

1 ARTICLE

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3 **Loss of Concurrent Regulation of the Expression of BIF-1, BAX and**
4 **Beclin-1 in Primary and Metastatic Melanoma**

5

6 **Running title: Dysregulated expression of BIF-1, BAX and Beclin-1 in melanoma**

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20 **Figures:** 5

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22

23 **Abstract**

24 Melanoma is one of the most aggressive and drug-resistant cancers. Despite novel promising
25 therapeutic strategies, the prognosis of metastatic melanoma patients remains poor and it is
26 often associated with high relapse rates. Endophilin B1, also known as BIF-1, is a
27 multifunctional protein involved in several biological processes such as autophagy and
28 apoptosis. BIF-1 promotes apoptosis through binding to BAX and its translocation to the
29 mitochondrial outer membrane. On the other hand, BIF-1 can interact with Beclin-1 through
30 UVRAG to promote autophagy. Several reports suggest an ambiguous role of BIF-1 in cancer
31 development and progression. For example, it has been demonstrated that the expression of
32 BIF-1 is reduced in both primary and metastatic melanoma and that the reduction of BIF-1
33 expression is associated with reduced overall survival of melanoma patients. Here we show
34 that the expression of Beclin-1 and the active form of BAX are also reduced in melanoma
35 patients. However, while we observed strong positive correlations between the expression of
36 BIF-1 and Beclin-1 as well as between BIF-1 and BAX in benign nevi, these correlations were
37 lost in primary and metastatic melanoma cells. These data indicate a dysfunctionality in the
38 proximal molecular mechanisms which regulate the expression of BIF-1, Beclin-1 and BAX in
39 primary and metastatic melanoma.

40

41 **Introduction**

42 Despite new promising therapeutic approaches, such as immunotherapy or targeted therapy,
43 the prognosis of metastatic melanoma patients remains poor and is often associated with high
44 tumor relapse rates. Unfortunately, prognostic factors that are currently used in clinics are not
45 sufficient to clearly identify high-risk melanoma patients. BIF-1, also known as endophilin B1
46 or BAX-interacting protein 1, is part of the endophilin family of proteins, which are
47 cytoplasmic proteins involved in numerous biological processes, such as mitochondrial
48 membrane dynamics, apoptosis, autophagy, synaptic vesicle retrieval as well as receptor
49 tyrosine trafficking and signaling [1]. We have previously demonstrated that the expression of
50 BIF-1 is reduced in primary and metastatic melanomas as compared to healthy tissues and that
51 the reduced BIF-1 levels are associated with a less favorable clinical outcome [2].

52 BIF-1 is mostly found in the cytosol and a fraction of the protein also resides on
53 intracellular membranous compartments, including the Golgi complex and mitochondria [3-6],
54 where it was shown to be required for the maintenance of mitochondrial morphology and
55 function [2, 4]. Since its discovery as an interacting partner of BAX, several reports focused
56 on its role in apoptosis. Moreover, depletion of BIF-1 expression in HeLa cells delays the
57 conformational change of BAX and BAK, cytochrome *c* release, and caspase-3 activation
58 induced by various intrinsic death signals [3, 6]. Besides being involved in BAX activation, it
59 was also shown that BIF-1 forms a complex with Beclin-1 through UVRAG and positively
60 modulates the formation of autophagosomes [7]. The involvement of BIF-1 in autophagy and
61 apoptosis led to several investigations of the role of BIF-1 in cancer. Low expression of BIF-1
62 was found, besides melanoma [2], in invasive urinary bladder and gallbladder cancers [8] as
63 well as in colorectal adenocarcinoma [9]. In contrast, high expression was reported in
64 hepatocellular carcinoma [10]. Moreover, loss of BIF-1 in melanoma cells led to increased

65 ATP production, metabolic acidification, and mitochondrial respiration which was associated
66 with higher proliferation rates both *in vitro* and *in vivo* [2].

