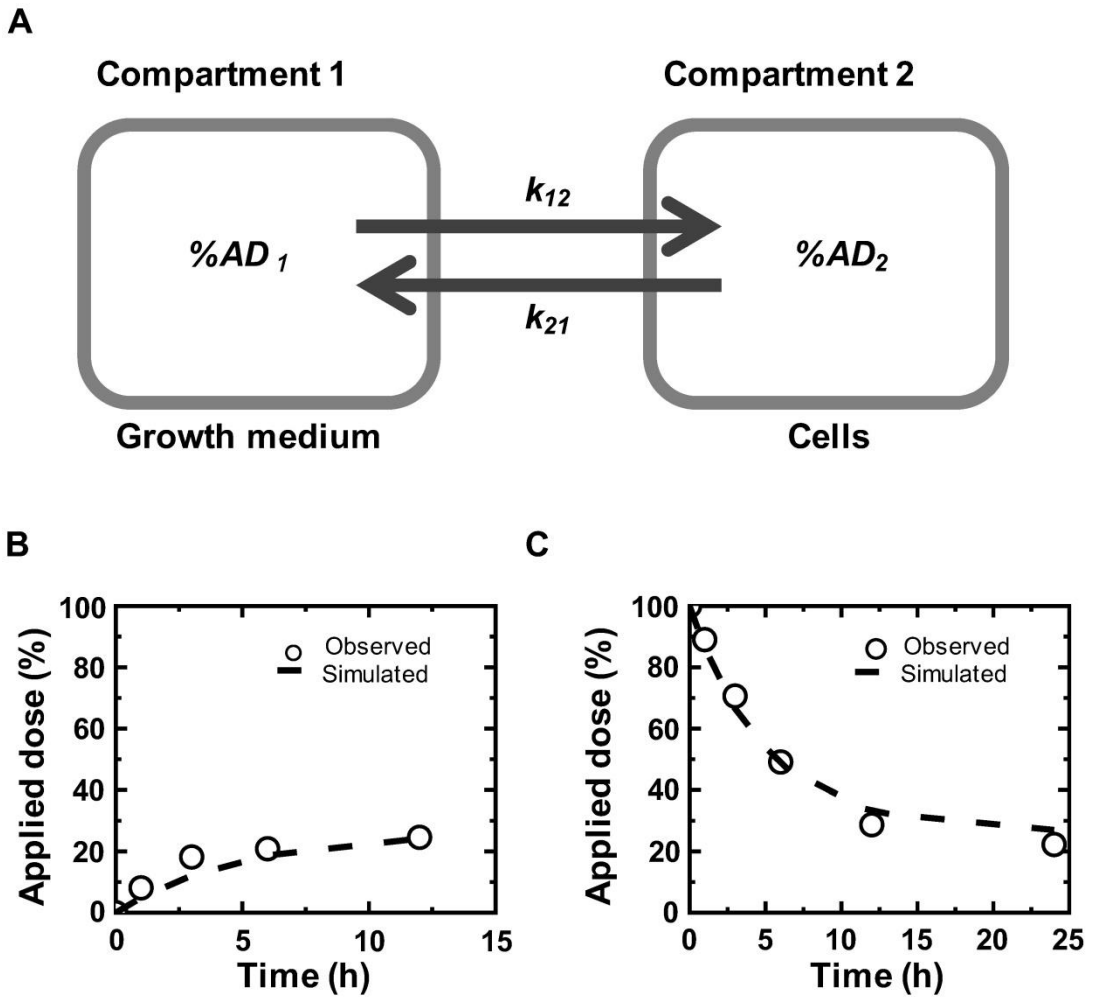

A
Compartment 1
Compartment 2
$k_{12}$
$k_{21}$
$\%AD_1$
$\%AD_2$
Growth medium
Cells
B
C
Applied dose (%)
Time (h)
Observed
Simulated
0
20
40
60
80
100
0
5
10
15
20
25

**Figure S2**

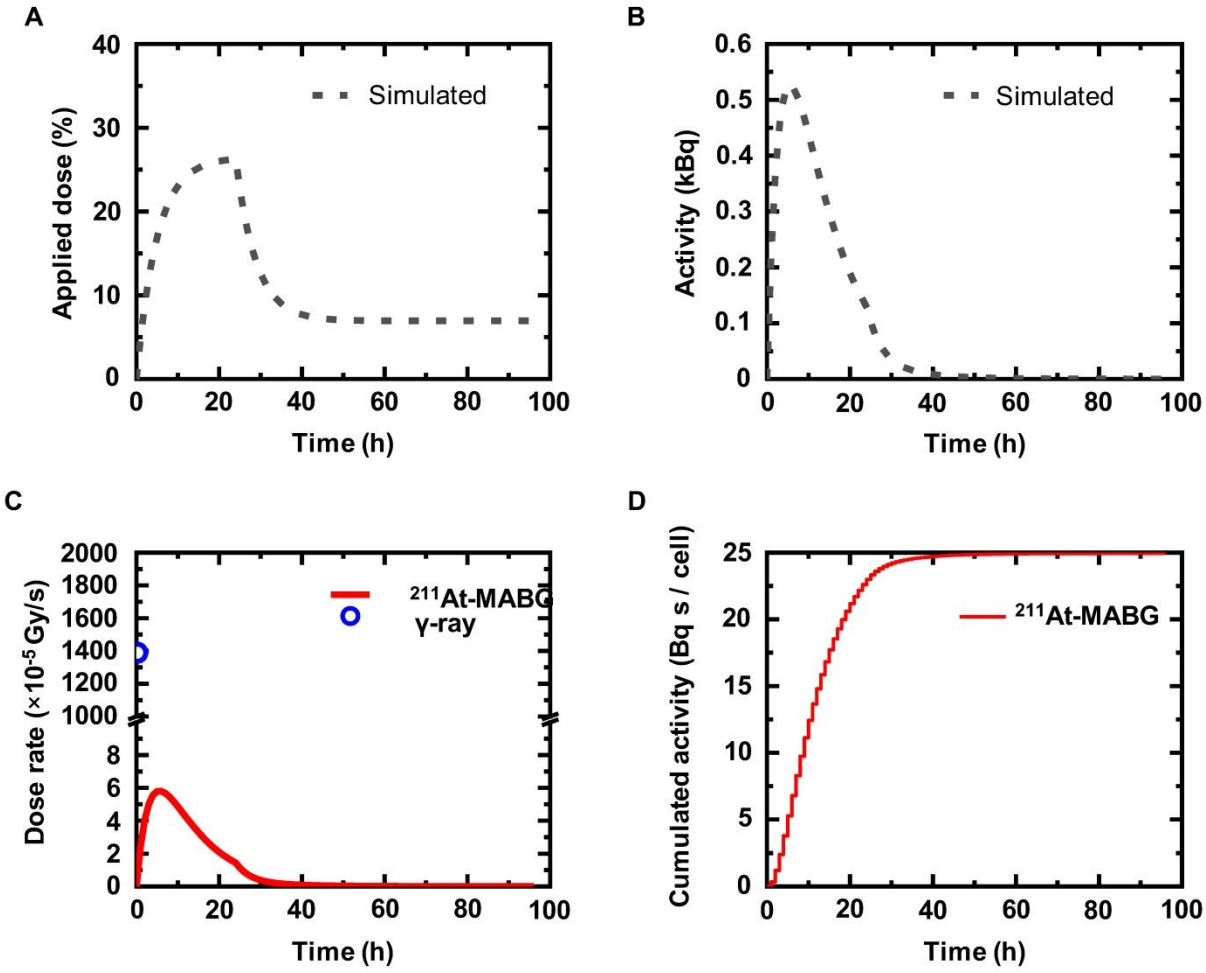

A
Applied dose (%)
Simulated
Time (h)
B
Activity (kBq)
Simulated
Time (h)
C
Dose rate (×10-5 Gy/s)
211At-MABG
γ-ray
Time (h)
D
Cumulated activity (Bq s / cell)
211At-MABG
Time (h)

