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ERR α promotes breast cancer cell dissemination to bone by increasing RANK expression in primary breast tumors

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Abstract

Bone is the most common metastatic site for breast cancer. Estrogen-related-receptor alpha (ERR α) has been implicated in cancer cell invasiveness. Here, we established that ERR α promotes spontaneous metastatic dissemination of breast cancer cells from primary mammary tumors to the skeleton. We carried out cohort studies, pharmacological inhibition, gain-of-function analyses in vivo and cellular and molecular studies in vitro to identify new biomarkers in breast cancer metastases. Meta-analysis of human primary breast tumors revealed that high ERR α expression levels were associated with bone but not lung metastases. ERR α expression was also detected in circulating tumor cells from metastatic breast cancer patients. ERR α overexpression in murine 4T1 breast cancer cells promoted spontaneous bone micro-metastases formation when tumor cells were inoculated orthotopically, whereas lung metastases occurred irrespective of ERR α expression level. In vivo, Rank was identified as a target for ERR α . That was confirmed in vitro in Rankl stimulated tumor cell invasion, in mTOR/pS6K phosphorylation, by transactivation assay, ChIP and bioinformatics analyses. Moreover, pharmacological inhibition of ERR α reduced primary tumor growth, bone micro-metastases formation and Rank expression in vitro and in vivo. Transcriptomic studies and meta-analysis confirmed a positive association between metastases and ERR α /RANK in breast cancer patients and also revealed a positive correlation between ERR α and BRCA1^{mut} carriers. Taken together, our results reveal a novel ERR α /RANK axis by which ERR α in primary breast cancer promotes early dissemination of cancer cells to bone. These findings suggest that ERR α may be a useful therapeutic target to prevent bone metastases.

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Introduction

Metastases are the primary cause of death [1] and in breast cancer (BCa), bone is the most common metastatic site [2]. Bone metastases (BM) detection often occurs in the

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symptomatic stage and when they are diagnosed, the prognosis for the patient is generally poor and associated with significant morbidity. Unfortunately, current treatments for BM that rely on anti-resorptive agents are only palliative underlining the need for early detection for early intervention [3–5].

Nuclear steroid receptors are transcription factors that comprise both ligand-dependent molecules, such as estrogen receptors (ERs) and a large number of so-called orphan receptors, for which no natural ligands have yet been identified [6]. The estrogen-related receptor alpha (ERR α) shares structural similarities with ER α/β , but it does not bind estrogens [6]. Indeed, sequence alignment of ERR α and the ERs reveals a high similarity (68%) in the 66 amino acids of the DNA-binding domain, but only a moderate similarity (36%) in the ligand-binding domain [7]. Although ERR α activity is decreased by synthetic molecules, such as the inverse-agonists XCT790 or C29, no natural ligand has yet been found [8, 9].

ERR α is known to regulate the adaptive bioenergetics response [10]. It is widely expressed in both healthy tissues and a range of cancerous cells [11–14]. Notably, ERR α -positive tumors were associated with more invasive disease and higher risk of recurrences [11–13]. In addition to angiogenesis, ERR α is mainly linked to tumor cell invasion [13–15]. ERR α is also highly expressed in skeletal tissues where it regulates osteoblast/osteoclast physiology [16–19]. In BM, we have reported that overexpression of ERR α in triple-negative BCa cells and castration-resistant prostate cancer cells results in either the inhibition or the activation of tumor cell progression in bone, respectively [20, 21]. Here, we assess whether ERR α in primary breast tumors regulates the molecular mechanisms that drive BCa cell homing to bone.

Results

High ERR α protein expression levels in primary tumors are associated with bone relapse in BCa patients

We assessed ERR α protein expression levels in primary tumors from patients of the ABCSG trial 6 cohort, a prospective, randomized clinical study of postmenopausal patients with hormone receptor-positive early-stage BCa, treated with tamoxifen-aminoglutethimide and followed for up to 15 years [22]. Kaplan–Meier analysis showed that high levels of ERR α protein expression (Fig. 1A, B) correlated with lower distant recurrence-free survival (%) ($n = 100$). After establishing conditions for ERR α detection using cell lines (Supplementary Fig. S1), we also detected

ERR α expression in circulating tumor cells (CTCs) (Fig. 1C), supporting the hypothesis that ERR α present in CTCs may be involved in the metastatic process. We then analyzed the ERR α transcriptional profile in the NKI-295 cohort that includes primary BCa patients with known location of the first distant metastasis site [23] (Supplementary Table S1). Univariate Cox proportional hazard model analysis indicated that ERR α expression is associated with metastases-free survival (MFS) and bone metastases free-survival (BMFS) but not with lung metastases free-survival (LMFS) (Table 1). Kaplan–Meier curves also demonstrated that high ERR α expression is correlated with a decrease in BMFS but not LMFS (Fig. 1D, E). Neither ERR β nor ERR γ mRNA expression levels correlated with MFS, BMFS, or LMFS (Table 1).

ERR α in BCa cells promotes spontaneous bone micro-metastases formation in vivo

To assess the role of ERR α in the early steps of metastatic dissemination of BCa, we used the murine 4T1 BCa cell line, from which cells spontaneously metastasize from primary tumor to lung and bone [24, 25]. Three independent 4T1 clones overexpressing-ERR α (4T1-ERR α) and two empty vector controls (4T1-CT) were isolated (Fig. 2Aa). In parallel, three 4T1-ERR α AF2 clones in which ERR α acts dominant-negatively, were obtained (Fig. 2Ab) [20, 21]. Analysis of primary breast tumors (Fat-Pad(FP)) indicated greater tumor progression in mice with 4T1-ERR α -FP compared to 4T1-CT-FP (Fig. 2Ba–b, C, D), and 4T1-ERR α AF2-FP tumors (Fig. 2Ba, c, E, F). In agreement with previous data [26], we found *Vegf-a* expression increased in 4T1-ERR α -FP tumors (Supplementary Fig. S2), as did expression of genes implicated in glycolysis and lactate metabolism (Supplementary Table S2). Culturing cells collected from crushed bones of mice kept for an additional 21 days post-primary tumor resection and counting colonies [25] revealed greater tumor cell colonization of bone (TCB) by 4T1-ERR α -FP (Fig. 2G, Supplementary Fig. S3A), with higher incidence of dissemination to bone with 4T1-ERR α cells (77% versus 12% (4T1-CT)) (Fig. 2H) and a higher number of TCB (Fig. 2I). No significant difference was observed between 4T1-ERR α AF2 cells and 4T1-CT cells (Fig. 2H, I, Supplementary Fig. S3B). In contrast, histological assessment of lung metastases (LM) revealed no difference in the number or incidence of lung colonies (Fig. 2J–L) or metastases surface amongst all groups (Supplementary Fig. S3C,D), in spite of maintenance of ERR α overexpression in 4T1-ERR α -LM versus 4T1-CT-LM (Supplementary Fig. S3E). These pre-clinical data confirm a correlation between ERR α expression level and BMFS but not LMFS (Fig. 1D, E; Table 1).