67 Because of the role of BIF-1 in apoptosis and autophagy, we decided to investigate the
68 correlation between the expression of apoptotic or autophagic proteins and BIF-1 in benign
69 nevi as compared to primary and metastatic melanoma tissues. We show that the expression of
70 active form of BAX is reduced in metastatic melanoma patients. Additionally, we could
71 demonstrate that the expression of an essential autophagic protein Beclin-1 is also reduced in
72 metastatic melanoma patients as compared to benign nevi. Moreover, the positive correlation
73 between BIF-1 and the active form of BAX, Beclin-1 or the autophagic marker LC3B was lost
74 in primary and metastatic melanoma cells. These data suggest that the proximal events
75 regulating the expression of BIF-1, Beclin-1 and BAX are dysregulated in primary and
76 metastatic melanoma cells.

77

78

79 **Materials and methods**

80 **Study design and patients**

81 The Tissue Micro Array (TMA) was constructed by the Department of Pathology, Bern. The
82 study was approved by the Ethics Committee of the Canton of Bern. This cohort study aimed
83 at investigating the role of BIF-1, BAX, LC3B and Beclin-1 in the pathogenesis of cutaneous
84 melanoma. TMA included archived tissue samples of consecutive 65 melanocytic nevi, 41
85 primary and 30 metastatic melanomas obtained between the years 2003 and 2015 from patients
86 at the Department of Dermatology, Inselspital, Bern, Switzerland.

87

88 **Immunohistochemistry**

89 IHC was performed as previously described [2, 11, 12]. Briefly, paraffin-embedded tissue
90 sections were deparaffinized and rehydrated with graded ethanol dilutions which was followed
91 by the antigen retrieval. Immunohistochemical staining was performed with the Dako REAL
92 Detection System, using the Alkaline Phosphatase/RED kit, which also provided the secondary
93 antibodies, according to the manufacturer's instructions (Agilent Technologies, K5005). The
94 following primary antibodies were used: monoclonal anti-endophilin B1/BIF-1 antibody
95 (Novus Biologicals, NBP2-24733; 1:100), polyclonal rabbit anti-Beclin-1 antibody (Abgent,
96 San Diego, CA, USA; 1:100), monoclonal anti-BAX (clone 6A7, Santa Cruz Biotechnology,
97 Dallas, TX, USA; 1:100) and polyclonal rabbit anti-LC3B antibody (Abgent, 1:100). The
98 intensity of staining was evaluated by QuPath software [2, 13] and is presented as the mean
99 optical density (OD) or by Image Pro Plus software and presented as integrated optical density
100 (IOD) [11, 12].

101

102 **Statistical analysis**

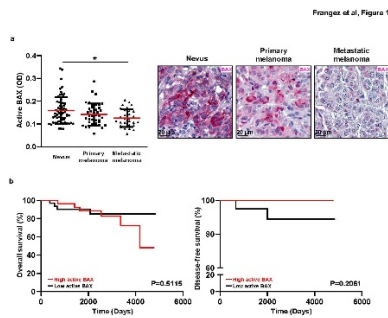
103 Data were presented as means \pm SD using the Prism Software v6 (GraphPad, La Jolla, CA,
104 US). Significant values were represented according to the following convention: $p \geq 0.05^{\text{ns}}$, p
105 $\leq 0.05^*$, $p \leq 0.01^{**}$, $p \leq 0.001^{***}$, and $p \leq 0.0001^{****}$. The follow-up data on primary and
106 metastatic melanoma patients were divided for analysis into two groups according to the
107 median expression of active form of BAX or Beclin-1 in their tumors. The group with “High
108 BAX or Beclin-1” included patients with BAX or Beclin-1 levels that were higher than the
109 median value of the whole population. The group with “Low BAX or Beclin-1” included
110 patients with BAX or Beclin-1 levels that were lower than the median value of the whole
111 population. Overall survival (OS) was defined as the time from random assignment to death
112 from any cause. Disease-free survival (DFS) was defined as the time from random assignment
113 to disease reoccurrence or progression. It was analyzed with the log-rank test and plotted as
114 Kaplan-Meier survival curves.

