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1 **ECT2 associated to PRICKLE1 are poor-prognosis markers in triple-negative breast**
2 **cancer**

3 Avais M. Daulat^{1,*}, Pascal Finetti², Diego Revinski^{1,4}, Mônica Silveira Wagner¹, Luc
4 Camoin³, Stéphane Audebert³, Daniel Birnbaum², Laurent Kodjabachian⁴, Jean-Paul
5 Borg^{1,3,#,*}, and François Bertucci^{2,#}

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9

10 ¹Centre de Recherche en Cancérologie de Marseille, Aix Marseille Univ UM105, Inst Paoli
11 Calmettes, UMR7258 CNRS, U1068 INSERM, «Cell Polarity, Cell signalling and Cancer -
12 Equipe labellisée Ligue Contre le Cancer », Marseille, France

13

14 ²Centre de Recherche en Cancérologie de Marseille, Aix Marseille Univ UM105, Inst Paoli
15 Calmettes, UMR7258 CNRS, U1068 INSERM, «Predictive Oncology team », Marseille,
16 France

17 ³Aix Marseille University, CNRS, INSERM, Institut Paoli-Calmettes, CRCM, Marseille
18 Protéomique, Marseille, France

19 ⁴ Aix Marseille Univ, CNRS, IBDM, Marseille, France

20

21 * To whom correspondence should be addressed: avais.daulat@inserm.fr/
22 paul.borg@inserm.fr/ Phone 33-4-8697-7201, Fax 33-4-8697-7499

23 # Co-lead authors

24 **Keywords:** PRICKLE1, ECT2, signaling, therapeutic targets, Triple Negative Breast Cancer

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26

27 **Abstract**

28 Triple negative breast cancer (TNBC) is the most aggressive breast cancer subtype and the
29 lack of specific signature makes difficult the development of targeted therapeutic strategy. We
30 previously found that PRICKLE1, an evolutionary conserved protein acting as a regulator of
31 vertebrate development, is upregulated in TNBC. Proteomic approaches allowed us to
32 decipher the protein complex associated to PRICKLE1 in TNBC. Within that complex, we
33 identified a large subset of proteins involved in the regulation of Rho-GTPase family
34 members. We build a metagene with regulators of small G-protein activity and we found that
35 this metagene is overexpressed in TNBC and is a poor prognosis marker. We analyzed the
36 combination of the metagene expression and *PRICKLE1* expression and identified that
37 combined expression of *ECT2* and *PRICKLE1* provides a worst prognosis than *PRICKLE1*
38 expression alone in TNBC. ECT2 is a GEF for Rac1 and we showed that PRICKLE1 regulate
39 the enzymatic activity of ECT2. Finally, we also observed that Ect2 and Prickle1 are
40 functionally connected during evolution since both act synergistically to coordinate cellular
41 movement during vertebrate gastrulation. Our results demonstrate the pivotal role of
42 PRICKLE1 in TNBC and build the path for development of targeted therapeutic strategies to
43 heal TNBC patients.

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51 **Introduction**

52 Triple-negative breast cancer (TNBC) is the most aggressive molecular subtype of breast
53 cancer(Tong et al 2018). In contrast with mammary cancers of other subtypes (HR+/HER2-
54 and HER2+), TNBCs do not express hormone receptors and HER2 oncogene and thus are not
55 candidate to hormone therapy and anti-HER2 therapy (Tong et al 2018). Chemotherapy is the
56 only systemic therapy currently approved for this subtype. However, TNBC is highly invasive
57 with strong metastatic propensity (Tong et al 2018). We recently identified *PRICKLE1* as
58 poor-prognosis marker in breast cancer(Daulat et al 2016). *PRICKLE1* is a member of a
59 conserved group of proteins involved in planar cell polarity (PCP) pathway(Butler and
60 Wallingford 2017). This pathway is well characterized in epithelial tissue morphogenesis
61 during embryonic development of invertebrates and vertebrates. The organization of PCP
62 relies on the spatial distribution of proteins at the plasma membrane such as Wnts, Frizzled,
63 Vang Gogh, Flamingo, Dishevelled, Diego, and Prickle. In vertebrates, homologous genes are
64 involved in the regulation of convergent-extension during the early stages of gastrulation
65 which leads to the organization of cells to organize the head-to-tail axis(Butler and
66 Wallingford 2017, Sokol 2015). Prickle1 plays a pivotal role to regulate PCP in
67 *Drosophila*(Gubb and Garcia-Bellido 1982), as well as convergent-extension in
68 *Zebrafish*(Veeman et al 2003) and *Xenopus*(Takeuchi et al 2003). *PRICKLE1* is an
69 evolutionary conserved cytoplasmic protein and contains from the amino-terminal end a PET
70 followed by three LIM domains and a C-terminal farnesylation site(Jenny et al 2003).
71 Recently, we and others have demonstrated the prominent role of *PRICKLE1* during cancer
72 progression(Daulat et al 2016, Lim et al 2016, Luga et al 2012, Zhang et al 2016). *PRICKLE1*
73 is a prometastatic molecule and regulates oriented cell migration in various cell lines
74 including the MDA-MB-231 prototypal TNBC cell line(Daulat et al 2016, Zhang et al 2016).
75 At the molecular level, *PRICKLE1* regulates subcellular localization of its associated proteins

76 such as VANGL2(Daulat et al 2012, Jenny et al 2003), RICTOR(Daulat et al 2016),
77 ARHGAP22/24(Zhang et al 2016), and LL5 β (Lim et al 2016) in order to coordinate oriented
78 cellular migration.

79 Here, we identified the proteome associated to PRICKLE1 in MDA-MB-231 cells. Among
80 the proteins associated to PRICKLE1, our attention was attracted by a large subset of small
81 G-protein regulators. Since regulation of cancer migration depends of the activation of small
82 G-proteins such as Rac, Rho, and Cdc42, we further explored the role of this subset of small
83 G-protein regulators using transcriptomic analysis from publically available data set obtained
84 from patients with breast cancer. We gathered all the identified small G-protein regulators in a
85 metagene to allow an in-depth analysis and showed that *PRICKLE1* was not only
86 overexpressed in TNBC, but its associated proteins were also up-regulated in TNBC and were
87 poor-prognosis markers. To further explore the protein complex associated to PRICKLE1, we
88 focused our attention on the Rho-Guanylyl Exchange Factor (GEF) called Epithelial cell
89 transforming sequence 2 (ECT2). In non-transformed cells, ECT2 regulates cytokinesis by
90 regulating Rac1 activity(Huff et al 2013, Justilien and Fields 2009, Justilien et al 2011,
91 Justilien et al 2017). *ECT2* is frequently up-regulated in various cancers such as ovarian(Huff
92 et al 2013), lung(Zhou et al 2017) and breast cancer(Wang et al 2018). Knockdown of ECT2
93 inhibits Rac1 activity and block transformed growth, invasion and tumorigenicity(Justilien
94 and Fields 2009, Justilien et al 2011). Here we showed that PRICKLE1 was associated to
95 ECT2 in MDA-MB-231 cells to regulate Rac1 activity and therefore promote cell motility.
96 Using *Xenopus laevis* embryos, we showed that Prickle1 and Ect2 acted synergistically during
97 embryonic development. Together these data demonstrate the importance of Prickle1 and its
98 associated protein complex as poor-prognosis markers in TNBC and give evidence that
99 PRICKLE1 can be a suitable therapeutic target for treatment of still lacking targeted therapy
100 for this aggressive subtype.