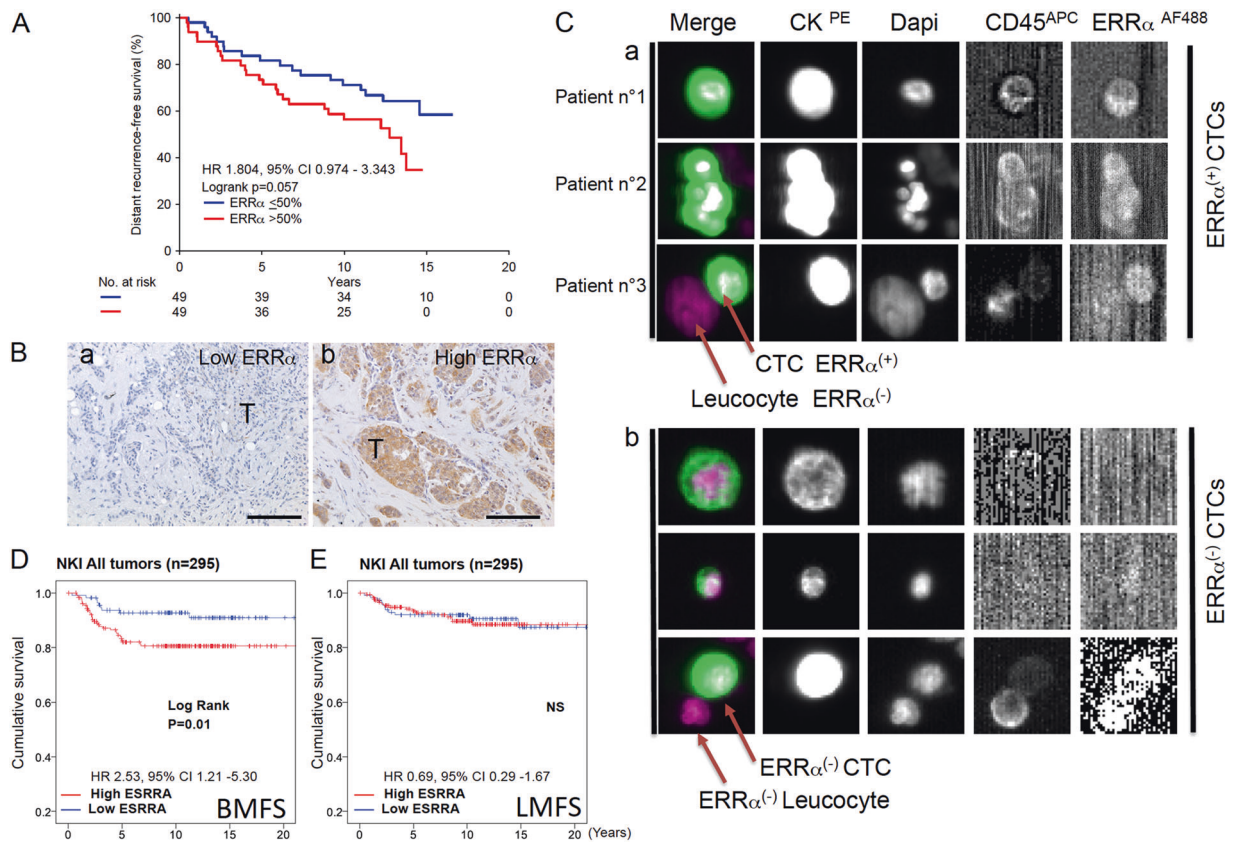


Fig. 1 ERR α is a bone metastases-associated gene in BCa. **A** Kaplan–Meier curves show correlation between high expression of ERR α , categorized with median value, and distant recurrence-free survival in patients treated with tamoxifen alone or aminoglutethimide with tamoxifen ($n = 100$). **B** Visualization of ERR α protein expression at low (a) and high level (b) by IHC on sections of primary breast tumor. **C** Representative EpCAM⁽⁺⁾, pan-keratin⁽⁺⁾ (K-PE), CD45⁽⁻⁾ CTCs and ERR α -AF488⁽⁺⁾/⁽⁻⁾ from metastatic BCa patients detected by the

CellSearch[®] System. DAPI was used for nuclear counter staining. **D** Kaplan–Meier curves constructed after segmentation into two groups on the basis of the median value for ERR α expression show correlation between high expression of ERR α and lower bone metastases free-survival (BMFS) in NKI cohort ($n = 295$, $P = 0.01$); Low $\leq 50\%$ quartile; high $\geq 50\%$. **E** No correlation was found between high level of ERR α and lung metastases free-survival (LMFS)

ERR α regulates Rank expression in BCa cells

To explore the mechanism underlying the ability of ERR α to promote homing of cancer cells to bone in vivo, we quantified in FP-tumors transcriptional expression levels of several markers known to be involved in the metastatic process (Fig. 3A; Supplementary Table S2). As expected, 4T1-ERR α -cells undergo epithelial–mesenchymal transition (EMT) (Supplementary Table S2) [13]. Interestingly, expression of receptor activator of nuclear factor κ B (*Rank*) mRNA was higher in 4T1-ERR α -FP [27–29], whereas no difference was observed in the mRNA expression of *Cxcr4* which had been implicated in lung tropism (Fig. 3A). Consistent with these data, Rank protein expression was also higher in 4T1-ERR α -FP (Fig. 3Ba, b). Rank protein expression was also much higher in 4T1-ERR α cells in bone metastases (BM) (Fig. 3Ca, c, Cb, d) induced by intra-arterial (IA) injection of cells, but was barely detectable in

the LM elicited by either cell population (Fig. 3Da, c, Db, d; Supplementary Fig. S4A,B,C), suggesting a major role of Rank for BCa cell anchorage and colonization in bone. Culturing cells collected from crushed bones and lungs confirmed *Rank* mRNA up-regulation in 4T1-ERR α -BM but not in LM (Fig. 3E). The fact that Rank-ligand (*Rankl*) expression was significantly higher in bone versus lung (Fig. 3F) further suggests their involvement in BCa cell colonization of bone but not lung. *RANK* regulation by ERR α was confirmed in two human BCa cell lines: the MCF7 line engineered to overexpress full-length (MCF7-ERR α) or truncated (MCF7-ERR α AF2) human *ERR\alpha* compared to the empty vector control (MCF7-CT) (Fig. 3G, H) and the MDA-MB-231-B02, known to metastasize only to bone (Fig. 3I) [20]. Moreover, C29 inhibited *Rank* mRNA expression in 4T1 and MCF7 parental cells (Fig. 3J–M) [9, 30]. *ERR\alpha* and *RANK* mRNA were also co-expressed in the human BC-M1 disseminated tumor cell

Table 1 ERR α is a bone metastases-associated gene in BCa

Parameter	Metastasis free survival			Bone metastasis free survival			Lung metastasis free survival		
	P-value	HR	FDR	P-value	HR	FDR	P-value	HR	FDR
ESRRA	0.008	5.80	0.025	0.003	23.72	0.009	0.215	5.192	0.55
ESRRG	0.08	0.55	0.118	0.56	0.72	0.838	0.366	0.531	0.55
ESRRB	0.39	2.5	0.392	0.94	1.15	0.941	0.673	0.346	0.673

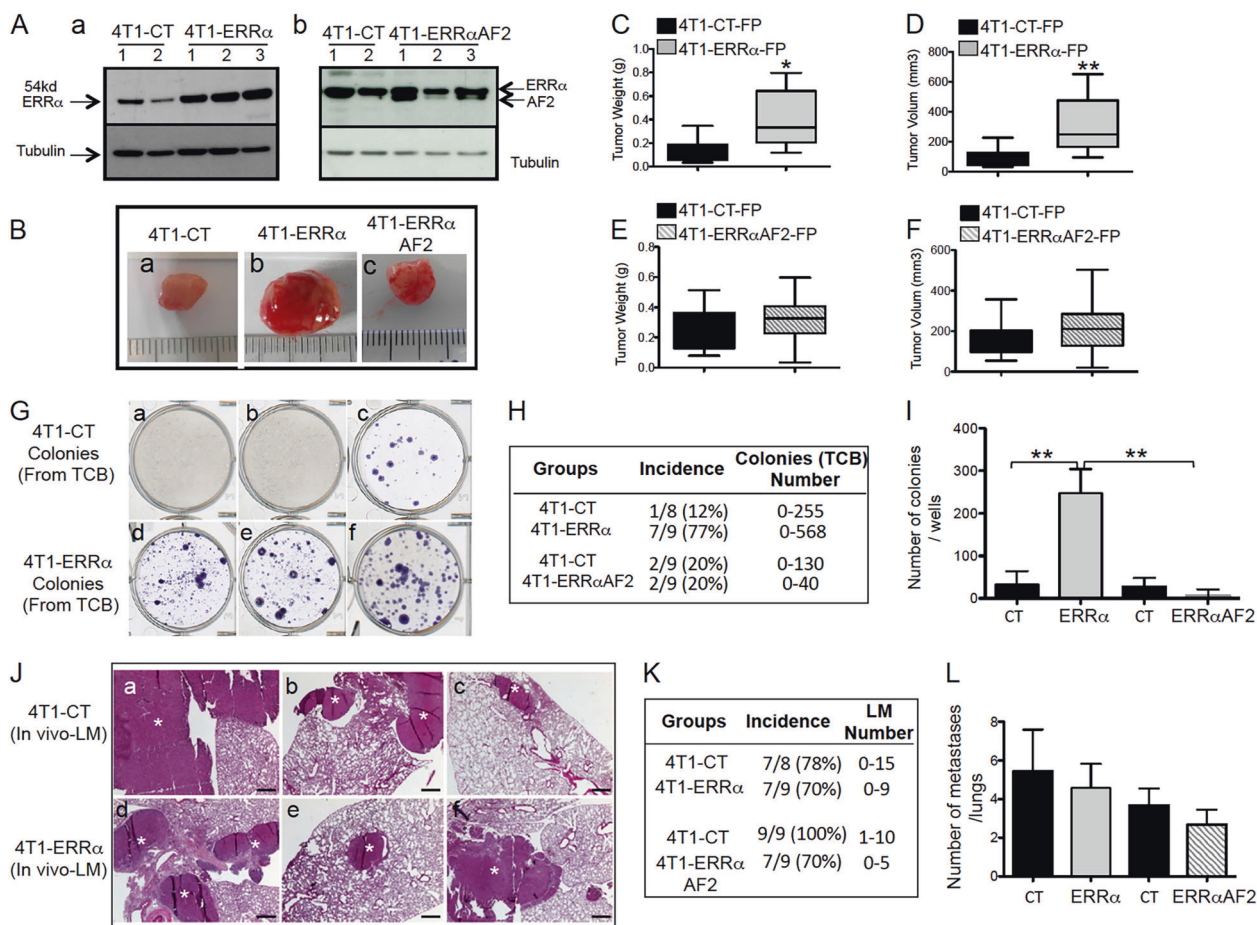
Cohort NKI ($n = 295$), Univariate Cox proportional hazards model, Wald statisticUnivariate Cox proportional hazard model analysis revealed that ERR α mRNA expression is associated with metastases free survival (MFS) and bone metastases free survival (BMFS) ($n = 295$, $P = 0.008$ and $P = 0.001$, respectively) while no link was found in lung metastases free-survival (LMFS)