115

116 **Results**

117 **Reduced active BAX expression in metastatic melanoma patients**

118 Our previous findings suggested that BIF-1 is a tumor suppressor in melanoma because it limits
119 tumor growth by inhibiting mitochondrial functions. Several studies demonstrated that BIF-1
120 enhances apoptosis through promoting the conformational changes of BAX and BAK and the
121 translocation of BAX to the mitochondria [3, 6]. Apoptosis is a crucial cell death mechanism
122 by which damaged or transformed cells, which could be potentially cancerous, are eliminated.
123 We decided to first investigate the expression of the conformational active form of BAX across
124 several stages of melanoma development using a custom made tissue microarray (TMA) [2].
125 TMA contained 64 benign nevi, 41 primary melanoma and 30 metastatic melanoma tissue
126 samples. Immunohistochemistry analysis of the TMA revealed a step wise decrease in the
127 expression of active BAX from benign nevi via primary melanoma to metastatic melanoma
128 (Fig. 1a). The expression of active BAX was significantly decreased in metastatic melanoma
129 (mean OD = 0.1116 ± 0.03897) as compared to benign nevi (mean OD = 0.1588 ± 0.05966).
130 To our surprise, further stratification of patients according to their median BAX expression in
131 groups with high (OD > 0.1344 for primary melanoma and OD > 0.1274 for metastatic
132 melanomas) or low active BAX expression showed that the active form of BAX does not have
133 an impact on the OS or DFS of melanoma patients (Fig. 1b).



134

Fig. 1. Expression of BAX active monomeric form is reduced in metastatic melanomas compared with benign melanocytic nevi. a) Immunohistochemistry. Quantification of the BAX active monomeric form signal intensity. Intensity (mean optical density (OD)) values for individual patients are presented. The red lines represent the mean of all values. Statistical differences were analyzed by one-way ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the basis of the median expression of BAX in their tumors. Kaplan-Meier curves for overall and disease-free survival are shown.

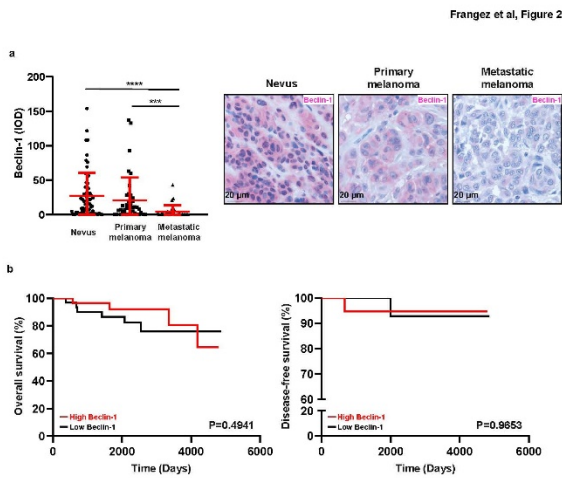
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137 **Reduced Beclin-1 expression in metastatic melanoma patients**

138 In addition to apoptosis, BIF-1 has also been associated with autophagy. BIF-1 interact with
 139 Beclin-1 through binding to UVRAG and acts as a positive mediator of the class III PI(3) kinase
 140 and autophagy [7]. We next investigated the expression of Beclin-1 in benign nevi and
 141 melanoma patients. We observed a slight decrease in Beclin-1 expression in primary melanoma
 142 and a robust decrease of Beclin-1 expression in metastatic melanoma patients compared to
 143 benign nevi (Fig. 2a). Interestingly, stratification of patients according to their Beclin-1 median
 144 expression in groups with high (IOD > 9316.11 for primary melanoma and IOD > 808.272 for

145 metastatic melanomas) or low Beclin-1 expression revealed that Beclin-1 expression does not
146 influence the OS or DFS of melanoma patients (Fig. 2b).



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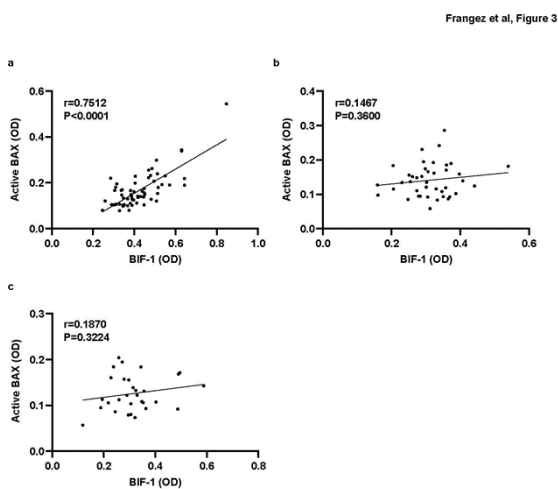
148 **Fig. 2. Expression of Beclin-1 is reduced in metastatic melanomas compared with benign**
149 **melanocytic nevi.** a) Immunohistochemistry. Quantification of the Beclin-1 signal intensity.
150 Intensity (integrated optical density (IOD)) values for individual patients are presented. The
151 red lines represent the mean of all values. Statistical differences were analyzed by one-way
152 ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative
153 images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right
154 panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the
155 basis of the median expression of Beclin-1 in their tumors. Kaplan-Meier curves for overall and
156 disease-free survival are shown.