101

102 **Results**

103 **Mass spectrometry analysis of the PRICKLE1 complex shows that PRICKLE1 is** 104 **associated with small G-protein regulators and modulates Rac and Rho activity**

105 We and others have shown that PRICKLE1 contribute to cancer cell dissemination in various
106 cancers(Daulat et al 2016, Lim et al 2016, Luga et al 2012, Zhang et al 2016). To investigate
107 the molecular mechanisms underlying the role of PRICKLE1 in tumorigenicity, and notably
108 cell motility and dissemination, we generated a stable cell line expressing GFP-PRICKLE1 in
109 the MDA-MB-231 highly invasive TNBC cell line. To identify protein complexes associated
110 to PRICKLE1 in these cells, we performed anti-GFP immunoprecipitation followed by mass
111 spectrometry analysis. We identified previously known PRICKLE1 interactors such as
112 VANGL1, MINK1, RICTOR, LL5 β , PLK1, and USP9x, validating our approach (**Fig. 1A**).
113 Cell migration is a complex and dynamic process that involves continuous remodeling of the
114 cellular architecture and relies on spatiotemporal modulation of signaling networks including
115 Rho-family GTPases. Our attention was attracted by the large number of regulators of Rho-
116 family GTPases such as Rac1, Rho and Cdc42 (**Fig. 1B**), known to be notably involved in the
117 regulation of cell motility and considered as interesting drug targets to prevent cancer
118 dissemination.

119

120 **Prognosis value of PRICKLE1-interacting small G-protein regulators in TNBC**

121 Based on our generated proteomic data describing the protein complex associated to
122 PRICKLE1, we focused our attention on the 10 regulators of small G-proteins (i.e. Rho-GEF
123 and Rho-GAP) identified, including ARHGAP21, ARGHAP22, ARHGAP23, ARHGEF2,
124 ARHGEF40, BCR, ECT2, IQGAP3, MYO9B and STARD13. We assessed the mRNA
125 expression level of the corresponding genes in a retrospective series of 8,982 clinically

126 annotated patients with invasive primary breast cancer gathered from several public data
127 bases (**Table S1**). Within these 10 genes, *ECT2*, *IQGAP3* and *MYO9B* were the most
128 overexpressed in tumors as compared to normal breast (**Fig. 2A**), whereas *ARHGEF40* and
129 *STARD13* showed the lowest expression levels. We built a metagene including these 10 genes
130 and compared its expression level in three molecular subtypes of breast cancer (RH+/HER2-,
131 HER2+, and TN). The metagene was significantly up-regulated in the TN subtype
132 comparatively to the two others subtypes ($p < 1.0 \times 10^{-250}$, Anova) (**Fig. 2B**).

133 We then searched for correlations between the GAP-GEF metagene expression (as binary
134 variable) and the clinicopathological features of samples, including MFS. Within the 8,982
135 breast cancer samples analyzed, 4,491 tumors (50%) showed metagene upregulation when
136 compared with normal breast (ratio T/NB ≥ 2 ; “metagene-up” group), and 4,491 (50%) did
137 not (ratio < 2 ; “metagene-down” group) (**Table 1**). We found significant correlations between
138 the metagene status and patients’ age ($p < 0.001$), grade ($p < 0.001$), ER ($p < 0.001$), PR
139 ($p < 0.001$), and HER2 ($p = 0.012$) statuses and as shown above with molecular subtypes. MFS
140 data were available for 2,030 patients: the 5-year MFS was 75% (95 CI, 72-79) in the
141 “metagene-down” group *versus* 67% (95CI, 63-71) in the “metagene-up” group ($p = 0.00023$,
142 log-rank test; **Fig. 2C**). In fact, such prognostic correlation was only observed in TNBC
143 patients, and not in the non-TNBC ones ($p = 0.461$, log rank test; **Fig. 2D**). In TNBC patients,
144 the 5-year MFS was 77% (95 CI, 66-90) in the “metagene-down” group *versus* 60% (95CI,
145 54-66) in the “metagene-up” group ($p = 0.029$, log-rank test; **Fig. 2E**).

146

147 **Cooperation between PRICKLE1 and ECT2 as poor-prognosis marker in TNBC**

148 We have previously shown that *PRICKLE1* upregulation is associated with poor MFS in basal
149 breast cancer (Daulat et al 2016), a molecular subtype mainly composed of TNBC. In the
150 present series of TNBC, we confirmed that *PRICKLE1* upregulation was associated with

151 shorter MFS, with 70% 5-year MFS (95CI, 61-79) *versus* 55% (95CI, 48-63) in the
152 *PRICKLE1*-down group and the *PRICKLE1*-up group respectively (p=0.0147, log-rank test)
153 (**Fig. 2F**). Since *PRICKLE1* and the 10 genes of the metagene interact together, we searched
154 for an eventual cooperation of their association in prognostic term. First, we analyzed the
155 combination of the metagene expression and *PRICKLE1* expression. Interestingly, patients
156 with upregulation of both markers displayed shorter 5-year MFS (53%, 95CI, 46-62) than
157 patients without upregulation of both markers (72%, 95CI 60-88; p=0.017, log-rank test),
158 whereas patients with intermediate status (up and no-up, and vice-versa) showed intermediate
159 5-year MFS not significantly different from the same patients (p=0.757, and p=0.495
160 respectively, log-rank test; **Fig. 2G**). This data suggest that metagene expression and
161 *PRICKLE1* expression might provide complementary prognostic value. Such
162 complementarity between the two prognostic variables was tested in the TN patients using the
163 likelihood ratio (LR) test. As shown in **Table 2A**, the metagene tended to add prognostic
164 information to that provided by *PRICKLE1* expression (LR- $\Delta X^2=2.75$, p=0.097).

165 Second, because *ECT2* was one of the most prominent hit identified by mass spectrometry
166 analysis (**Fig. 1B**) and the gene most overexpressed in TNBCs among members of the
167 metagene (**Fig. 2A**), we investigated whether *ECT2* expression alone (without the nine other
168 genes of the metagene) would be sufficient to improve the prognostic value of *PRICKLE1*
169 expression in TNBC patients. As shown in **Figure 2H**, patients with *ECT2* upregulation
170 displayed shorter 5-year MFS (56%, 95CI 50-64) than patients without upregulation (70%,
171 95CI 60-81; p=0.0243, log-rank test). More interestingly, *ECT2* expression status increased
172 the prognostic value of *PRICKLE1* expression when combined. Patients with upregulation of
173 both genes displayed 50% 5-year MFS (95CI, 46-62) *versus* 67% for patients with
174 intermediate status (up and down, and vice-versa) *versus* 76% (95CI, 64-90) for patients
175 without upregulation of both markers (p=0.0134, log-rank test; **Fig. 2I**). The model

176 comparison (**Table 2B**) showed that such *ECT2* prognostic information added to that of
177 *PRICKLE1* expression was statistically significant (LR- Δ X²=4.74, p=0.029), indicating that
178 *ECT2* expression improved the prognostic value of *PRICKLE1* expression in TNBC.