Fig. 2 ERR α promotes BCa cell homing to bone in vivo. **A** (a) Three independent clones (4T1-ERR α 1–3) overexpressing full-length ERR α and two control clones with empty vector (4T1-CT 1–2) were obtained after stable transfection of 4T1 cells. ERR α (~54kDa) expression was assessed by Western blotting and compared to α -tubulin as a protein loading control showing the ERR α protein band increased in 4T1-ERR α (1–3) compared to 4T1-CT (1, 2) cells. (b) Three independent 4T1-ERR α AF2 clones (ERR α dominant-negative) and two control clones (4T1-CT clones 1,2) (empty vector) were obtained and a band with slightly lower molecular weight, corresponding to the expected size for truncation of the AF2-AD domain (42aa) (co-activators binding domain), was present in 4T1-AF2 cells. **B** 4T1-ERR α (pool of three clones), 4T1-CT (pool of two clones), or 4T1-ERR α AF2 (pool of three clones) cells were inoculated into the mammary gland fat pad of BALB/c mice. Greater tumor expansion was observed in mice with

4T1-ERR α -FP. **C–F** Weight and volume of tumors dissected at endpoint ($n = 10$, Mann–Whitney, weight: $P = 0.0156$; volume: $P = 0.0074$ for 4T1-ERR α), no difference was observed in mice with 4T1-ERR α AF2-FP ($n = 9$). **G** After resection, animals were kept for 21 days, bones were then crushed and released cells were cultured for 3 weeks. **H, I** Counting of tumor cells that colonized the bone (TCB) revealed a high incidence with 4T1-ERR α cells (**H**) that was associated with an increase in TCB number (ANOVA: $p < 0.0001$ and Mann–Whitney, $P = 0.0083$ ($n = 9$), 262 ± 175 (4T1-ERR α) versus 32 ± 55 (4T1-CT); $P = 0.006$, 4T1-ERR α versus 4T1-ERR α AF2) (**I**). Incidence and TCB number were similar in 4T1-CT and 4T1-ERR α AF2 cells. **J–L** Histological assessment of lung metastases (LM) (**J**) shows no difference between LM incidence (**K**) and LM number/lungs (**L**) ($n = 9$). Asterisk represents lung metastases

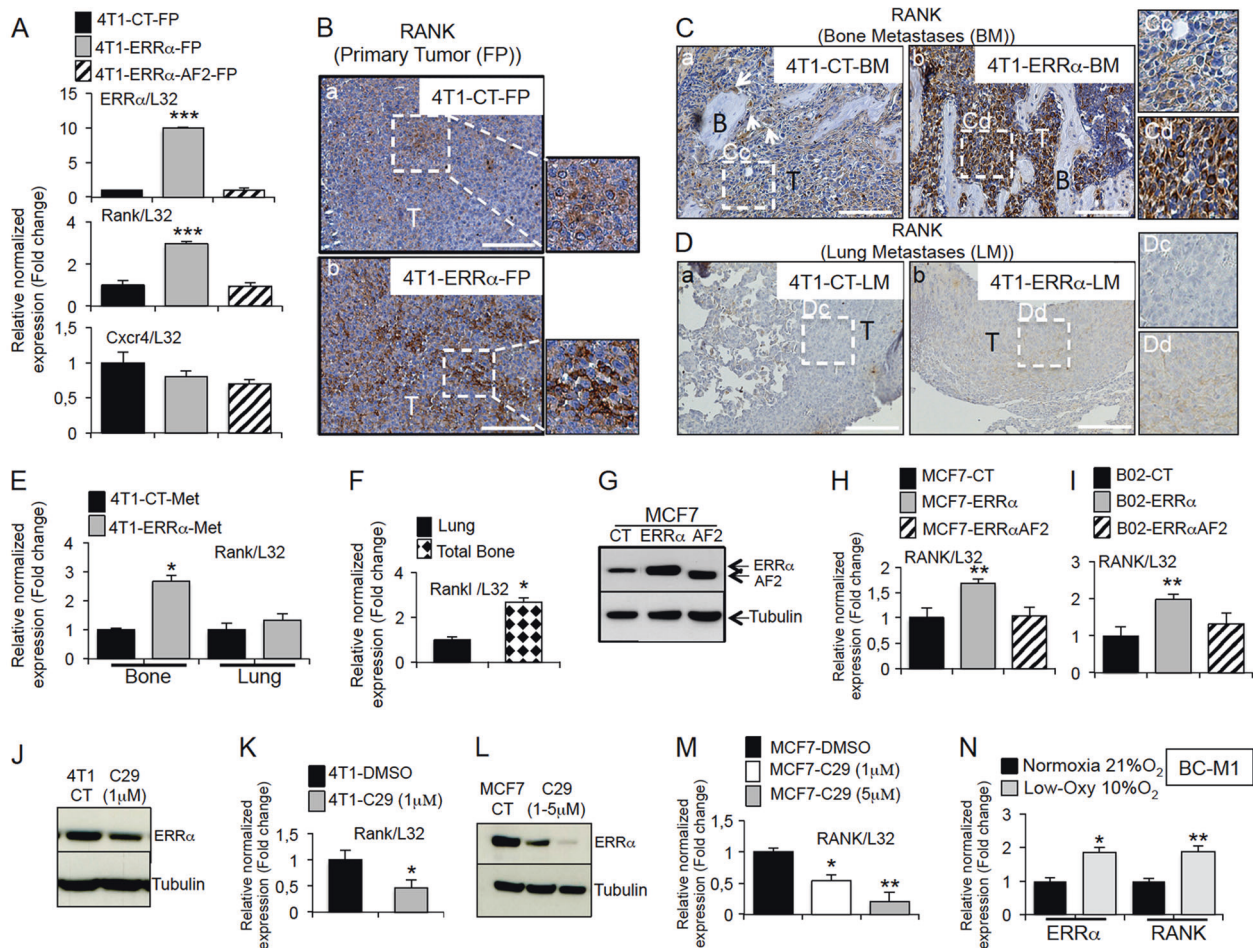


Fig. 3 Correlation between ERR α and Rank expression in BCa cells. **A** Real-time PCR performed on mRNA extracted from 4T1 primary tumors ($n = 3$ for all conditions) confirmed increased expression of ERR α in 4T1-ERR α -FP and show increased expression of Rank by ERR α (ANOVA: $P < 0.0001$ and unpaired t -test $P < 0.0001$ for both ERR α and Rank). *Cxcr4* mRNA expression was not altered. **B** Increased expression of Rank was visualized by IHC in 4T1-ERR α -FP (**b**) compared to 4T1-CT-FP (**a**) and in bone metastases (BM) (**C**) after IA injection of 4T1-ERR α (**b, d**) compared to 4T1-CT (**a, c**) cells. Rank-positive osteoclasts were used as a positive control (see White arrow). Representative examples of three and two mice for FP tumor and BM, respectively. **D** Rank expression was barely detected by IHC in either 4T1-ERR α -LM (**b, d**) or 4T1-CT-LM (**a, c**). Representative example of $n = 2$. **E, F** Real-time RT-PCR was performed on triplicate samples and normalized against the ribosomal protein gene *L32* to evaluate (**E**) Rank expression in bone and lung metastases extracted from 4T1-ERR α versus 4T1-CT tumor-bearing animals ($n = 2$) (ANOVA: $P < 0.0001$, unpaired t -test $P = 0.0123$ for bone metastases) and (**F**) Rankl expression in total normal bone and lung (unpaired t -test $P = 0.0112$). **G** MCF7 clones overexpressing ERR α or ERR α AF2 were obtained after retroviral transfection. ERR α overexpression was

assessed by Western blotting and compared to α -tubulin. **H** and **I** Real-time RT-PCR was performed on triplicate samples and normalized against the ribosomal protein gene *L32* to evaluate RANK expression in (**H**) MCF7 clones (ANOVA: $P = 0.0135$, unpaired t -test $P = 0.0041$) and (**I**) MDA-MD231-B02(B02) clones (ANOVA: $P = 0.0031$, unpaired t -test $P = 0.0032$). **J–M** C29 treatment induced a decrease in Rank expression in both parental 4T1 and MCF7 cells lines. **J, K** ERR α protein by Western blot (**J**) and Rank mRNA by real-time PCR (**K**) were inhibited by C29 at 1 μ M after 48 h (unpaired t -test, $P = 0.0311$) in 4T1 cells. (**L, M**) Inhibition of ERR α protein (**L**) induced by C29 at 1 and 5 μ M after 48 h in MCF7 cells is associated with a decrease of RANK mRNA expression (**M**) (ANOVA: $P = 0.0016$, unpaired t -test $P = 0.0127$ and $P = 0.0062$). (**N**) Real-time PCR was performed on triplicate samples to evaluate ERR α and RANK expression in the BC-M1 cells under low O $_2$ conditions compared with normoxia. ERR α and RANK expression was higher under low O $_2$ conditions (unpaired t -test $P = 0.0143$ and $P = 0.0016$ for ERR α and RANK, respectively). All in vitro assays were repeated at least two times. Data are plotted as mean \pm SEM. Bar = 200 μ m, T: Tumor, B: Bone

line and co-stimulated in BC-M1 cells cultured under low O $_2$ versus normoxia conditions (Fig. 3N) [31].