157

158 **The correlations of BIF-1 with active BAX, Beclin-1 or LC3B expression are lost in**
159 **primary and metastatic melanomas**

160 Since BIF-1 has been implicated in autophagy and apoptosis and since those processes are
161 crucial in preventing tumor development or progression, we decided to investigate whether

162 proteins involved in autophagy or apoptosis have a similar expression pattern as BIF-1 in
163 benign nevi and melanoma patients. For this purpose, we analysed the correlation between
164 BIF-1 and the active form of BAX and Beclin-1. We observed a strong correlation between
165 BIF-1 and the active form of BAX in benign nevi (Fig. 3a), suggesting a similar or the same
166 mechanism regulating these two proteins in melanocytes. Interestingly, the correlation between
167 BIF-1 and the active form of BAX was lost in primary and metastatic melanoma tissues (Fig.
168 3b and 3c).



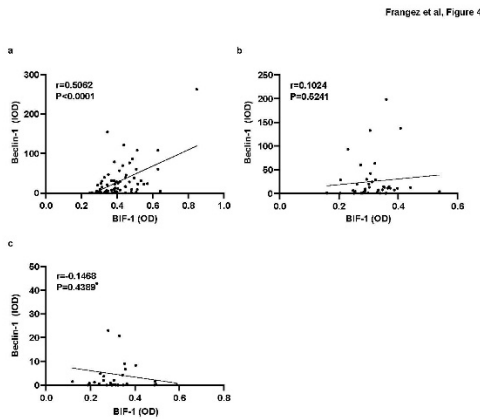
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170 **Fig. 3. BIF-1 positively correlates with BAX active monomeric form in benign nevi but**
171 **not in primary or metastatic melanoma samples.** (a-c) The scatter plot shows the correlation
172 between the mean OD values of BAX active monomeric form with BIF-1 in benign nevi (a),
173 primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show
174 the Pearson correlation.

175

176 In addition, to the interaction with BAX, BIF-1 also forms a complex with Beclin-1
177 through UVRAG, influencing the induction of autophagy [7]. Beclin-1 is a central regulator of

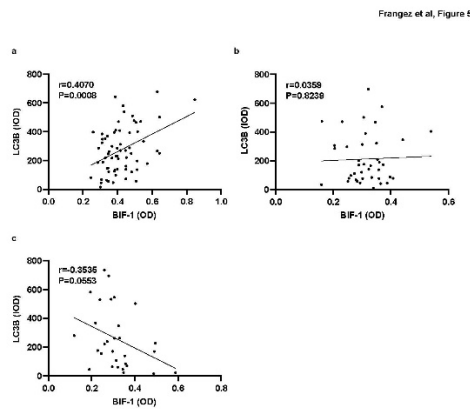
178 autophagy and acts in the initiation phase of autophagy by forming the isolation membrane that
179 engulfs the cytoplasmic material [14]. Therefore, we also investigated the expression of Beclin-
180 1 and observed a strong correlation between active BAX and Beclin-1 in benign nevi (Fig. 4a)
181 and loss of this correlation in primary and metastatic melanoma tissue (Fig. 4b and 4c).



182 **Fig. 4. BIF-1 positively correlates with Beclin-1 in benign nevi but not in primary or**
183 **metastatic melanoma samples.** (a-c) The scatter plot shows the correlation between the IOD
184 values of Beclin-1 with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b)
185 and metastatic melanoma (c) samples. The reported r and p -values show the Pearson
186 correlation.
187
188

189 Since BIF-1 is a positive regulator of autophagy, we also investigated the correlation
190 between the expression of BIF-1 and a commonly used autophagic marker LC3B. Upon
191 cleavage and lipidation with phosphatidylethanolamine (PE), LC3 is found on the inner and
192 outer autophagosomal membrane [15]. In agreement with the role of BIF-1 in autophagy, we
193 observed a strong positive correlation between the levels of BIF-1 and LC3B in benign nevi
194 (Fig.5a) that was lost in primary and metastatic melanoma tissues (Fig. 5b and 5c). These

195 findings indicate that the proximal regulation of the expression of BIF-1, Beclin-1 and BAX is
196 dysregulated in primary and metastatic melanoma cells.