179

180 *PRICKLE1 binds to ECT2 through its PET domain and modulates Rac1 activity*

181 We then sought to investigate the molecular mechanisms potentially associated to this
182 cooperation of *PRICKLE1* and *ECT2* expressions to confer poor prognosis. *ECT2* is a Rho-
183 GEF and acts in non-cancerous cells as regulator of cytokinesis by exchanging GDP to GTP
184 on the small GTPases, RhoA, Rac1 and Cdc42(Basant and Glotzer 2018). *ECT2* is
185 upregulated in human cancers and acts as oncogene(Jin et al 2014). In lung and ovarian
186 cancer, *ECT2* has a distinct role than cytokinesis and acts in the nucleus by recruiting Rac1
187 and effectors which are required for tumour initiation and transformation(Justilien and Fields
188 2009, Justilien et al 2011, Justilien et al 2017). *ECT2* knockdown inhibits Rac1 activity
189 leading to a decrease of tumorigenicity and invasion in lung adenocarcinoma(Justilien and
190 Fields 2009). Recently, *ECT2* has been described to be upregulated in breast cancer(Wang et
191 al 2018). At the cellular level, *ECT2* is localized in the cytoplasm of cancerous cells(Justilien
192 et al 2011). To confirm our mass spectrometry analysis, we immunoprecipitated GFP-
193 *PRICKLE1* stably expressed in MDA-MB-231 cells using GFP-targeted antibody and we
194 assessed the presence of *ECT2* associated to *PRICKLE1* immunoprecipitate by western blot
195 analysis complex (**Fig. 3A**). We confirmed that *ECT2* is associated with *PRICKLE1* in MDA-
196 MB-231 cells. We further confirmed that *ECT2* colocalizes in actin-enriched structures of
197 lamellipodia along with *PRICKLE1* using MDA-MB-231 stably expressing GFP-*PRICKLE1*
198 (**Fig. 3B**). We next decided to map the domain of interaction between *PRICKLE1* and *ECT2*.
199 We thus generated deleted versions of *PRICKLE1* lacking the PET and/or the LIM domains
200 and a construct encompassing the *PRICKLE1* C-terminal region. We co-transfected

201 HEK293T cells with the indicated flag tagged PRICKLE1 mutants with Cherry-ECT2. After
202 Flag immunoprecipitation, we assessed the presence of Cherry-ECT2 by western blot
203 analysis. We observed that the PET domain of PRICKLE1 was required for the formation of
204 the PRICKLE1-ECT2 protein complex (**Fig. 3C**).

205 We further assessed PRICKLE1 contribution on Rac activity. We used previously
206 characterized siRNAs(Daulat et al 2016) to specifically downregulate PRICKLE1 expression
207 in MDA-MB-231 cells. We observed that PRICKLE1 modulated Rac1 activity, suggesting a
208 prominent role of PRICKLE1 in the regulation of Rho-GEF and Rho-GAP (**Fig. 3D**). We next
209 set up an assay to monitor the role of PRICKLE1 on ECT2 Rho GEF activity. We expressed
210 cherry-ECT2 in HEK293T cells and observed an increase of active Rac1 (lane 2). However,
211 when flag-PRICKLE1 was co-expressed with cherry-ECT2, we observed an inhibitory effect
212 of PRICKLE1 (lane 3). This observation was confirmed by the co-expression of PRICKLE1
213 delta PET delta LIM1 which is unable to bind ECT2 and does not affect the gain of activity of
214 ECT2 in our system (lane 4) (**Fig. 3E**). Altogether, our data suggest that PRICKLE1 is
215 associated with ECT2 in actin-rich structures within the lamellipodia of the cells in order to
216 modulate the activity of the ECT2 on Rac1.

217

218 ***Prickle1 and Ect2 functionally interact in Xenopus during embryonic development***

219 PRICKLE1 is an evolutionary conserved protein and plays a pivotal role during gastrulation
220 to modulate convergent-extension movements (CE), which are crucial to shape the body
221 plan(Takeuchi et al 2003, Wallingford et al 2002a). To test whether Ect2 is required for the
222 previously characterized function of Prickle1 during CE, we first compared and analyzed the
223 RNA-seq profile of *prickle1* and *ect2* reported on the public XenBase repository(Session et al
224 2016) (data not shown). We noticed a sharp peak of zygotic *ect2* expression at stage 9,
225 which decreases abruptly at stage 10, just before gastrulation and CE movements take place.

226 Zygotic *prickle1* expression also begins to increase at stage 9, reaching a maximum at stage
227 12 (mid gastrula), and gradually decreasing until the end of neurulation. We next performed
228 *in situ* hybridization and detected expression of *ect2* RNA in the animal hemisphere up until
229 stage 9 (**Fig. 4A**). Thus, *ect2* transcription appears to terminate when *prickle1* transcription
230 starts. However, inspection of genome-wide proteomic data(Peshkin et al 2015) indicated that
231 Ect2 protein levels were maintained during gastrulation, suggesting that Ect2 could cooperate
232 with Prickle1 to regulate morphogenetic movements. To test this hypothesis, we performed
233 Prickle1 and Ect2 knockdown through antisense morpholinos (MO) injections, and assessed
234 CE problems (**Fig. 4B**). Injection of 40ng MO Prickle1 led to CE defects in 73% of embryos,
235 in comparison to non-injected embryos (98%) or embryos injected with RFP as control
236 (83%). This data is consistent with previously published results(Daulat et al 2012, Takeuchi et
237 al 2003). We then injected 20ng of MO targeting Ect2 and we observed CE problems at a rate
238 of 71%, phenocopying the effect observed with MO Prickle1 with narrower and shorter
239 embryos at tailbud stage 28. We then defined subthreshold doses of individual Mo-Prickle1
240 (≤ 10 ng) and Mo-Ect2 (≤ 10 ng) that yielded moderate CE defects in this assay when
241 injected separately into two blastomeres at 2-cell stage (18% and 12% CE defects,
242 respectively). In contrast, co-injecting both MOs at subthreshold doses caused strong
243 disruption of CE movements (67%), suggesting that Prickle1 and Ect2 functionally interact
244 during *Xenopus* embryonic development.

245

246 **Discussion**

247 We and others have demonstrated the prominent role of PRICKLE1 during cancer
248 progression(Daulat et al 2016, Lim et al 2016, Luga et al 2012, Zhang et al 2016). In this
249 study, we identified the protein complex associated to PRICKLE1 and we aimed to evaluate
250 the impact of PRICKLE1 and its associated protein complex in TNBC. Our results show that

251 PRICKLE1 acts as a scaffold protein due to the large number of associated proteins with
252 enzymatic activity. Among the PRICKLE1-associated proteins, we focused our attention on
253 small G-protein regulators since their impact on cell motility and cancer cell dissemination
254 has been well characterized(Abreu-Blanco et al 2014, Cook et al 2014, Machacek et al 2009).
255 Exploiting our transcriptomic breast cancer database, we showed that this subset of genes is
256 up-regulated in TNBC. Among this group of genes, we identified *ECT2* as the most
257 prominent contributor to *PRICKLE1* prognostic value. Indeed TNBC patient with up-
258 regulated expression of both *PRICKLE1* and *ECT2* expression have a shorter MFS than other
259 patients. We further characterized PRICKLE1 and ECT2 interaction and showed that
260 PRICKLE1 controlled ECT2 function on Rac1 activation. We finally defined that Prickle1
261 and Ect2 interaction was evolutionary conserved, since both proteins contribute to *Xenopus*
262 embryonic development and are involved in convergent-extension movements.

263 Among breast cancers, TNBC are considered as the most aggressive form and no targeted
264 therapy is currently available due to a lack of specific targets(Tong et al 2018). Here, we show
265 that *PRICKLE1* is overexpressed in TNBC and is a poor-prognosis marker. PRICKLE1 is a
266 protein highly regulated by post-translational modifications, particularly through
267 ubiquitination/deubiquitination. PRICKLE1 is indeed the target of SMURF1, an ubiquitin
268 ligase, which allows its rapid degradation(Narimatsu et al 2009). PRICKLE1 is also protected
269 from degradation by USP9x which de-ubiquitinates the protein(Paemka et al 2015).
270 Interestingly USP9x is also up-regulated in several cancers and is considered as a poor-
271 prognosis marker(Murtaza et al 2015). PRICKLE1 is also regulated through phosphorylation
272 by the serine/threonine kinase called MINK1, which promotes its function, its membrane
273 localization and association with signaling molecules(Daulat et al 2012). Together, this shows
274 that PRICKLE1 is a pivotal protein in cancer cell dissemination and a candidate for setting up
275 novel therapeutic strategies.