That Rank stimulation has functional consequences is supported by the observation that Rankl significantly stimulated migration of 4T1-ERR α cells (4T1-ERR α -versus-4T1-ERR α +Rankl) (Fig. 4A, B). Unexpectedly, the

migratory ability of untreated 4T1-ERR α cells was lower than that of untreated 4T1-CT cells but this was reversed by Rankl treatment (Fig. 4B), revealing the high sensitivity of 4T1-ERR α cells to Rankl. Rankl had no effect on migration of 4T1-ERR α AF2 (4T1-ERR α AF2-versus-4T1-ERR α AF2+Rankl) (Fig. 4C, D). Consistent with ERR α -Rank

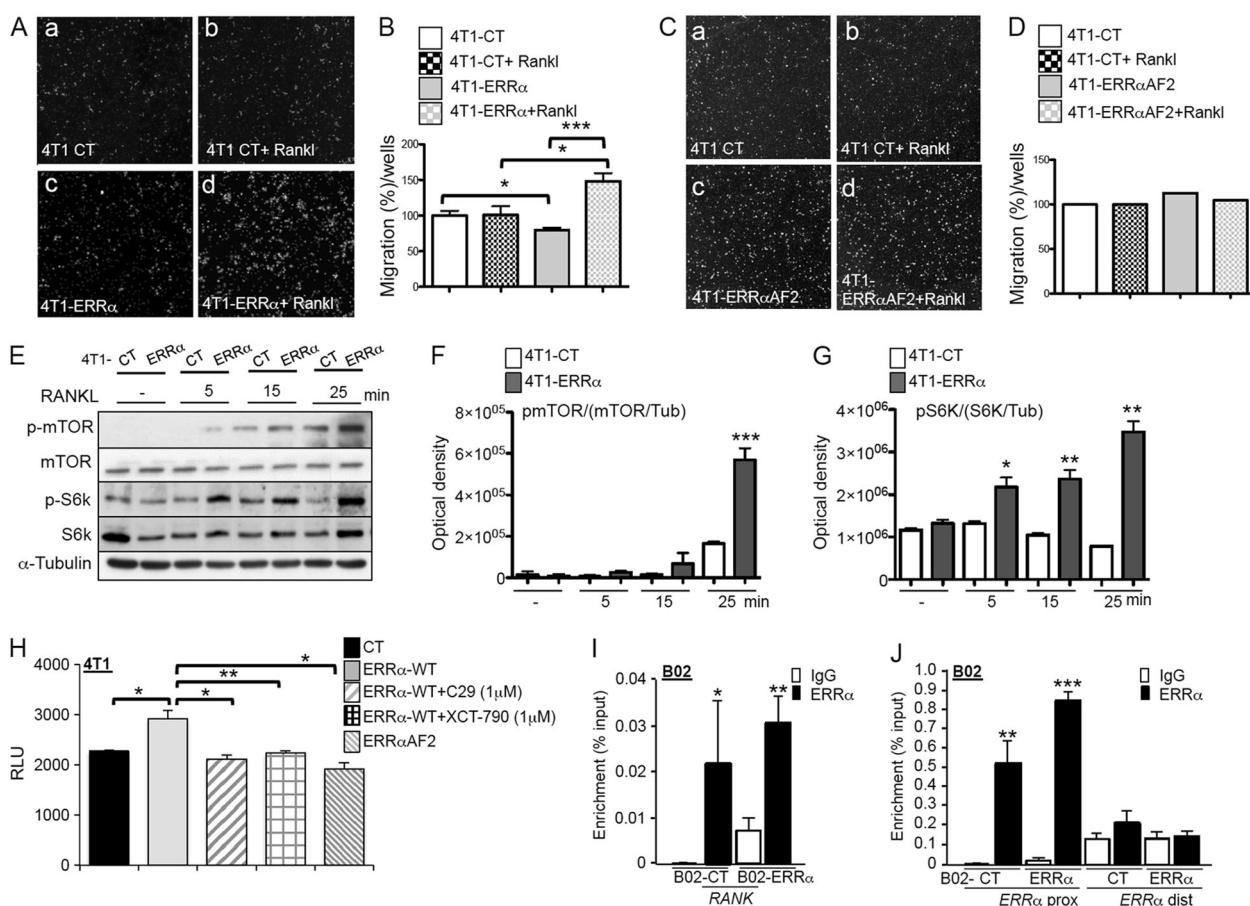


Fig. 4 Increased migration capacity of BCa cells overexpressing ERR α after Rankl treatment. **A, B** Migration was increased in 4T1-ERR α cells (clones 1–3) after Rankl treatment (50 ng/mL) for 12 h (4T1-ERR α versus 4T1-ERR α +Rankl: ANOVA: $P=0.0005$, unpaired t -test $P=0.0001$). The percentage of migration of 4T1-CT cells (clones 1–2) was unchanged after Rankl treatment (4T1-CT versus 4T1-CT+Rankl). In the absence of added Rankl, migration of 4T1-ERR α cells was lower than that of 4T1-CT cells ($P=0.0019$), but the opposite was observed after Rankl treatment ($P=0.002$: 4T1-CT+Rankl versus 4T1-ERR α +Rankl). **C, D** Migration was not modulated in 4T1-ERR α AF2 cells (clones 1–3) after Rankl treatment (50 ng/mL) for 12 h (4T1-ERR α AF2 versus 4T1-ERR α AF2+Rankl). **E–G** Rankl signaling was assessed by treating 4T1-ERR α cells (clones 1–3) and 4T1-CT cells (clones 1–2) cells with Rankl for different times (0, 5, 15, 25 min). Activation of mTOR and S6K was assessed with α -tubulin as loading control. mTOR and S6K were markedly activated in 4T1-ERR α cells at 5–25 min. **H** Reporter activity shows the activation

of the Rank promoter activity by ERR α in 4T1 cells (CT versus ERR α -WT ANOVA: $P=0.0004$, unpaired t -test $P=0.0347$) that was blocked by C29 (1 μ M) and XCT-790 (1 μ M) and which is dependent on a functional ERR α AF2 domain (cf. ERR α -WT versus ERR α -WT+C29 or ERR α -WT+XCT790, or ERR α AF2) ($P=0.00275$; $P=0.0058$; and $P=0.0022$, respectively). **I** ERR α binding to the human RANK locus was analyzed using ChIP-qPCR in control B02-CT(CT) and B02-ERR α (ERR α) cells (IgG versus ERR α in B02-CT ($P=0.0237$) and in B02-ERR α ($P=0.0015$)). **J** ERR α binding to a proximal (prox), but not to distal (dist) element of its own human promoter was used as positive (IgG versus ERR α in B02-CT ($P=0.0023$) and in B02-ERR α ($P<0.0001$)) and negative controls, respectively. Percent enrichments relative to input of a representative experiment are shown using anti-ERR α or non-specific rabbit-IgG antibody as a negative control. All in vitro assays were repeated two times. ChIP assay data are representative of two independent ChIP experiments. Data are plotted as mean \pm SEM

signaling, mTOR/S6K phosphorylation was higher in Rankl-treated 4T1-ERR α cells (Fig. 4E–G) [27]. C29 and the XCT-790 [8] inhibited Rank mRNA expression in 4T1 parental cells after 24 h treatment and the endogenous ERR α activity decreased by C29 treatment (Supplementary Fig. S5AB) suggesting that the effect of ERR α on Rank expression is direct. This is supported by the finding that ERR α transactivates the Rank promoter in 4T1 cells (CT-versus-ERR α WT) and that this transactivation is dependent

on a functional ERR α AF2 domain (ERR α -versus-ERR α WT++C29, ERR α WT+XCT790 or ERR α AF2) (Fig. 4H). By using ChIP-qPCR in B02-CT and B02-ERR α cells, we also show ERR α binding on an ERRE site (TGAAGGTCA) localized on the human RANK locus (Fig. 4I) (Supplementary Fig. S5C) [20]. ERR α binding to a proximal (prox), but not to distal (dist) element of its own human promoter was used as positive and negative controls, respectively (Fig. 4J) [32, 33].

Pharmacological inhibition of ERR α reduces mammary tumor growth, bone marrow micro-metastases formation, and RANK expression in vivo

To determine whether inhibition of ERR α abrogates FP-tumor progression and BCa cell dissemination to bone, mice were inoculated with 4T1-ERR α cells and treated with the C29 or vehicle from the time of tumor appearance to tumor resection. Tumor weight/volume was reduced in 4T1-ERR α -FP tumor-bearing animals treated with C29 (Fig. 5A–C), as was TCB (Fig. 5D, E) but not LM (Supplementary Fig. S6E), without C29 toxicity (Supplementary Fig. S6A–D). ERR α and *Rank* mRNA and protein expression were reduced in FP-tumors in C29-treated mice (Fig. 5F) (Supplementary Fig. S7A,B).