197

198 **Fig. 5. BIF-1 positively correlates with LC3B in benign nevi but not in primary or**
199 **metastatic melanoma samples.** (a-c) The scatter plot shows the correlation between the IOD
200 values of LC3B with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b) and
201 metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.
202

203 Discussion

204 BIF-1 has been associated with several biological processes, in particular apoptosis, during
205 which it interacts with BAX and promotes its conformational changes during cell death [3, 6].
206 We therefore wanted to investigate the BIF-1-BAX axis in our cohort of melanoma patients.
207 Using a conformation-specific BAX antibody recognizing an activated form of BAX, we found
208 a consistent reduction in active BAX associated with lower BIF-1 levels in metastatic
209 melanoma tissue sections as compared to benign nevi. Moreover, we observed a positive
210 correlation between the expression of BIF-1 and active BAX in nevi in which the mitochondrial
211 apoptotic pathway is active [16]. In contrast, this correlation did no longer exist in melanoma

212 cells, suggesting that the apoptosis resistance, frequently arising in melanoma tumors, could
213 be, at least partially, explained by downregulation of BIF-1.

214 Autophagy and apoptosis are both crucial cellular processes in determining the cellular
215 fate and have a high impact on the development and progression of neoplasms [17]. Apoptosis
216 is the most common physiological form of cell death in the absence of inflammation [18]. The
217 intrinsic apoptotic pathway is regulated by the BCL-2 family of proteins and involves
218 mitochondrial outer membrane permabilization (MOMP) which leads to the release of the pro-
219 apoptotic factors such as cytochrome *c* and SMAC/DIABLO from the mitochondria to the
220 cytosol. MOMP is followed by the activation of the caspase cascade which leads to cell death
221 [19]. In healthy cells, the core pro-apoptotic regulators BAX and BAK shuttle between the
222 outer mitochondrial membrane (OMM) and the cytosol [20, 21]. Under apoptotic conditions,
223 BAX and BAK are activated and undergo dimerization and assemble at the OMM where they
224 form multimeric pores [21]. The balance of interaction between pro-apoptotic and anti-
225 apoptotic proteins, such as BCL-2 or BCL-XL, ensures appropriate apoptotic regulation in
226 response to cellular stresses and other cell death triggering factors [19].

227 Loss of BAX expression has been reported as a negative prognostic marker in several
228 cancers, such as breast, ovarian, pancreatic, and esophageal cancer [22-25]. Additionally,
229 Fecker et al. have previously shown that loss of BAX in primary superficial-spreading
230 melanomas was associated with tumor progression and reduced survival rates [26]. Moreover,
231 downregulation of BAX in stage IIa melanomas was associated with an increased risk of
232 development of metastasis and poor prognosis [27].

233 Interestingly, Beclin-1 has been identified as an interaction partner of BCL-2, BCL-XL
234 and MCL-1. Those interactions may regulate the crosstalk between apoptotic and autophagic
235 signalling pathways [17]. Besides being involved in BAX activation, BIF-1 also forms a
236 complex with Beclin-1 through UVRAG and positively regulates the formation of

237 autophagosomes [7]. Here we show that the expression of Beclin-1 is reduced in melanoma
238 tissues as compared to benign nevi. Moreover, besides the positive correlation between BIF-1
239 and BAX, we also observed a strong correlation between the expression of BIF-1 and Beclin-
240 1 in melanocytes that was lost in primary and metastatic melanoma tissues. Furthermore, we
241 observed the same correlation pattern between BIF-1 and the autophagic marker LC3B.