276

277 During developmental processes and cancer progression, PRICKLE1 is required for oriented
278 cell migration (Chiapparo et al 2016, Daulat et al 2016, Lim et al 2016, Luga et al 2012). At
279 the molecular level, we and others have shown that PRICKLE1 contributes to localize
280 VANGL at the plasma membrane (Daulat et al 2012, Jenny et al 2003), LL5 β at the +ends of
281 the microtubules (Lim et al 2016), and to restrict localization of the Rho-GAP at the edge of
282 the migrating cancer cells (Zhang et al 2016). PRICKLE1 also regulates spatial localization of
283 several active proteins such as mTORC2 to allow local activation of Akt at the leading edge
284 of migrating cells (Daulat et al 2016), PHLDB2 to disassemble focal adhesions (Lim et al
285 2016) and to restrict RhoA activity by regulating subcellular localization of Rho-GAP (Zhang
286 et al 2016). Together the contribution of PRICKLE1 to localization of its interacting partners
287 allows the cells to coordinate cellular movements to create a cellular imbalance and promote
288 directed cell migration. Here we showed that PRICKLE1 also contribute to regulate the
289 activity of ECT2, a GEF for Rac1, which is essential for cell motility.

290 ECT2 is a Rho-GEF controlling Rac1 activity (Justilien and Fields 2009). Although ECT2 has
291 been extensively studied for its role in the nucleus and during cytokinesis, reports have shown
292 that ECT2 can also be localized in the cytoplasm of cancerous cells (Justilien et al 2011). We
293 observed that ECT2 is localized in actin-rich structures within the lamellipodia. As described
294 for other PRICKLE1 interactors, PRICKLE1 might contribute to ECT2 spatial localization in
295 order to modulate its Rac activity. Moreover, our data show that overexpression of ECT2 in
296 HEK293T cells contributes to an increase of Rac activity, and that PRICKLE1 overexpression
297 leads to a decrease of this gain of function, suggesting an inhibitory role of PRICKLE1 on
298 ECT2 activity. Altogether, this depicts PRICKLE1 as a master regulator of localized
299 expression and regulation of signaling events in migratory cancer cells.

300 Our data also identified a role for the PET domain of PRICKLE1, as ECT2 is to date the only
301 protein identified to be associated with this domain. At the molecular level, it has been shown
302 that PRICKLE1 exists in an open and closed conformation(Sweede et al 2008). It has been
303 suggested that in closed conformation, the three LIM domains of PRICKLE1 mask the
304 PRICKLE1 PET domain. In open conformation, the PET domain is unmasked, thus activating
305 PRICKLE1. We can speculate that the interaction between PRICKLE1 and ECT2 can be
306 modulated by switching between these two conformations providing a still uncharacterized
307 molecular mechanism of PRICKLE1 activation.

308 Finally, our study identified that ECT2 acts during *Xenopus* embryonic development. Prickle1
309 has been extensively characterized for its contribution during convergent-extension(Takeuchi
310 et al 2003, Veeman et al 2003) movements and has been shown to be asymmetrically
311 distributed within cells in order to organize their movement(Ciruna et al 2006, Yin et al
312 2008). A previous study indicated that *Prickle1* mRNA accumulates within the blastopore lip
313 from the onset of gastrulation(Wallingford et al 2002b). Here, we showed that *ect2* mRNA
314 and presumably Ect2 protein are expressed prior to and in a broader pattern than
315 Prickle1(Wallingford et al 2002b). Knockdown experiments strongly suggest that Prickle1
316 and Ect2 act together to allow convergence-extension movements during gastrulation.
317 Altogether, our data support the view that Ect2 might represent a permissive factor for
318 Prickle1 activity. This study demonstrates the importance of the evolutionary conserved
319 interaction between Prickle1 and Ect2, which appears to be reactivated during tumorigenesis
320 to promote cancer cell dissemination and metastasis.

321

322 **Materials and Methods**

323

324 **Rac1 activity assay**

325 Cells were lysed with ice cold lysis buffer (50 mM Tris, pH7.6, 150mM NaCl, 0.1% Triton X-
326 100, 20mM MgCl₂ supplemented with protease inhibitor (Sigma)). Supernatant were collected
327 after 10 min of centrifucation at 10,000xg at 4°C. Protein concentration is measured from the
328 solubilized fraction and adjusted to 2mg/mL. 10% of the lysates are conserved as loading
329 controls. 100µg of GST-CRIB are added to 2mg of lysate and incubate with rotation during
330 30 min at 4°C. Beads are then washed with 10 volumes of lysis buffer. Rac-GTP forms are
331 eluted from the beads using 2x Leammli buffer. 30% of the sample are run on 15%SDS-
332 PAGE gel and transfer to PVDF and blot with the indicated antibody.

333

334 **Breast cancer samples and gene expression profiling**

335 Our institutional series included 353 tumor samples from pre-treatment invasive primary
336 mammary carcinomas either surgically removed or biopsied.(Sabatier et al 2011) The study
337 was approved by our institutional review board. Each patient had given a written informed
338 consent for research use. Samples had been profiled using Affymetrix U133 Plus 2.0 human
339 microarrays (Santa Clara, CA, USA). We pooled them with 35 public breast cancer data sets
340 comprising both gene expression profiles generated using DNA microarrays and RNA-Seq
341 and clinicopathological annotations. These sets were collected from the National Center for
342 Biotechnology Information (NCBI)/Genbank GEO, ArrayExpress, European Genome-
343 Phenome Archive, The Cancer Genome Atlas portal (TCGA) databases, and authors' website
344 (**Supplementary Table 1**). The final pooled data set included 8982 non-redundant non-
345 metastatic, non-inflammatory, primary, invasive breast cancers.

346

347 **Gene expression data analysis**

348 Before analysis, several steps of data processing were applied. The first step was the
349 normalization of each set separately. It was done in R using Bioconductor and associated

350 packages; we used quantile normalization for the available processed data from non-
351 Affymetrix-based sets (Agilent, SweGene, and Illumina), and Robust Multichip Average
352 (RMA) with the non-parametric quantile algorithm for the raw data from the Affymetrix-
353 based sets. In the second step, we mapped the hybridization probes across the different
354 technological platforms represented as previously reported.(Bertucci et al 2014) When
355 multiple probes mapped to the same GeneID, we retained the most variant probe in a
356 particular dataset. We log₂-transformed the available TCGA RNA-Seq data that were already
357 normalized. In order to avoid biases related to trans-institutional IHC analyses and thanks to
358 the bimodal distribution of respective mRNA expression levels, the ER, progesterone receptor
359 (PR), and HER2 statuses (negative/positive) were defined on transcriptional data of *ESR1*,
360 *PGR*, and *HER2* respectively, as previously described.(Lehmann et al 2011) The molecular
361 subtypes of tumors were defined as HR+/HER2- for ER-positive and/or PR-positive and
362 HER2-negative tumors, HER2+ for HER2-positive tumors, and triple-negative (TN) for ER-
363 negative, PR-negative and HER2-negative tumors. Next, expression levels of *PRICKLE1* and
364 10 genes of interest from the protein complex associated to Prickle1 (namely, *ARHGAP21*,
365 *ARGHAP22*, *ARHGAP23*, *ARHGEF2*, *ARHGEF40*, *BCR*, *ECT2*, *IQGAP3*, *MYO9B*, and
366 *STARD13*) were extracted from each of the 36 normalized data sets. Before analysis, gene
367 expression levels were standardized within each data set using the PAM50 luminal A
368 population as reference. This allowed to exclude biases due to laboratory-specific variations
369 and to population heterogeneity and to make data comparable across all sets. *PRICKLE1* and
370 *ECT2* upregulation in a tumor was defined by an expression level above median expression
371 the other cases being defined as downregulation. GEF/GAP activity was based on metagene
372 approach and computed on the mean of the 10 related genes standardized. GEF/GAP activity
373 “up” was defined by a metagene score value above the global median of the metagene
374 whereas other cases were defined as “down”.