Bioinformatics analysis reveals an extensive RANK–ESRRA regulatory interactome

To address further a RANK and ESRRA connection, we combined bioinformatics analyses of protein interaction networks and transcriptional regulator databases. We first built an extended network (Supplementary Fig. S8, Table S3) from public datasets and then defined a minimal network that included well-known ESRRA regulators, *e.g.* HDAC8, AIB1, SIRT1, SHP1, and linked ESRRA–RANK signaling, as exemplified by the presence of TRAFs and c-SRC (Fig. 5G) [34]. More than half (32 out of 62 proteins) of the regulators within the network are associated with extracellular matrix (ECM) regulation, cell adhesion and migration (see gray boxes, Fig. 5G) and a further half of these (16 of 32) are proteins encoded by genes that are potentially directly regulated by ESRRA (Fig. 5H, Supplementary Table S4). By shortest path analysis, we also identified two new potential ESRRA–RANK-associated regulators, such as MEX67 and SP1 (Fig. 5G).

ERR α /RANK are linked in BCa patients who developed BM

Meta-analysis of the GSE14020 dataset confirmed a positive association of ERR α and *RANK* in patients who developed BM but not in patients who developed lung and liver metastases (Table 2A) [35]. Moreover, in the Curie Institute cohort ($n = 446$), stratification of BCa patients into subtypes revealed a positive correlation between ERR α and *RANK* in Triple Negative patients (NNN) and Luminal BCa patients (HR⁺ERBB2) but not ERBB2+patients (HR– and HR+) (Table 2B; Supplementary Fig. S9A,B; Table S5) [36]. Reinforcing our clinical data obtained in the NKI-294 cohort (Fig. 1D, E), Kaplan–Meier curves demonstrated that high ERR α expression is correlated with a decrease in BMFS but not LMFS in the Curie Institute cohort (Fig. 6A,

B). No correlation was observed between ERR α and *RANKL* (*TNFSF11*) (Table 2B). Expression levels of ERR α and *RANK* were positively correlated in the TCGA dataset (Supplementary Fig. S9C) and in primary BCa samples (HR⁺ERBB2–) in combined microarray datasets (GSE12276–GSE2034–GSE2603) ($n = 248$) (Table 2C; Supplementary Table S6). The strongest correlation was observed in patients who had developed only BM (Pearson $R = 0.5145$) and no correlation was observed in non-metastatic patients (No Mets).

ERR α is highly expressed in primary tumors from BRCA1-mutated carriers

Recently, *RANK/RANKL* were shown to control *BRCA1* mutation-driven mammary tumor formation and progressions [37], prompting us to ask whether ERR α and *BRCA1* expression is correlated. Using microarray datasets (GSE12276–GSE2034–GSE2603), we found a positive correlation between ERR α and *BRCA1* in BCa samples in patients who had developed both visceral+bone and only BM, and with *BRCA2* expression in patients with metastases at all sites (Table 2C). Since these datasets were not informative on the *BRCA1* and 2 mutations status, we used the METABRIC cohorts that include 2051 radically resected primary BCa, where *BRCA1*^{mut} or 2^{mut} were identified. ERR α expression level was higher in *BRCA1*^{mut} carriers but not *BRCA2*^{mut}-carriers compared to those with no mutations (Fig. 6C). By bioinformatics, our minimal network was structured over three main protein hubs (ESRRA, ESR, and BRCA1) (Fig. 5G). Systematic shortest path analysis revealed that ESRRA could control RANK and BRCA1 signaling (Fig. 6D) and, more specifically, BRCA1 through SRC1, AIB1, SP1, RIP140, and ESR which are ESRRA regulators [13, 14, 38, 39]. ESRRA may also directly regulate BRCA1 (with five potential ESRRA-binding sites) (Fig. 6F) and ESR, AIB1, SRC1, MEX67, and SP1 (Fig. 6F).

Discussion

Our findings indicate that ERR α is expressed in CTCs and promotes BCa cell homing to bone. The identification of *Rank* as an ERR α -regulated gene in our pre-clinical 4T1 cell model and ERR α /RANK-positive association in BCa patient cohorts provide new mechanistic insights into ERR α -regulated pathways mediating early BCa cell colonization of bone.

Elevated ERR α expression has been associated with tumor aggressiveness [13]. The fact that we have observed ERR α expression in CTCs of BCa metastatic patients and documented changes in BCa cell migration with changes

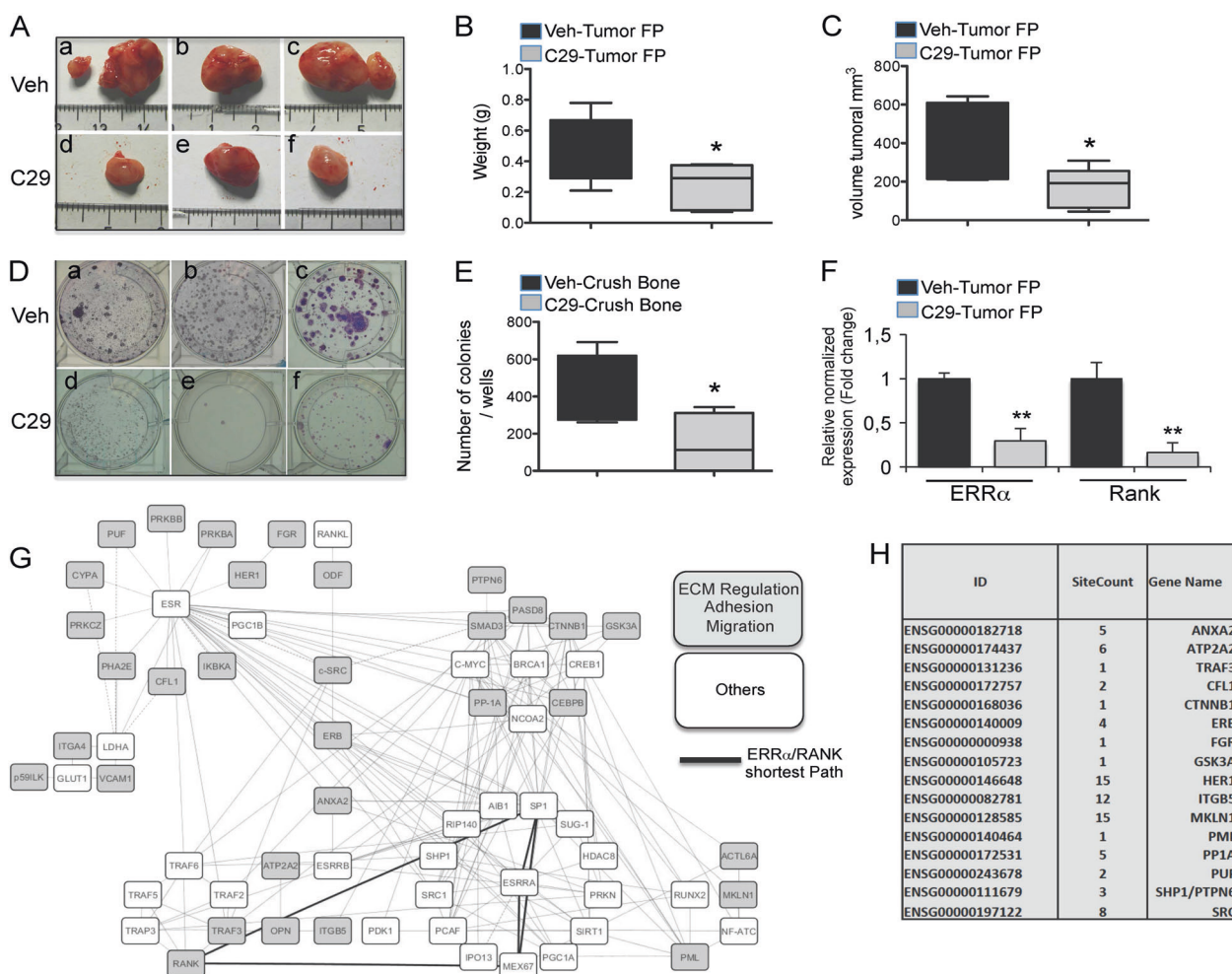


Fig. 5 Pharmacological inhibition of $ERR\alpha$. **A** 4T1- $ERR\alpha$ (clones 1–3) cells were inoculated into the FP of the mammary gland of BALB/c mice and mice were treated with C29 (10 mg/kg) for 10 days from the time of tumor appearance to tumor resection (day 14). C29 (C29-Tumor-FP) (**d–f**) decreased tumor expansion compared to tumor growth in vehicle treated mice (**a–c**). **B**, **C** Weight and volume of tumors dissected at endpoint ($n = 9$, unpaired t -test, weight: $P = 0.014$; Mann and Whitney; volume: $P = 0.019$). **D**, **E** After tumor resection, animals were kept for an additional 21 days, bones were then crushed and released cells were cultured for 3 weeks. C29 treatment (**d–f**; **E**) decreased TCB number compared Veh (**Da–c**; **E**) ($n = 5$, 142 ± 138 versus 416 ± 138 , unpaired t -test, $P = 0.035$). **F** Real-time PCR

performed on RNA extracted from C29 (C29-Tumor-FP) versus vehicle-treated (Veh-Tumor-FP) mice ($n = 3$, unpaired t -test $P = 0.004$ for $ERR\alpha$ and $P = 0.0032$ for $Rank$) confirmed a decrease in the expression of $ERR\alpha$ and $Rank$ in treated animals. **G** By bioinformatics analysis, a protein interaction and transcriptional regulation network was built. Potential new regulators, including MEX67 and SP1, were identified through systematic shortest path definition of cross-talks (see black bold line). 32 out of 62 regulatory proteins are associated with ECM regulation, cell adhesion and migration (see gray boxes). **H** GTRD database analysis indicated that 16 of the 32 regulatory proteins are encoded by genes that could be directly regulated by $ESRRA$

in $ERR\alpha$ activity strengthens the notion that $ERR\alpha$ may be involved into CTCs survival and metastases.