242 Several reports have pointed to a tumor suppressing role of autophagy. Beclin-1 has
243 been described as a tumor suppressor gene because it was found to be monoallelically deleted
244 in 40% to 75% of human breast, ovarian, and prostate tumors [28]. Moreover, allelic loss of
245 Beclin-1 in mice led to high occurrence of tumors such as B cell lymphoma, hepatocellular
246 carcinoma and lung adenocarcinoma [29]. A link between the role of Beclin-1 in melanoma
247 was described in recently published work demonstrating that decreased Beclin-1 expression
248 correlates with invasiveness and a decrease in 5-year survival after surgery [30].

249 In summary, our findings indicate that the proximal regulation responsible for a
250 coordinated expression of BIF-1, Beclin-1 and BAX in melanocytes appears to be lost in
251 primary and metastatic melanoma cells. It is likely that the reduced expression of BIF-1,
252 Beclin-1 and BAX and its anticipated dysfunctional proximal regulatory mechanism
253 contributes to tumorigenesis and drug resistance in melanoma.

254

255

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261

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263 Ž.F. conceived, planned and performed the study, analyzed and interpreted data and wrote the
264 paper. S.M.S.J. performed experiments; R.E.H. took clinical care of the melanoma patients;
265 H.U.S. provided overall guidance, experimental advice and laboratory infrastructure and edited
266 the paper; all authors read and approved the final manuscript.

267

268 **Conflict of interests.**

269 No potential conflict of interest was reported by the authors.

270

271 **Compliance with Ethical Norms**

272 All the procedures carried out in the research with participation of humans were in compliance
273 with the ethical standards of the institutional and/or national ethics committee and with the
274 Helsinki Declaration of 1964 and its subsequent changes or with comparable ethics standards.
275 Informed voluntary consent was obtained from every participant of the study.

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382 **Figure legends**

383 **Fig. 1. Expression of BAX active monomeric form is reduced in metastatic melanomas**
384 **compared with benign melanocytic nevi.** a) Immunohistochemistry. Quantification of the
385 BAX active monomeric form signal intensity. Intensity (mean optical density (OD)) values for
386 individual patients are presented. The red lines represent the mean of all values. Statistical
387 differences were analyzed by one-way ANOVA using a Kruskal-Wallis test and Dunn's *post*
388 *hoc* test (left panel). Representative images of 65 benign nevi as well as 41 primary and 30
389 metastatic melanomas are shown (right panel). b) The same follow-up patients were divided
390 into two groups ("high" and "low") on the basis of the median expression of BAX in their
391 tumors. Kaplan-Meier curves for overall and disease-free survival are shown.

392
393 **Fig. 2. Expression of Beclin-1 is reduced in metastatic melanomas compared with benign**
394 **melanocytic nevi.** a) Immunohistochemistry. Quantification of the Beclin-1 signal intensity.
395 Intensity (integrated optical density (IOD)) values for individual patients are presented. The
396 red lines represent the mean of all values. Statistical differences were analyzed by one-way
397 ANOVA using a Kruskal-Wallis test and Dunn's *post hoc* test (left panel). Representative
398 images of 65 benign nevi as well as 41 primary and 30 metastatic melanomas are shown (right
399 panel). b) The same follow-up patients were divided into two groups ("high" and "low") on the
400 basis of the median expression of Beclin-1 in their tumors. Kaplan-Meier curves for overall and
401 disease-free survival are shown.

402
403 **Fig. 3. BIF-1 positively correlates with BAX active monomeric form in benign nevi but**
404 **not in primary or metastatic melanoma samples.** (a-c) The scatter plot shows the correlation
405 between the mean OD values of BAX active monomeric form with BIF-1 in benign nevi (a),
406 primary melanoma (b) and metastatic melanoma (c) samples. The reported r and p -values show
407 the Pearson correlation.

408
409 **Fig. 4. BIF-1 positively correlates with Beclin-1 in benign nevi but not in primary or**
410 **metastatic melanoma samples.** (a-c) The scatter plot shows the correlation between the IOD
411 values of Beclin-1 with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b)
412 and metastatic melanoma (c) samples. The reported r and p -values show the Pearson
413 correlation.

414
415 **Fig. 5. BIF-1 positively correlates with LC3B in benign nevi but not in primary or**
416 **metastatic melanoma samples.** (a-c) The scatter plot shows the correlation between the IOD
417 values of LC3B with mean OD values of BIF-1 in benign nevi (a), primary melanoma (b) and
418 metastatic melanoma (c) samples. The reported r and p -values show the Pearson correlation.

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