375

376 **Statistical analysis**

377 Correlations between tumor classes and clinicopathological variables were analyzed using the
378 one-way analysis of variance (ANOVA) or the Fisher's exact test when appropriate.
379 Metastasis-free survival (MFS) was calculated from the date of diagnosis until the date of
380 distant relapse. Follow-up was measured from the date of diagnosis to the date of last news
381 for event-free patients. Survivals were calculated using the Kaplan-Meier method and curves
382 were compared with the log-rank test. The likelihood ratio (LR) tests were used to assess the
383 prognostic information provided beyond that of PRICKLE1 model, GEF/GAP metagene or
384 ECT2 model, assuming a χ^2 distribution. Changes in the LR values ($LR-\Delta X^2$) measured
385 quantitatively the relative amount of information of one model compared with another. All
386 statistical tests were two-sided at the 5% level of significance. Statistical analysis was done
387 using the survival package (version 2.30) in the R software (version 2.15.2;
388 <http://www.cran.r-project.org/>). We followed the reporting REcommendations for tumor
389 MARKer prognostic studies (REMARK criteria)(McShane et al 2005).

390

391 **Xenopus embryo injections, plasmids, RNAs, and Mos**

392 Eggs obtained from NASCO females were fertilized in vitro, dejellied and cultured as
393 described previously(Marchal et al 2009). Wild-type embryos were obtained using standard
394 methods(Franco et al 1999) from adult animals and staged according to Nieuwkoop and Faber
395 (1994).

396 Ect2 riboprobe was generated from *Xenopus laevis* full-length Ect2 cDNA, obtained from
397 Dharmacom™ (Plasmid XGC ect2 cDNA, Clone ID: 5083828; pCMV-SPORT6.ccdb). The
398 cDNA was subcloned in pBS-SK vector. For *Ect2* sense probe the plasmid was linearized by

399 NotI and transcribed with T7 RNA polymerase. For *Ect2* antisense probe the plasmid was
400 linearized by EcoRV and transcribed with T3 RNA polymerase.

401 Synthetic capped mRFP mRNA was produced using Ambion mMESSAGING mMACHINE Kit.
402 pCS2-mRFP was linearized with NotI and mRNA was synthesized with Sp6 polymerase.
403 0,5ng of mRFP capped mRNA was used as injection control and tracer.

404 Morpholino antisense oligonucleotides (MO) were obtained from Genetools®, and the
405 sequences were the following: Prickle1 (Pk1) 5'-CCTTCTGATCCATTTCCAAAGGCAT-3'
406 (Dingwell and Smith 2006); ECT2 5'-TACTGGGAGAGCCATGTTTGATTT-3'. Embryos at
407 2-cell stage were injected in each blastomere with various doses of MOs. Embryos were
408 cultured in modified Barth's solution until stage 28, when they were photographed.

409

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422 use of samples and data, patients were appropriately informed and asked to express their
423 consent in writing, in compliance with French and European regulations. The project was

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426

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569 independent prognostic factor for poor overall survival and recurrence-free survival in non-
570 small cell lung adenocarcinoma. *PLoS One* **12**: e0187356.

571

572

573 **Figure legends:**

574 **Figure 1: Mass spectrometry analysis of the PRICKLE1 protein complex from a TNBC**
575 **cell line**

576 A) Schematic representing the proteins associated to PRICKLE1 identified by mass
577 spectrometry analysis from MDA-MB-231 cell extracts. Proteins have been classified
578 following their function in several groups: Small G-proteins regulators, associated to
579 cytoskeleton, Kinases, involved in Ubiquitination process, Membrane integrated, Scaffold
580 proteins and others. B) Volcano plot showing the significance two-sample *t*-test (-Log p-
581 value) *versus* fold-change (Log₂ (GFP-PRICKLE1 *versus* GFP as control)) on the y and x
582 axes, respectively. The full line is indicative of protein hits obtained at a permutation false
583 discovery rate of 1% (pFDR). Data results from two different experiments processed three
584 times. PRICKLE1 (the bait) is represented in red and ECT2 one of the most abundant
585 PRICKLE1 associated partner is represented in green.

586

587 **Figure 2: Prognosis value of PRICKLE1-interacting small G-protein regulators in**
588 **TNBC and cooperation between PRICKLE1 and ECT2 as poor prognosis markers**

589 A) Boxplot of GEF/GAP regulators expression across breast cancers. B) Boxplot of
590 GEF/GAP regulators expression across triple negative (TN) *versus* HR+/HER2- or HER2+
591 breast cancer. C) Kaplan-Meier curves of metastasis-free survival among breast cancers
592 patients according to overexpression (Up) *versus* underexpression (Down) of GEF/GAP
593 metagene mRNA. D) Kaplan-Meier curves of metastasis-free survival among non-TNBC
594 patients for GEF/GAP metagene mRNA expression. E) Kaplan-Meier curves of metastasis-
595 free survival among TNBC patients for GEF/GAP metagene mRNA expression. F) Kaplan-
596 Meier curves of metastasis-free survival among TNBC patients for PRICKLE1 mRNA
597 expression. G) Kaplan-Meier curves of metastasis-free survival among TNBC patients for

598 *PRICKLE1* mRNA and GEF/GAP metagene expression. H) Kaplan-Meier curves of
599 metastasis-free survival among TNBC patients for *ECT2* mRNA expression. I) Kaplan-Meier
600 curves of metastasis-free survival among TNBC patients for *PRICKLE1* and *ECT2* mRNA
601 expression.

602

603 **Figure 3: PRICKLE1 is associated to the Rho-GEF ECT2 and controls its activity**

604 A) Immunopurification of GFP-PRICKLE1 from MDA-MB-231 cell lysate using GFP
605 nanobodies coupled to sepharose beads allows the identification of ECT2 associated to
606 PRICKLE1. B) Immunofluorescence of MDA-MB-231 cells stably expressing GFP-
607 PRICKLE1 shows that ECT2 (endogenous) is colocalized with PRICKLE1 and enriched in
608 actin structures within the lamellipodia. C) Mapping of PRICKLE1 domain of interaction
609 with ECT2. HEK293T cells were co-transfected with the indicated form of PRICKLE1 (see
610 on the left for topology details) and Cherry-ECT2. After FLAG immunopurification, presence
611 of ECT2 is detected using anti-cherry antibody. D) Downregulation of PRICKLE1 expression
612 using siRNA targeting *PRICKLE1* shows an increase of Rac activity in MDA-MB-231 cells.
613 E) PRICKLE1 modulates ECT2 activity. Using HEK293T cells, we expressed or co-
614 expressed ECT2 with full length PRICKLE1 or a deleted version of PRICKLE1 lacking its
615 domain of interaction with ECT2. Overexpression of ECT2 leads to an increase of Rac
616 activity which was inhibited when PRICKLE1 is co-expressed. Co-expression of a mutant
617 form of PRICKLE1 did not modify the gain of function observed by ECT2 overexpression.