We found that a high level of $ERR\alpha$ was associated with bone but not LM in our mouse experimental model of spontaneous metastatic BCa and in two independent ($n = 740$) BCa patient cohorts, suggesting that $ERR\alpha$ / $RANK$ expression in primary tumors may be a predictive marker of BM occurrence in BCa patients. With respect to bone colonization, our identification of $RANK$ as a new $ERR\alpha$ -regulated gene is notable. $RANK$, the cognate receptor of $RANKL$, was described as being predictive of poor prognosis in patients with BM but not in patients with visceral

metastases [28, 29, 40]. One explanation comes from evidence that $RANKL$ is a chemoattractant that is more highly expressed in bone than lung, thus promoting bone colonization (Fig. 3F): $RANKL$ produced by osteoblasts has been shown to attract $RANK$ -expressing cancer cells and induce their migration in the bone microenvironment [41]. This mechanism has been observed not only in BCa but also in other cancers [29] indicating that $RANKL$ is one of the important “soil” factors that promote BCa- $RANK$ +cell bone colonization. Additionally, knockdown of $Rank$ expression in metastatic BCa-reduced BM indicating that $RANK$ is required for BCa migratory response toward $RANKL$ -

Table 2 Correlation between ERRα and Rank expression in BCa patients

A					
GSE14020 <i>n</i> = 65	All ESRRA 65	Bone Mets ESRRA 18	Lung Mets ESRRA 20	Liver Mets ESRRA 5	
<i>Pearson-correlation coefficient</i>					
<i>TNFRSF11A</i> (RANK)	<i>R</i> = 0.510 <i>P</i> < 0.0001	<i>R</i> = 0.515 <i>P</i> = 0.0287	<i>R</i> = 0.457 <i>P</i> = 0.0504(NS)	<i>R</i> = 0.009 <i>P</i> = 0.884(NS)	
B					
Curie <i>n</i> = 446	All ESRRA 446	NNN ESRRA 68	HR-ERBB2 +ESRRA 42	HR+ERBB2- ESRRA 285	HR+ERBB2 +ESRRA 51
<i>Spearman-correlation coefficient</i>					
<i>TNFRSF11A</i> (RANK)	<i>R</i> = 0.318 <i>P</i> < 0.0000001	<i>R</i> = 0.299 <i>P</i> = 0.013	<i>R</i> = 0.25 <i>P</i> = 0.11(NS)	<i>R</i> = 0.303 <i>P</i> = 0.00000099	<i>R</i> = 0.203 <i>P</i> = 0.15(NS)
<i>TNFSF11</i> (RANKL)	<i>R</i> = 0.054 <i>P</i> = 0.25(NS)	<i>R</i> = 0.146 <i>P</i> = 0.23 (NS)	<i>R</i> = 0.058 <i>P</i> = 0.71(NS)	<i>R</i> = -0.001 <i>P</i> = 0.99(NS)	<i>R</i> = -0.017 <i>P</i> = 0.90(NS)
C					
GSE12276 2034-2603 <i>n</i> = 248	All ESRRA 248	No Mets ESRRA 121	Visceral +bone ESRRA 53	Bone only ESRRA 74	
<i>Pearson-correlation coefficient</i>					
<i>TNFRSF11A</i> (RANK)	<i>R</i> = 0.4063 <i>P</i> < 0.0001	<i>R</i> = 0.1050 <i>P</i> = 0.2517(NS)	<i>R</i> = 0.4667 <i>P</i> = 0.0005	<i>R</i> = 0.5145 <i>P</i> < 0.0001	
<i>BRCA1</i>	<i>R</i> = 0.2949 <i>P</i> < 0.0001	<i>R</i> = 0.1470 <i>P</i> = 0.1075(NS)	<i>R</i> = 0.332 <i>P</i> = 0.0152	<i>R</i> = 0.2846 <i>P</i> = 0.0140	
<i>BRCA2</i>	<i>R</i> = 0.5113 <i>P</i> < 0.0001	<i>R</i> = 0.3559 <i>P</i> < 0.0001	<i>R</i> = 0.425 <i>P</i> = 0.0015	<i>R</i> = 0.5026 <i>P</i> < 0.0001	

A Meta-analysis of the public dataset GSE14020 (*n* = 65) revealed correlation between *ERRα* and *RANK* in all tumors and in patients with bone but not lung or liver metastases. **B** Meta-analysis of the Curie BCa cohorts (*n* = 446) revealed correlation between *ERRα* and *RANK* (*TNFRSF11A*) mRNA expression in the total tumor population, in the triple negative group and in the luminal group (HR+ERBB2-). No correlation was found in ERBB2+patients (HR- and HR+) or with *RANKL* (*TNFSF11*). **C** Meta-analysis of public datasets (GSE12276, GSE2034, and GSE2603) (*n* = 248) revealed correlation between *ERRα* and *RANK*, *BRCA1* and *BRCA2* expression levels in all tumors, in patients with visceral and bone metastases or only bone metastases. Correlation scores were calculated using the Spearman correlation coefficient for the Curie cohort and the Pearson correlation coefficient for GSE14020 and GSE12276-GSE2034-GSE2603. *P*-values < 0.05 were considered statistically significant

expressing bone cells in vivo [41, 42]. This, together with the fact that the 4T1-CT cells that colonized bone highly express *Rank* while *Rank* was barely detectable in lung (Fig. 3Ca, Da), strongly suggests that RANK is a prerequisite factor for cancer cell colonization of bone. Consistent with RANK-RANKL signaling [27], EMT, mTOR/S6K, and cell migration were stimulated in 4T1-ERRα.

RANKL has recently been linked to mitochondrial mass in osteoclasts [17, 43], and glucose uptake and glycolysis with increased PGC1α, GLUT1, LDHA, and VEGF

expression in osteoblasts resembling the Warburg effect [44, 45]. *ERRα* and *Pgc1α*, which is up-regulated in 4T1-ERRα-FP, are known to play a major role in coordinating oncometabolic programs [13]. Interestingly, these genes which are all direct targets of *ERRα*, were all up-regulated in vivo in 4T1-ERRα-FP, suggesting that RANKL-RANK may also participate in cancer cell metabolic reprogramming to meet the rapid energy requirement for survival and metastases. Interestingly, by bioinformatic, we identified MEX67 and SP1 two potential ESRRA-RANK-associated