**Figure S3**

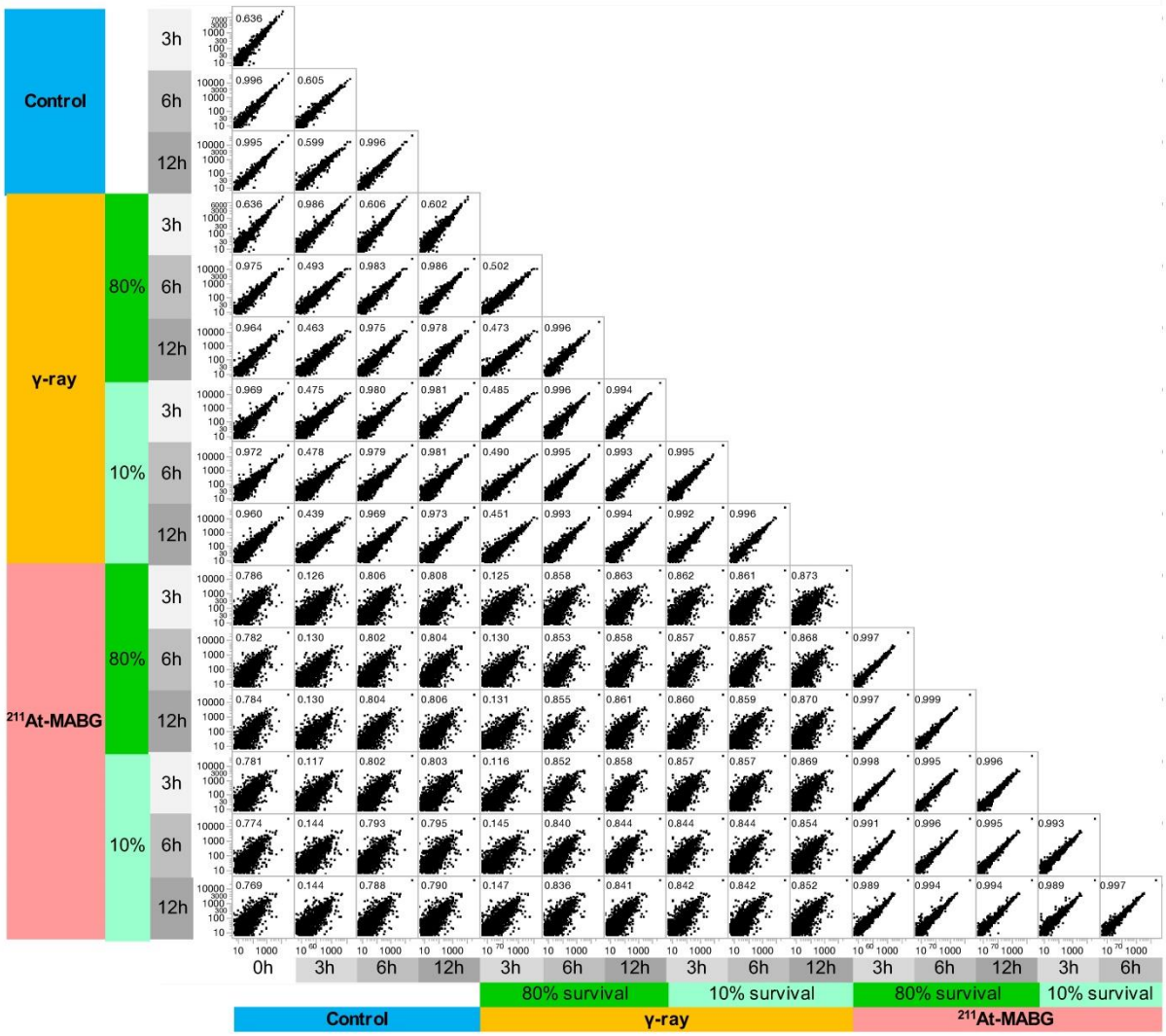
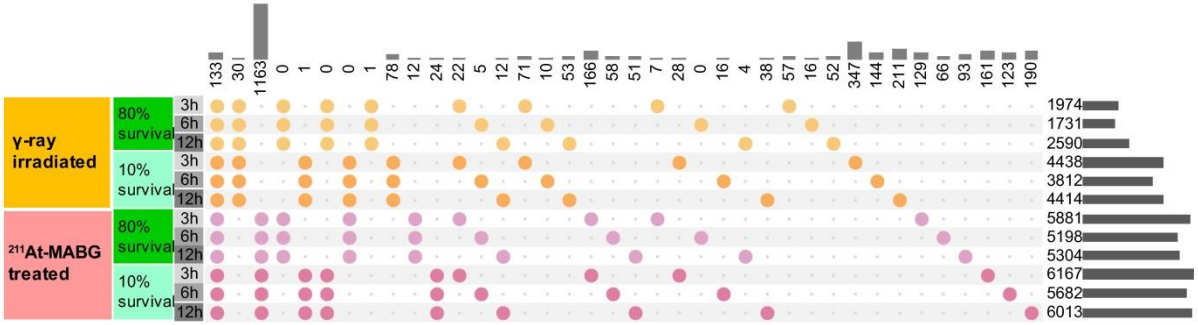

Control
γ-ray
211At-MABG
80%
10%
3h
6h
12h
0h
80% survival
10% survival
Control
γ-ray
211At-MABG

**Figure S4**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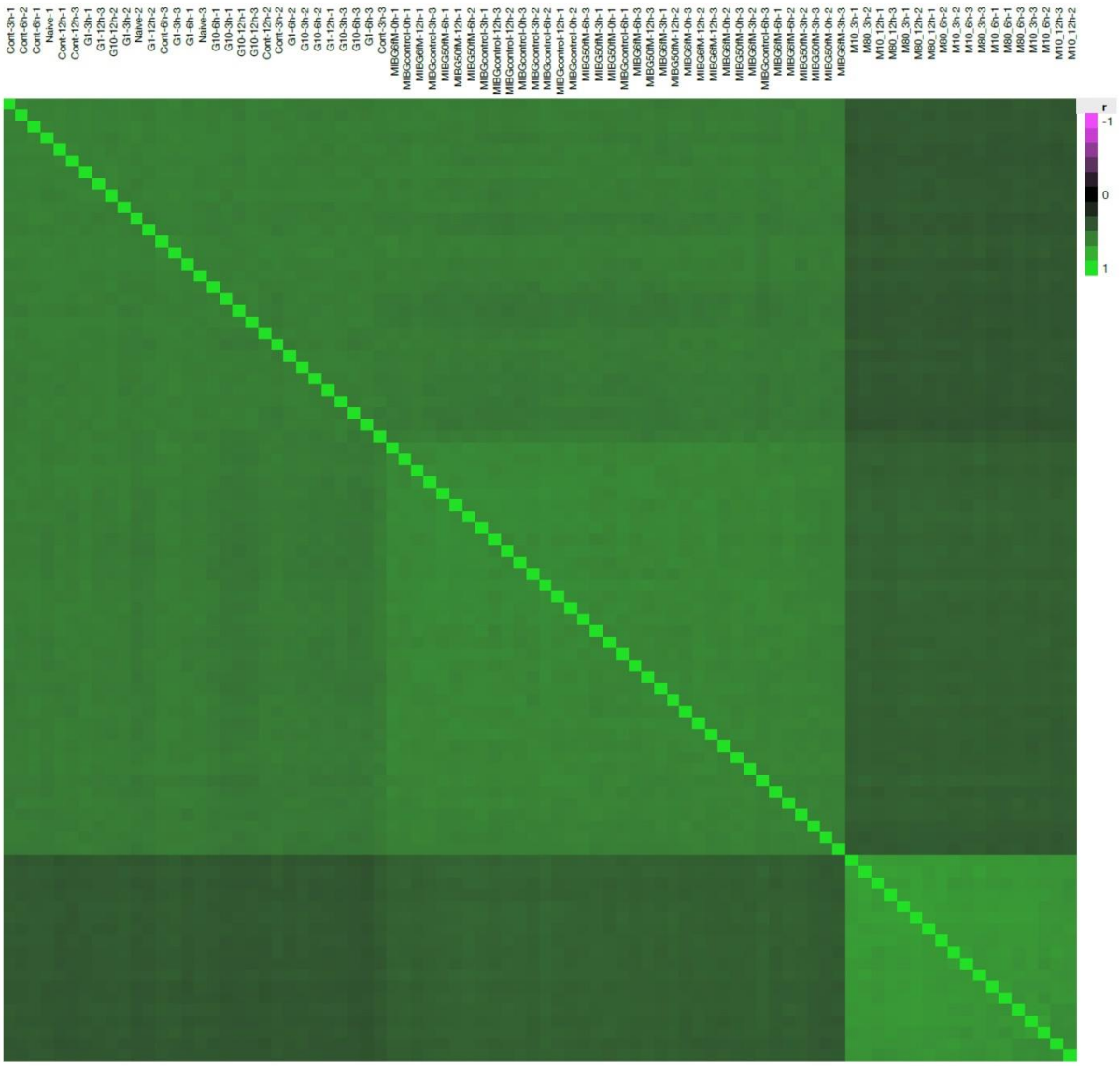
1163
0
1
0
0
1
78
12
24
22
5
12
71
10
53
166
58
51
7
28
0
16
4
38
57
16
52
347
144
211
129
66
93
161
123
190
γ-ray irradiated
80% survival
10% survival
211At-MABG treated
80% survival
10% survival