618

619 **Figure 4: Prickle1 and Ect2 functionally interact in Xenopus during embryonic**
620 **development**

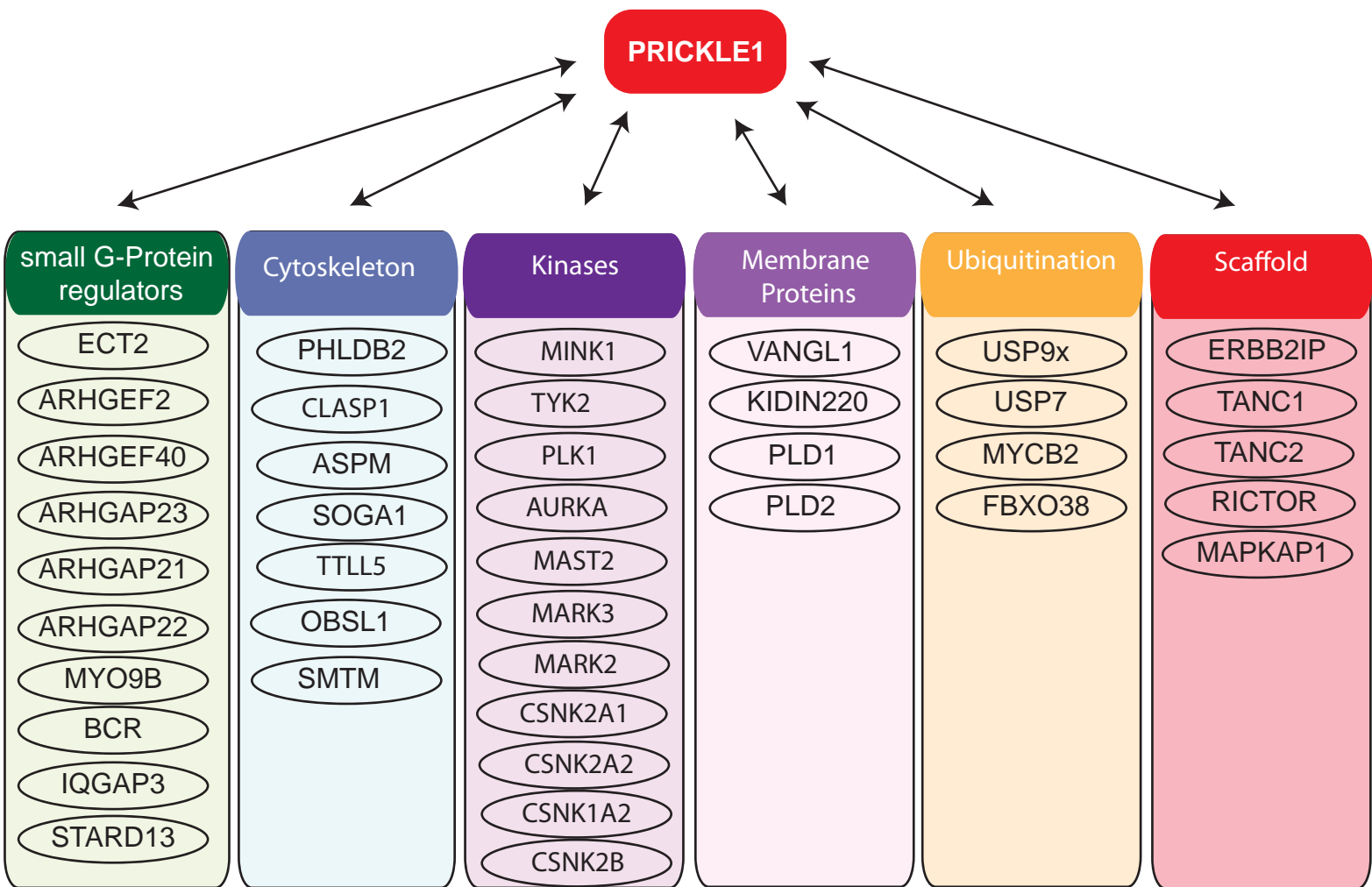
621 A) *In situ* hybridization against *ect2* transcripts at stage 8, 9 and 10. *ect2* RNA is detectable in
622 the animal pole (animal view and lateral view) but not in the vegetal pole (vegetal view) at

623 stages 8 and 9, but no longer at stage 10. Schematic representations of embryos at the stages
624 analyzed are shown on the right. B) Embryos at 2-cell stage were injected into two
625 blastomeres with Prickle1 and Ect2 MOs as indicated. In all cases 0,5ng of *mRFP* mRNA was
626 injected as control and tracer. Suboptimal doses (10ng) of either MO did not cause CE
627 problems. However, when both Prickle1 and Ect2 MOs were co-injected at suboptimal doses
628 (5ng each), embryos displayed CE problems at a rate comparable to high doses of each MO
629 injected separately (40ng Prickle1-MO or 20ng Ect2-MO). A total of 60 embryos per
630 condition were analyzed in two independent experiments. Pictures illustrate representative
631 phenotypes. (SR=survival rate; ND=percentage of surviving embryos developing normally;
632 CED= percentage of surviving embryos showing convergent-extension defects). Scale bars: A
633 = 0,25 mm; B = 0,5mm.

634

FIGURE 1:

A



B

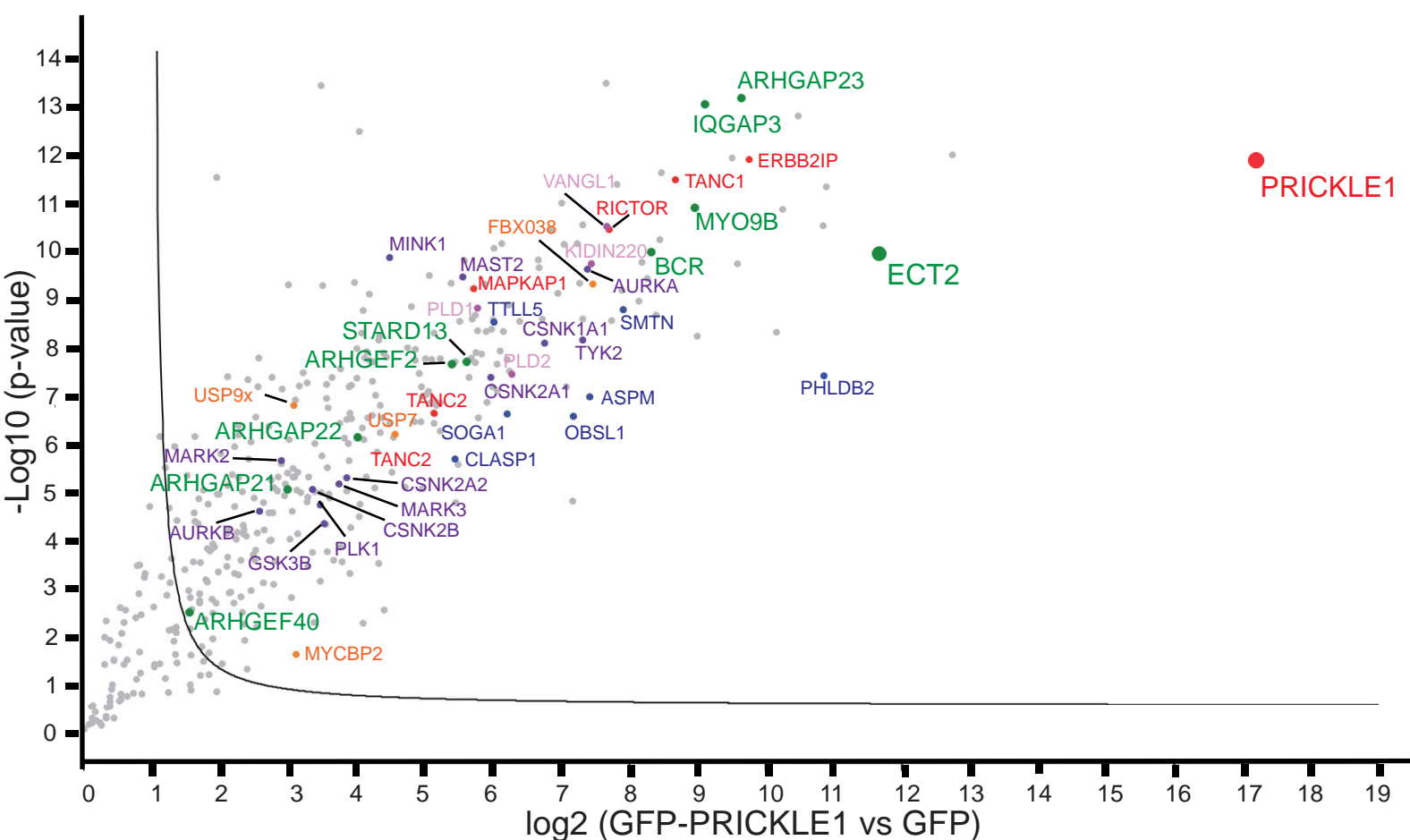


FIGURE 2:

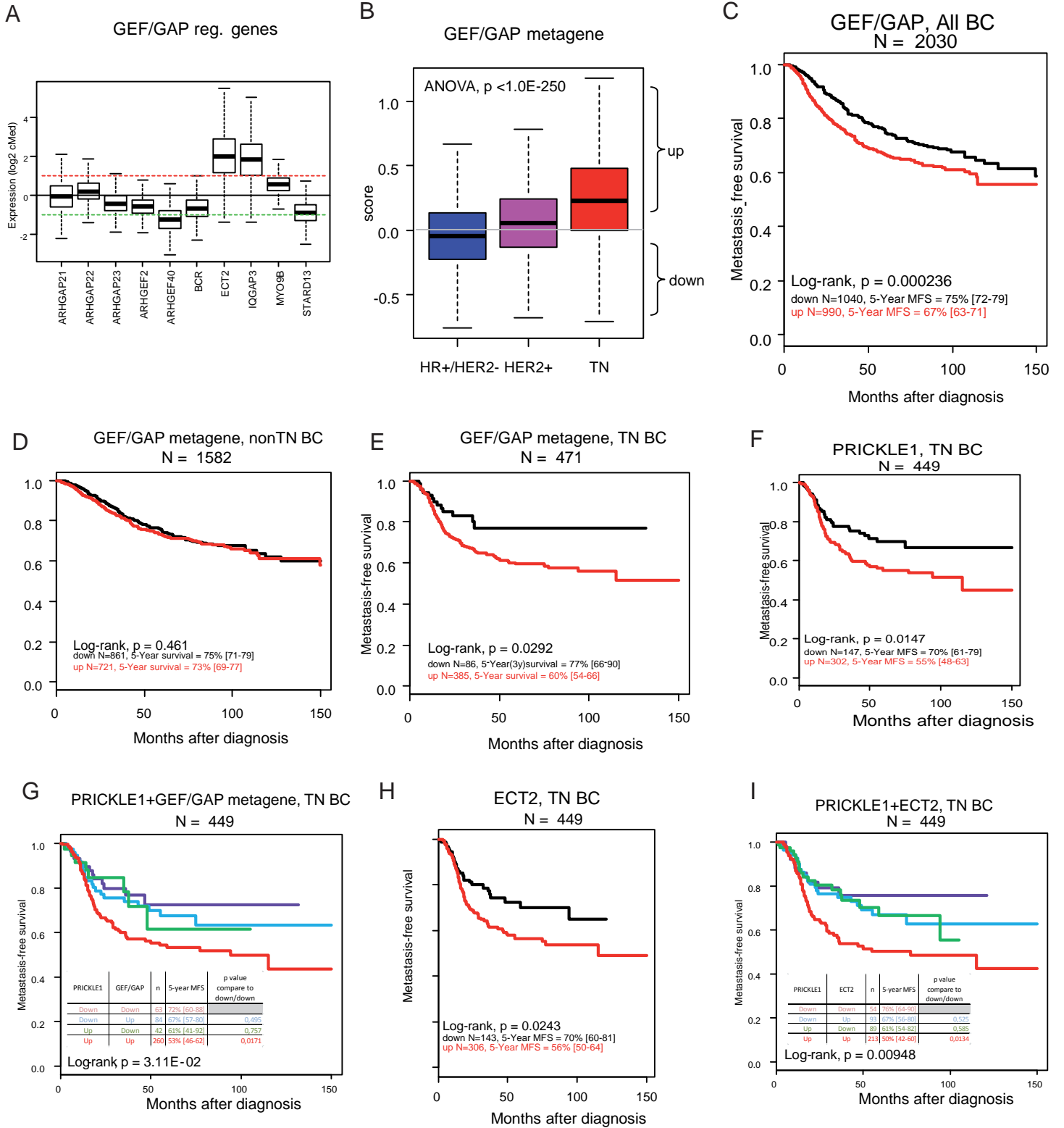


FIGURE 3:

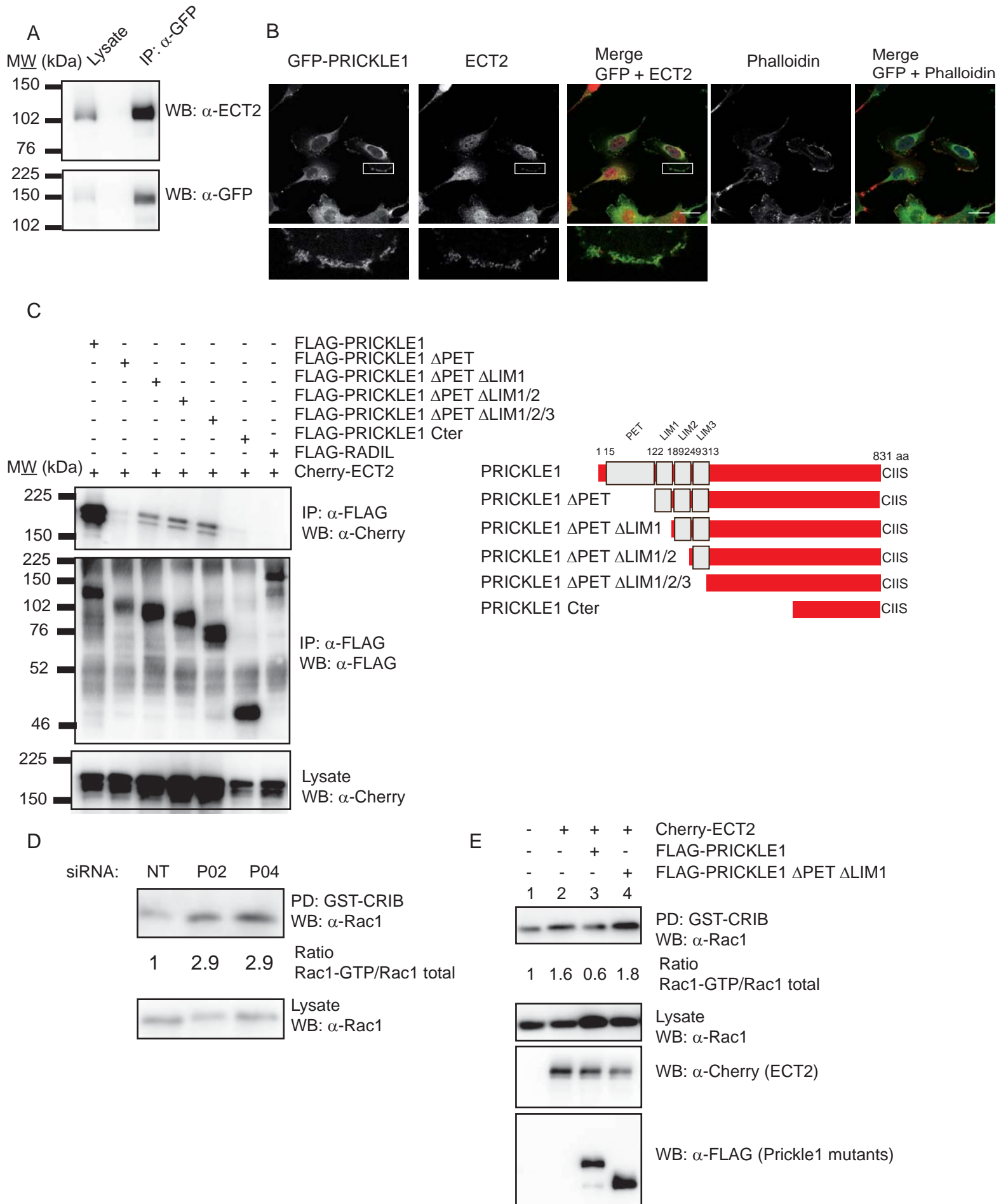


FIGURE 4:

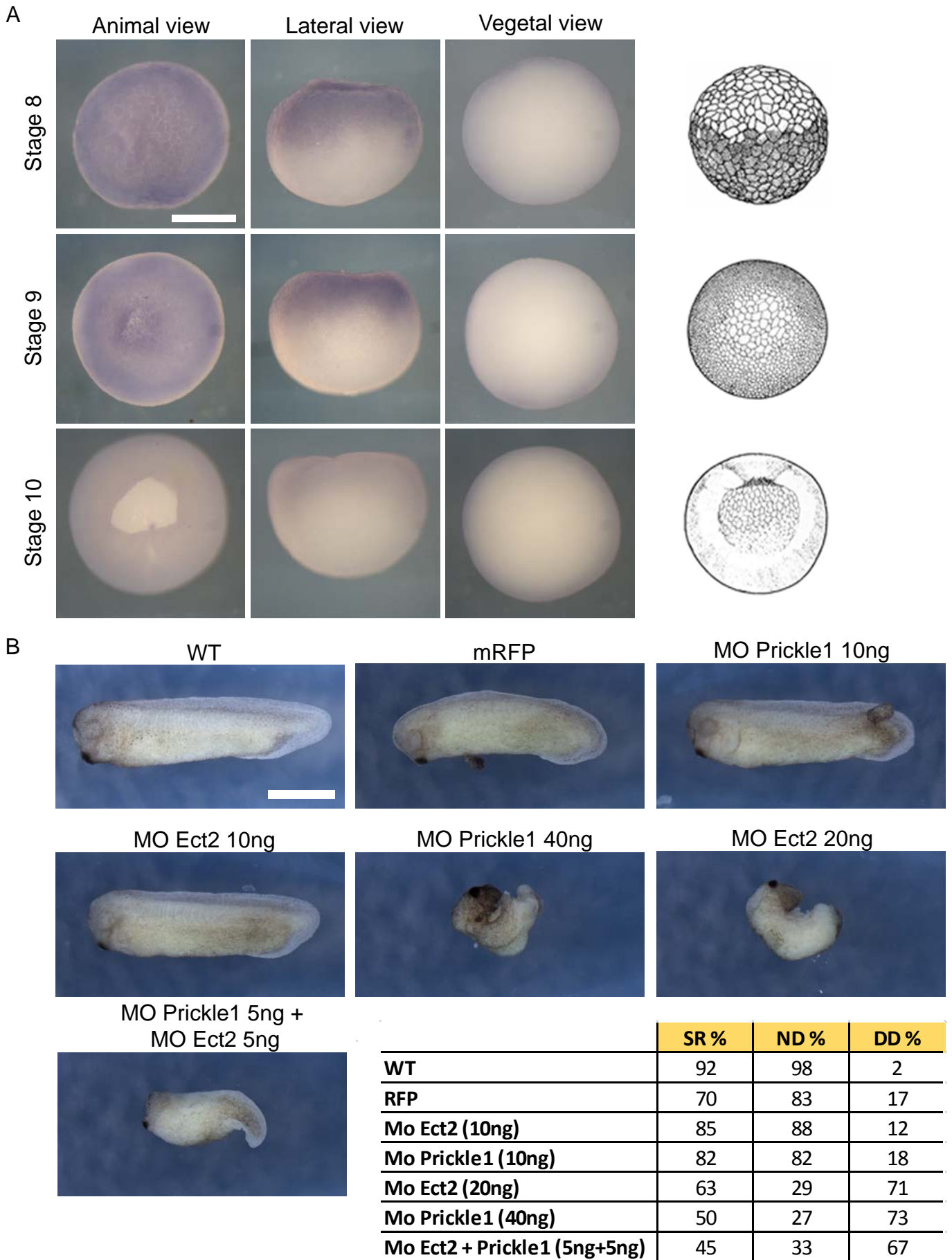


Table 1:

Description GEF/GAP, all BC

Characteristics	N (%)	GEF/GAP		p-value
		down	up	
Age at diagnosis (years)				3,46E-14
≤50	2540 (36%)	1112 (32%)	1428 (40%)	
>50	4488 (64%)	2388 (68%)	2100 (60%)	
Pathological type				1,21E-02
ductal	3979 (79%)	1998 (77%)	1981 (80%)	
lobular	498 (10%)	263 (10%)	235 (10%)	
other	574 (11%)	325 (13%)	249 (10%)	
Pathological tumor size (pT)				0,133
pT1	2113 (38%)	1100 (39%)	1013 (36%)	
pT2	2923 (52%)	1439 (51%)	1484 (53%)	
pT3	595 (11%)	304 (11%)	291 (10%)	
Pathological axillary node status (pN)				0,239
0	3446 (56%)	1741 (56%)	1705 (55%)	
1	2743 (44%)	1344 (44%)	1399 (45%)	
Pathological grade				1,00E-06
1	721 (11%)	442 (14%)	279 (9%)	
2	2573 (41%)	1478 (48%)	1095 (34%)	
3	2986 (48%)	1181 (38%)	1805 (57%)	
ER mRNA status				2,29E-153
negative	2764 (31%)	811 (18%)	1953 (43%)	
positive	6218 (69%)	3680 (82%)	2538 (57%)	
PR mRNA status				8,62E-53
negative	4670 (52%)	1976 (44%)	2694 (60%)	
positive	4255 (48%)	2489 (56%)	1766 (40%)	
ERBB2 mRNA status				0,00021
negative	7884 (88%)	4000 (89%)	3884 (86%)	
positive	1098 (12%)	491 (11%)	607 (14%)	
Molecular subtype				1,00E-06
HR+/HER2-	5929 (66%)	3532 (79%)	2397 (54%)	
HER2+	1098 (12%)	491 (11%)	607 (14%)	
TN	1936 (22%)	463 (10%)	1473 (33%)	
Metastasis				6,81E-06
no	3127 (77%)	1606 (80%)	1521 (74%)	
yes	923 (23%)	396 (20%)	527 (26%)	
follow-up	42 (1-232 71% [69- 74])	38 (1-221)	37 (1-232)	0,48
5-year MFS		75% [72-79]	67% [64-71]	5,09E-04

Table 2: Model of comparison**A, PRICKLE1 & GEF/GAP**

<u>MFS,TN BC</u>	statistic		p-value
PRICKLE1	LRX ²	6,23	1,25E-02
PRICKLE1 + GEF/GAP	LRX ²	8,98	1,12E-02
GEF/GAP+PRICKLE1 vs. PRICKLE1	Δ LRX ²	2,75	0,097

B, PRICKLE1 & ECT2

<u>MFS,TN BC</u>	statistic		p-value
PRICKLE1	LRX ²	6,23	1,25E-02
PRICKLE1 + ECT2	LRX ²	11	4,14E-03
ECT2+PRICKLE1 vs. PRICKLE1	Δ LRX ²	4,74	2,90E-02