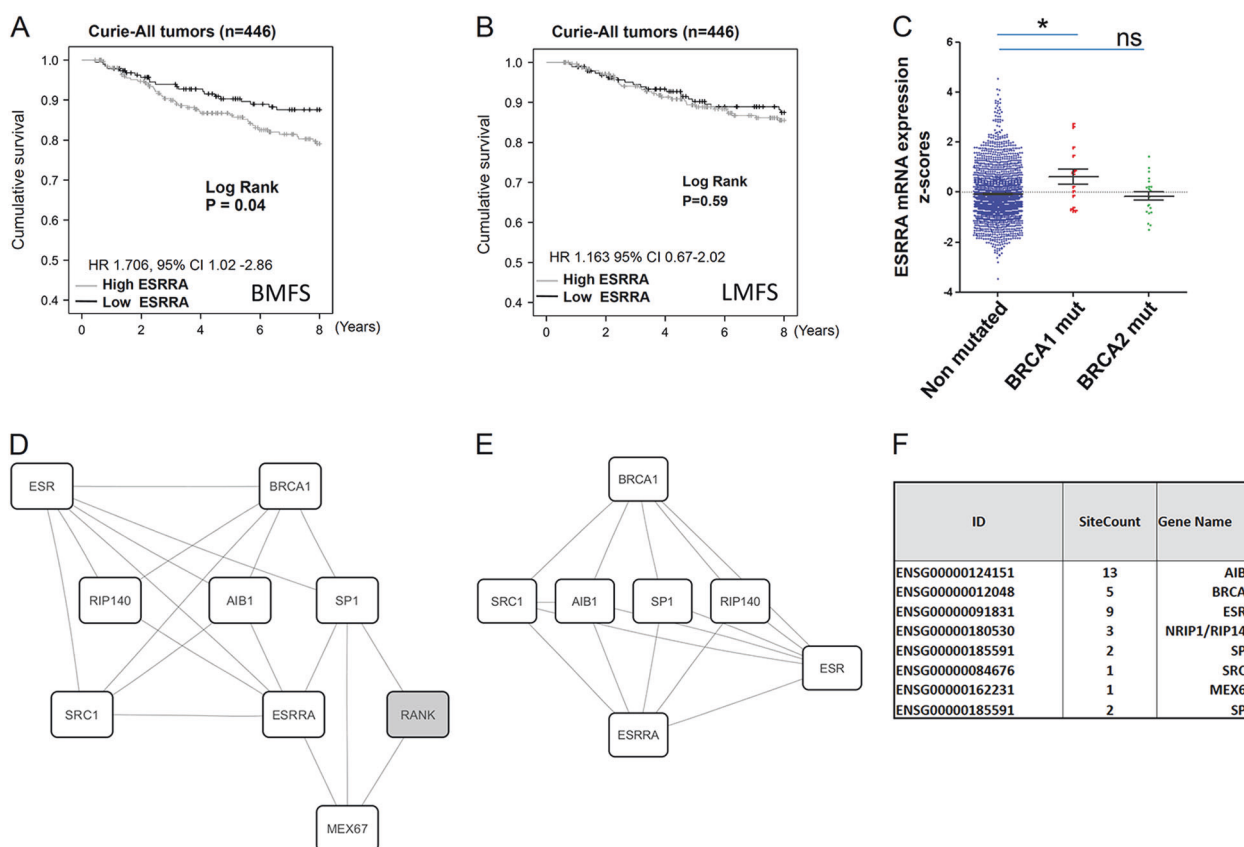


Fig. 6 **A** Kaplan–Meier curves constructed after segmentation into two groups on the basis of the median value for *ERRα* expression show correlation between high expression of *ERRα* and lower bone metastases-free survival (BMFS) in Curie BCa cohorts ($n = 446$, $P = 0.04$); Low $\leq 50\%$ quartile; high $\geq 50\%$. **B** No correlation was found between high expression of *ERRα* and lung metastases free-survival (LMFS). **C** Meta-analysis of the METABRIC cohorts that include 2051 radically resected primary BCa, where BRCA1 and 2 mutations

were identified, revealed correlation between high *ERRα* and BRCA1 mutation (Mann–Whitney $P = 0.02$). No link was found between *ERRα* expression and BRCA2 mutation. **D** Analysis of our minimal network (Fig. 5G) showed that *ESRRR* could control RANK and BRCA1 signaling and, more specifically, (**E**) BRCA1 through SRC1, AIB1, SP1, RIP140 and ESR. (**F**) GTRD database analysis indicated that *ESRRR* may directly regulate BRCA1; *ESRRR*-binding sites are also present in ESR, AIB1, SRC1, MEX67, and SP1

regulators that had been detected in invasive ductal BCa and as an *ERRα* transcription control factor-coregulator, respectively [46, 47] (Expression-Atlas-EMBL—EBI). Moreover, the RANK/RANKL axis is a critical regulator of BRCA1-mutation-driven BCa and anti-RANKL therapy is now proposed as a preventive strategy for women carrying BRCA1 mutations [37, 48]. We found a significant correlation between high *ERRα* and BRCA1^{mut} in BRCA1^{mut} carriers, implying that *ERRα* may participate directly in the RANKL/RANK control of progenitor cell expansion and tumorigenesis in inherited BCa and/or via the modulation of BRCA1 (by, e.g., the *ERRα* coregulators, SRC-1, AIB1, and RIP140) (Fig. 6D–F). The presence of ESR in our shortest path analysis also reinforces the link between *ERRα/ERα* in BCa. Similarly to the suggestion that inhibition of the RANKL/RANK axis may be a therapeutic target for BRCA1^{mut} carriers, inhibition of an upstream RANK regulator, *ERRα*, may also constitute a useful new therapeutic approach for these

patients. In conclusion, we report for the first time a RANK/*ERRα* axis in BCa that contributes new mechanistic understanding of the negative outcome associated with *ERRα* in BCa.

Materials and methods

Ethics approval

BALB/c female mice were purchased from Janvier and handled according to the French Ministerial Decree no. 87–848 of 19 October 1987. Experimental protocols were approved by the Institutional Animal Care and Use Committee at the Université-Lyon1 (France)(CEEA-55 Comité d’Ethique en Expérimentation Animale-DR2014-44-DR2015-28). Studies involving human primary specimens were carried out according to the principles embodied in the Declaration of Helsinki.

Study on BCa patient primary tumor specimens

Immunohistochemistry (IHC) was performed on formalin-fixed paraffin-embedded (FFPE) tumor tissue sections ($n = 100$) from the Austrian-Breast and Colorectal-Cancer-Study Group (ABCSCG) Trial-6 [22]. For IHC, FFPE tumor tissue and a mouse monoclonal anti-ERR α antibody (1/50) (sc-65715, Santa-Cruz) were used. Sections were incubated with HRP-conjugated anti-mouse (Dako), stained using 3,3'-diaminobenzidine (Dako) and Mayer's hematoxylin (Merck). ERR α immunostaining was assessed by the *H*-score method [49].

Samples of 446 unilateral invasive primary breast tumors excised from women managed at Institut-Curie-Hôpital-René-Huguenin (France) for which grading information was available (Supplementary Table S5) were analyzed [36, 50]. The mean age of the patients was 61.8 years, and the median follow-up was 103.1 months. Since 2007, patients have signed informed consent. This study was approved by the review board and ethics committee of the institute. A tumor sample was considered suitable for our study if the proportion of tumor cells exceeded 70% [36]. Real-time-PCR was performed using specific primers (Supplementary Table S7) for human housekeeping gene *TBP* (TATA-box-binding protein), *ERR α* , *RANK*, and *RANKL* (ABI-Prism-7900 sequence-detection-system) (Perkin-Elmer-Applied-Biosystems, CA), and the PE-Biosystems analysis software according to the manufacturer's manuals [36]. Results were expressed as fold differences equal to $2^{-\Delta\Delta C_t}$.

Detection of ERR α in CTC

For ERR α detection in CTC detection, patients after signed informed consent, were recruited at the Cancer Institute of Montpellier in 2017 (Bioethic-committee D-RCB 2015-A00119-40). We first performed experiments by adding 200 BCa cells (SKBR3 and MDA-MB-231) to 7.5 mL of PBS. To mimic blood of BCa patients, we spiked 200 BCa cells in 7.5 mL of blood from healthy donors. Tumor cells were detected by using the CellSearch[®] system (Silicon-Biosystem, Menarini) with the CellSearch CTC Kit. This FDA-cleared semiautomatic system contains a ferrofluid-based capture reagent targeting all EpCAM-positive cells, which could be further identified as CTCs by a pan-keratin (CK8-18-19) and nuclear staining. Contaminating leukocytes were excluded by CD45 staining. For ERR α detection, we used the fourth channel of the CellSearch[®] and the anti-human ERR α /NR3B1-A488-conjugated polyclonal antibody (Novus, USA) (25 μ g/mL). The corresponding Rabbit IgG-Isotype control (Alexa-Fluor[®] 488) (Novus) was used at the same final concentration. Blood of three metastatic BCa patients were analyzed for the presence of CTCs overexpressing ERR α .

Meta-analysis of ESSRA expression

For the BCa cohorts "NKI" ($n = 295$) [23] and "METABRIC" ($n = 2051$) [51], the genomic and transcriptomic data were obtained through the cBioPortal Web resource (<http://cbioportal.org>). All statistical calculations were performed using PASW Statistics (version 18.0; SPSS Inc., Chicago, IL) and a two-sided unpaired Student's *t*-test. By using of the receiver operating characteristic method, we determine whether *ESRRA*, *ESRRB*, and *ESRRG* expression levels were able to define low-metastases and high-metastases risk populations.

For correlation analysis, published datasets were downloaded from the Gene-Expression-Omnibus including primary tumors, bone, lung, and liver metastases (GSE14020) ($n = 65$) [35] and primary tumor, no-metastases, visceral +bone or only BM (GSE12276-GSE2034-GSE2603) ($n = 248$) [52–54]. Z-scores were obtained by normalizing data were \log_2 transformed and calculating by subtracting the population mean from individual expression values for each gene and then dividing the difference by the population standard deviation. Correlation scores were calculated using the Pearson-correlation-coefficient. *P*-values < 0.05 were considered statistically significant. Statistical significance was determined by GraphPad Prism v5.02 using the two-sided Student's *t*-test.

Cell lines, transfection, and immunoblotting

Human MCF7 (year 2012) and mouse 4T1 (year 2012) BCa cell lines were obtained from the American Type Culture Collection. 4T1, MDA-MB231-B02 [20] and MCF7 were cultured in DMEM or RPMI-1640 (Life-Technologies) medium, respectively, supplemented with 10% fetal bovine serum (FBS, Perbio) and 1% penicillin/streptomycin (Invitrogen) at 37 °C in a 5% CO₂ incubator. The human BC-M1 (Vimentin/CK5-positive, CK8/18-negative, HER2) cell line (generous gift from the Dr. K. Pantel lab) was obtained from a bone marrow aspirate of a BCa patient with no clinical signs of distant metastases at the time of the primary tumor resection [31, 55]. BC-M1 cells were cultured in complete RPMI-1640 medium (Life-Technologies) supplemented with 10% FBS, 200 U/mL penicillin/streptomycin (Life-Technologies), insulin-selenium-transferrin (IST) (Gibco, France), 50 ng/mL of epidermal growth factor (EGF), and 10 ng/mL of human basic-fibroblasts growth factor (bFGF) (Miltenyi-Biotec, France) at 37 °C in a 5% CO₂, low (10% of O₂), or normoxic (21% of O₂) conditions. Total mRNA was extracted using the RNeasy Mini Kit (Qiagen, Courtabeuf).