3h
6h
12h
3h
6h
12h
3h
6h
12h
3h
6h
12h
1974
1731
2590
4438
3812
4414
5881
5198
5304
6167
5682
6013

**Figure S5**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G10-12h-2
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G1-12h-2
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G1-6h-3
Cont-3h-3
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MIBGcontrol-0h-1
MIBG6IM-12h-1
MIBGcontrol-3h-3
MIBGcontrol-6h-1
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MIBG50IM-6h-2
MIBGcontrol-3h-1
MIBGcontrol-12h-3
MIBGcontrol-12h-2
MIBGcontrol-0h-3
MIBGcontrol-3h-2
MIBGcontrol-6h-2
MIBGcontrol-12h-1
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MIBG50IM-0h-3
MIBG6IM-3h-2
MIBGcontrol-6h-3
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MIBG6IM-6h-2
MIBG50IM-3h-2
MIBG50IM-3h-3
MIBG50IM-0h-2
MIBG6IM-3h-3
M10_3h-1
M80_3h-2
M10_12h-1
M80_12h-3
M80_3h-1
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M10_6h-1
M80_6h-1
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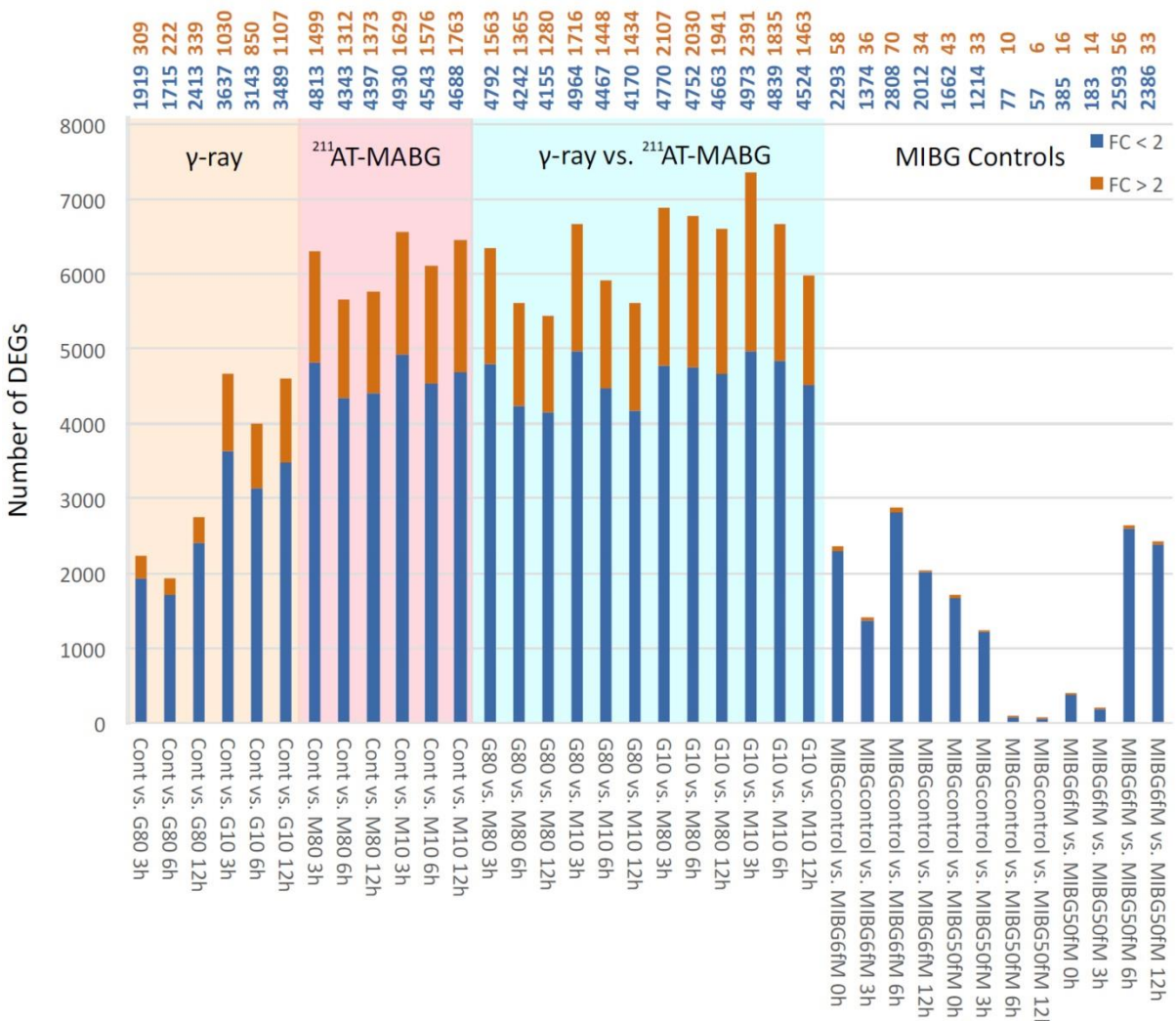
**Figure S6**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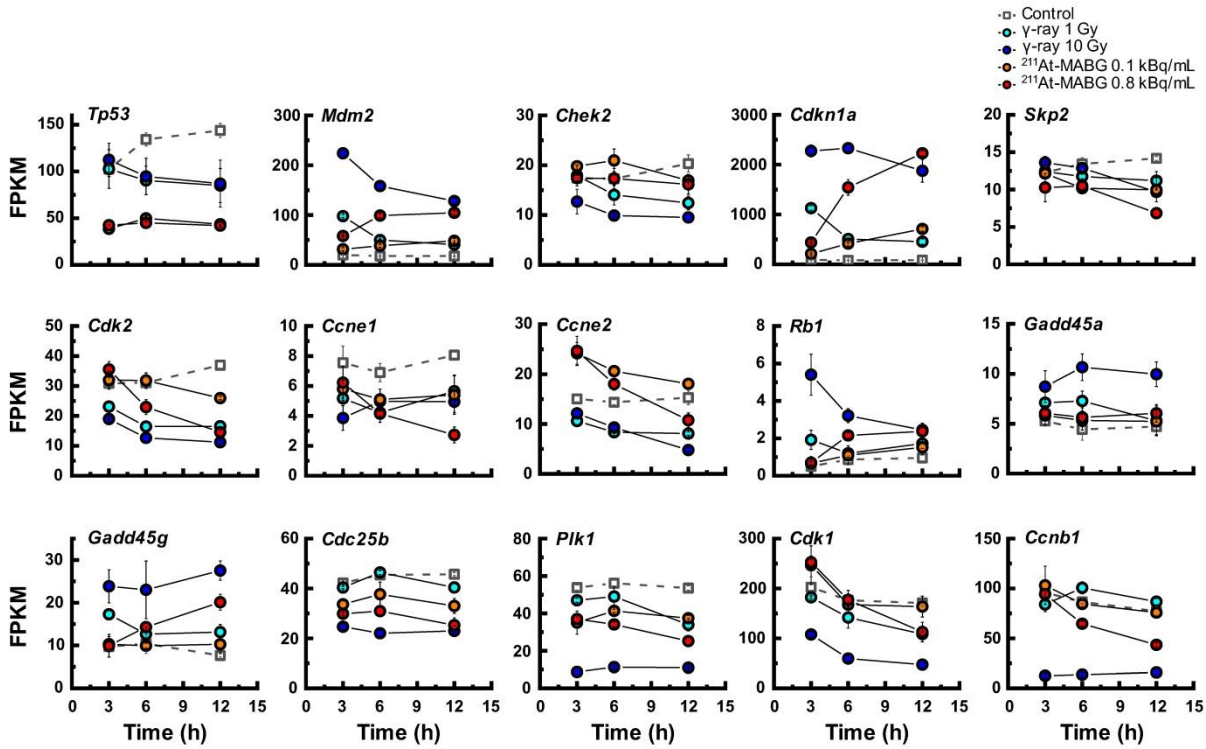
4792 1563
4242 1365
4155 1280
4964 1716
4467 1448
4170 1434
4770 2107
4752 2030
4663 1941
4973 2391
4839 1835
4524 1463
2293 58
1374 36
2808 70
2012 34
1662 43
1214 33
77 10
57 6
385 16
183 14
2593 56
2386 33
γ-ray
211AT-MABG
γ-ray vs. 211AT-MABG
MIBG Controls
FC < 2
FC > 2
Number of DEGs
8000
7000
6000
5000
4000
3000
2000
1000
0
Cont vs. G80 3h
Cont vs. G80 6h
Cont vs. G80 12h
Cont vs. G10 3h
Cont vs. G10 6h
Cont vs. G10 12h
Cont vs. M80 3h
Cont vs. M80 6h
Cont vs. M80 12h
Cont vs. M10 3h
Cont vs. M10 6h
Cont vs. M10 12h
G80 vs. M80 3h
G80 vs. M80 6h
G80 vs. M80 12h
G80 vs. M10 3h
G80 vs. M10 6h
G80 vs. M10 12h
G10 vs. M80 3h
G10 vs. M80 6h
G10 vs. M80 12h
G10 vs. M10 3h
G10 vs. M10 6h
G10 vs. M10 12h
MIBGcontrol vs. MIBG6fM 0h
MIBGcontrol vs. MIBG6fM 3h
MIBGcontrol vs. MIBG6fM 6h
MIBGcontrol vs. MIBG6fM 12h
MIBGcontrol vs. MIBG50fM 0h
MIBGcontrol vs. MIBG50fM 3h
MIBGcontrol vs. MIBG50fM 6h
MIBGcontrol vs. MIBG50fM 12h
MIBG6fM vs. MIBG50fM 0h
MIBG6fM vs. MIBG50fM 3h
MIBG6fM vs. MIBG50fM 6h
MIBG6fM vs. MIBG50fM 12h

**Figure S7**


Control
γ-ray 1 Gy
γ-ray 10 Gy
211At-MABG 0.1 kBq/mL
211At-MABG 0.8 kBq/mL
Tp53
Mdm2
Chek2
Cdkn1a
Skp2
Cdk2
Ccne1
Ccne2
Rb1
Gadd45a
Gadd45g
Cdc25b
Plk1
Cdk1
Ccnb1
FPKM
Time (h)