Murine and human ERR α cDNA (ERR α) and the dominant-negative co-activator domain AF2 (AF2) mutant were obtained previously [20, 21, 56]. pSR α -ERR α WT and

pSR α -ERR α AF2 or empty vector constructs were transfected into parental 4T1 cells and cultured for 4 weeks in puromycin (2 μ g/mL) (Life-Technologies). For MCF7 clones, a mix containing 1.5 μ g Retroviral pLPCX-HumanERR α WT, pLPCX-HumanERR α AF2, or empty vector and 0.5 μ g pCMV-VSV-G envelope vector (Cell-Biolabs) were transfected by calcium phosphate precipitation (CalPhos-mammalian-Transfection-Kit, Clontech), according to the manufacturer's instruction with selection in puromycin (2 μ g/mL) (Life-Technologies) for 3 weeks.

Parental 4T1 and MCF7 cells were treated with the ERR α inverse-agonist C29 (AGVdiscovery, France) at 1 and 1–5 μ M, respectively, for 24 and 48 h [9, 30, 33, 57]. XCT790 (Sigma) was also used at 1 μ M [8]. DMSO was used as vehicle. For Rankl studies, 4T1-CT (two clones), 4T1-ERR α (three clones) were plated at 5×10^5 cells/well in complete medium. The next day, cells were starved for 24 h and then treated with Rankl (50 ng/mL) (R&D) for different times (0–5–15–25 min). Protein extraction and immunoblotting was performed as described [20], using antibodies against ERR α (Abcam-EPR46Y) (1:400), mTOR and pmTOR (Ser2481) (Cell-Signaling) (1:1000), S6K and pS6K (Thr389) (Cell-Signaling) (1:1000), or α -tubulin (Cell-Signaling) (1:1000) as protein-loading control [20, 21]. The experiments were done in triplicated and repeated two times.

Animal studies

BALB/c female mice (6 weeks), were housed in barrier conditions under isolated laminar flow hoods. Mice bearing tumor xenografts were monitored for established signs of distress and were humanely euthanized. Tumor fat pad (FP) experiments were performed ($n = 10$ for each groups) using the 4T1-CT (two clones), 4T1-ERR α (three clones), or 4T1-AF2 (three clones) cell lines (10^5 cells in 10 μ L of PBS) injected into FP of the fourth mammary gland of mice as described [58]. Briefly, primary tumors were resected 2 weeks after cell injection and tumor weight and size were measured. For spontaneous metastases dissemination studies (lungs (macro-metastases), bone (micro-metastases)), mice were followed after resection for an additional 3 weeks at which time they were sacrificed. Lungs were collected for histological analysis or crushed (C29 experiment). Hind limbs were removed and crushed. Cells released from lungs (C29 experiment) and bone were incubated at 37 $^{\circ}$ C in the presence of DMEM supplemented with FBS and 6-Thioguanine (10 μ g/mL) for 3 weeks before tumor cells that colonized the bone (TCB) and lung colonies were stained by using Crystal Violet and counted.

FP experiments were performed using 4T1-ERR α cells (three clones) (10^5 cells in 10 μ L of PBS). When primary

tumors reached ~ 30 mm³, mice were randomized ($n = 10$), treated every 2 days for 10 days by intra-peritoneal injection with vehicle (DMSO 10%, 30% PEG) or C29 (10 mg/kg) [9, 57], then prepared and analyzed as above. Bone lesion experiments were performed using 4T1-CT (two clones) and 4T1-ERR α cells (three clones) cells (10^5 cells in 100 μ L of PBS) injected intra-arterially (IA). After 2 weeks, bones and lungs were collected for histological analysis ($n = 2$). Some hind limbs and lungs were removed and crushed for mRNA extraction.

IHC on mice FP-tumor sections

5 μ m paraffin sections were incubated overnight with RANK(1/50)(Abcam-64C1385), or Vegf-a(1/50)(Abcam-EP1176Y) or ERR α antibody (1/50)(Santa-Cruz-V19) [20], followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit (Dako) antibodies for 1 h and staining with 3,3'-diaminobenzidine (Dako) and Mayer's hematoxylin (Merck).

Cell migration assay

Migration assays were done with the HTS FluoroBlokTM-Multiwell-Insert-system (8 μ m corning) as described [20]. After 12 h of starvation, 4T1-CT (two clones), 4T1-ERR α (three clones), and 4T1-ERR α AF2 (three clones) (5×10^4 cells) were plated in the upper chambers and the chemoattractant (0.5%FBS) with or without Rankl (50 ng/mL) (R&D) in the lower chambers. After 12 h at 37 $^{\circ}$ C in 5% CO₂ incubator, cells that had migrated through the filters were fixed, stained with DAPI (Life-Technologies) and counted. All experiments ($n = 2$) were run in triplicate, migration was expressed in % of migration/wells.

Transactivation assay

4T1 cells (10^6 cells/well in six-well plates) were transfected with a mouse Rank promoter-driven *Gaussia*-Luciferase-reporter plasmid (pEZX-PG04 RankLuc:–1450/+31) (Genecopoeia) and TurboFect Transfection Reagent according to the reverse transfection protocol (Thermo-Scientific). 4T1 cells were cotransfected with plasmids expressing ERR α -WT or mutated ERR α AF2. The ratio of DNA/Turbofect was 4 μ g/6 μ L. After overnight incubation, transfection medium was replaced with fresh medium containing DMSO 0.1% or XCT790(1 μ M) or C29(1 μ M). Medium aliquots were withdrawn at 48 h for measuring *Gaussia* Luciferase with the Secrete-PairTM *Gaussia* Luciferase-Assay Kit in Buffer GL-S (Genecopoeia). The experiments were done in triplicated and repeated two times.

Real time-PCR

Total RNA was extracted with Trizol-reagent (Life-Technologies) and 2 μ g was reverse-transcribed using SuperscriptTM-II (Life-Technologies) [21]. Real-time PCR was performed on a Mastercycler-ep-Realplex (Eppendorf) with primers specific to human and mouse genes (Supplementary Table S7) using Quantifast-SYBR-Green (Life-Technologies) according to the manufacturer's instructions [21]. The ribosomal protein L32 gene was used as control and relative results expressed as fold differences equal to $2^{-\Delta\Delta C_t}$.

Protein–Protein interaction network reconstruction and analysis

We reconstructed the protein–protein interaction network with BIOGRID (release 3.4.160) protein–protein interaction data from *Homo sapiens* with Proteomics-Standard-Initiative-Common-QUery-InterfaCe (PSICQUIC) retrieval (05292018) and CytoScape environment [34]. A custom approach was used to define a protein interactome of the following proteins: ESRRA-ESR-RIP140-SRC1-SRC2-SRC3-PGC1A-PGC1B-TRAF6-TRAF5-TRAF2-PDK1-GLUT1-LDHA-TNFRSF11A. The resulting interactome encompasses more than 300 proteins (so called “Extended Network”). To determine the connectors between ESR, RANK, BRCA1, and ESRRA, a custom approach combining shortest path and connectivity degree analysis was applied to determine a “Minimal Network” acknowledging connections that may support ESRRA signaling. We overlaid information from Gene-Ontology-consortium to pinpoint proteins that are already known to be involved in the regulation of ECM assembly and degradation, as well as cellular adhesion and migration (GO-IDs: 1903053-0022617-012715-0007155-0016477). Generated network maps by using CyNetShare (cynetshare.ucsd.edu) can be up-loaded for public access (<https://doi.org/10.18119/N9PC7F>) (Supplementary Fig. S8) [59].

Determination of ESRRA-mediated transcriptional regulation

We determined the proteins encoded by human genes under the control of ESRRA and the number of regulatory sites within these genes using the GTRD ChIP-seq database (<http://gtrd.biouml.org/>) [60].

Chromatine immunoprecipitation (ChIP)

ChIP assays were performed as previously described [61] from MDA-MB231-B02-CT and -ERR α cells [20] using either a monoclonal rabbit anti-ERR α (13826) (Cell-

Signaling) or a control rabbit IgG(2729) antibody (Cell-Signaling). The immune-precipitated genomic DNA was purified using NucleoSpin Clean-up columns (Macherey-Nagel, Germany) and analyzed by qPCR. Quantification of ChIP enrichment was calculated relative to input values. Distal and proximal elements of ERR α gene were used as negative and positive controls, respectively (Supplementary Table S7) [32, 33].

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (San Diego, USA). Data were analyzed statistically by the non-parametric Mann–Whitney *U*-test or unpaired *t*-test for in vivo studies ($n = 10$ mice for each group (bioStaTGV), unblinding studies). In vitro assays were repeated at least two times and performed on triplicate samples. Data were analyzed using ANOVA and Student's *t*-test to assess the differences between groups. MFS, LMFS, and BMFS were determined as the interval between diagnosis and detection of the first metastases. Survival distributions were estimated by the Kaplan–Meier method, and the significance of differences between survival rates was ascertained using the log-rank test. Univariate and multivariable analyses using Cox proportional hazards models were used to assess the independent contribution of each variable to organ-specific MFS. All data are presented as means \pm SEM with variance were similar between the groups. Statistical tests, differences were considered significant at confidence levels $>95\%$ ($p < 0.05$).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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