PLK1 inhibition exhibits strong anti-tumoral activity in CCND1-driven breast cancer metastases with acquired palbociclib resistance

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A significant proportion of patients with oestrogen receptor (ER) positive breast cancers (BC) develop resistance to endocrine treatments (ET) and relapse with metastatic disease. Here we perform whole exome sequencing and gene expression analysis of matched primary breast tumours and bone metastasis-derived patient-derived xenografts (PDX). Transcriptomic analyses reveal enrichment of the G2/M checkpoint and up-regulation of Polo-like kinase 1 (PLK1) in PDX. PLK1 inhibition results in tumour shrinkage in highly proliferating CCND1-driven PDX, including different RB-positive PDX with acquired palbociclib resistance. Mechanistic studies in endocrine resistant cell lines, suggest an ER-independent function of PLK1 in regulating cell proliferation. Finally, in two independent clinical cohorts of ER positive BC, we find a strong association between high expression of PLK1 and a shorter metastases-free survival and poor response to anastrozole. In conclusion, our findings support clinical development of PLK1 inhibitors in patients with advanced CCND1-driven BC, including patients progressing on palbociclib treatment.
Oestrogen receptor-positive BC accounts for over 80% of primary breast malignancies. Classically, ER-positive BC patients are treated with ET which block ER signalling. Although the introduction of ET has significantly increased survival, resistance remains a significant problem.

A major challenge for the successful treatment of ER-positive BC has been the identification of new therapeutic targets for endocrine-resistant disease. Inhibitors of mTOR and cyclin-dependent kinases 4 and 6 (CDK4/6) substantially improve progression-free survival and are now standard of care for the treatment of advanced ER + BC. However, the mTOR inhibitor everolimus is poorly tolerated and intrinsic or acquired resistance to CDK4/6 inhibitors are frequent events, limiting the success of these treatments. Resistance to CDK4/6 inhibitors is emerging as a critical consideration in patients care and clinical drug development. Identifying novel therapies for the treatment of CDK4/6 inhibitor-resistant patients is of great importance.

Bone is the most common metastatic site in ER+ patients, however, bone metastases are technically challenging to biopsy and analyse. Difficulties concern both tumour tissue acquisition and techniques for analysis and DNA/RNA extractions. Patient-derived xenografts of bone metastases have not been reported yet. Here, we establish PDX models from bone metastatic biopsies of patients progressing on ET and treated by vertebroplasty. PDX models are analysed at the genomic and transcriptomic level and compared to patient’s early primary tumours to identify new therapeutic targets associated with endocrine resistance in the metastatic setting. Transcriptomics analysis show enrichment of G2/M cell cycle signalling with an increased expression of several targetable kinases. In particular, PLK1, a serine threonine kinase, which plays an essential role in centrosome maturation, mitotic chromosome segregation and mitosis, is among the top up-regulated genes in PDX models. Based on this finding we hypothesised that PLK1 could represent a therapeutic target in endocrine resistant advanced BC. We show that PLK1 inhibition results in dramatic tumour shrinkage in highly proliferating CCND1-driven PDX including different RB-positive PDX with acquired palbociclib resistance.

**Results**

**PDX of bone metastases show activation of the G2/M checkpoint.** To identify new therapeutic targets in endocrine resistant metastatic BC, we established PDX models from metastatic bone biopsies taken from ER-positive BC patients treated by vertebroplasty. Seven PDXs were initially established. The clinical characteristics of the corresponding patients are summarised in Table 1. Six out of seven ER-positive BC patients were progressing on ET (aromatase inhibitors (AI), tamoxifen or fulvestrant). HBCx-176 and HBCx-180 were recently established from patients progressing on palbociclib alone or combined with fulvestrant. 

PDXs were molecularly characterised at the genomic level by a targeted next generation sequencing (NGS) analysis of 95 genes (the most frequently mutated genes in BC). Copy number alterations (CNA) were predicted from whole-exome sequencing or by Cytoscan HD array. Significantly mutated genes and CNA in putative cancer drivers genes are shown in Table 1. Details on mutations and variant frequencies are shown in Supplementary Data 1 and CNA in PDX tumour samples are listed in Supplementary Data 2.

To identify activated signalling pathways in bone metastasis, we performed comparative transcriptomic analysis of the bone metastasis derived PDX compared to the patients’ primary BC. Gene set enrichment analysis (GSEA) showed augmentation of different hallmarks associated with cell proliferation and G2/M DNA damage checkpoint (E2F target, G2/M checkpoint, DNA damage, and mitotic spindle) in PDX as compared to primary tumours (Supplementary Table 1). The most significantly enriched gene sets in primary tumours were epithelial mesenchymal transition (EMT), NFKB/TNF, TGF beta signalling, and gene sets associated with the loss of human stroma (inflammatory response and interferon response). The integrative pathway and network analysis are shown in Supplementary Fig. 1. Figure 1a, b shows the hallmark of the G2/M checkpoint and mitotic spindle with the heat map of the top differentially expressed genes in the G2/M checkpoint gene set. Notably, different targetable kinases associated to mitosis were up-regulated in PDX, including PLK1, AURKA, AURKB, CDK1 and MPS1 (Fig. 1c), and PLK1 was among the top up-regulated genes in PDX models.

PLK1 is a therapeutic target in highly proliferating PDX models. Based on these findings, we hypothesised that PLK1 could represent a therapeutic target in PDX of metastatic BC. Therefore, we assessed the efficacy of the PLK1 inhibitor volasertib (BI 6727) in the PDXs. PDX was established from an aggressive ER + BC (patient’s clinical history is detailed in Fig. 1d and associated figure’s legend). Immunohistochemical analysis revealed a strong expression of PLK1 and Ki67 in the PDX, the patient’s bone metastasis and primary breast tumour. The CNA profile of the primary breast tumour, the bone metastasis and the PDX, were highly similar with focal amplification of FGFR1, FGFR2 and CCND1 and CN gains of CCNE2 (Fig. 1e, Supplementary Data 2). Focal amplification of FGFR1 also associated with high protein expression in PDX. The in vivo anti-tumour activity of volasertib was assessed in comparison with the CDK4/6 inhibitor palbociclib and the FGFR1/2/3 inhibitor AZD4547, as monotherapies or in combination with fulvestrant. Strikingly, volasertib caused rapid tumour shrinkage and complete response achieved in 4 weeks (Fig. 1f). The combination of volasertib with fulvestrant was less efficient than volasertib alone, although tumour regression was observed in all xenografts. Statistical analysis of tumour growth inhibitions are depicted in Supplementary Data 3. Within the same time frame, treatment by palbociclib alone or combined with fulvestrant resulted in stable disease. Two volasertib-treated xenografts relapsed 6 weeks after end of treatment but responded to a second round of treatment with volasertib resulting in rapid tumour shrinkage, indicative of tumour rewiring (Fig. 1f).

We hypothesised that PLK1 inhibition may influence ER ligand-dependent and/or ligand-independent transcription, as it was previously suggested in vitro using BC cell lines. We therefore analysed the expression of the ER-regulated genes (ERGs) TFF1, GREB1 and PR, together with PLK1, Ki67, PCNA and CENPE (a centromere associated protein that accumulates in G2 phase and is involved in chromosome alignment), in treated tumours. No significant alteration in the expression changes of the ERG was evident in volasertib-treated xenografts. Contrastingly, the expression of CENPE was significantly increased (Fig. 1g). Interestingly, the expression of PLK1 was decreased by fulvestrant. The expression of Ki67 and PCNA was unchanged in volasertib treated xenografts, in contrast to palbociclib-treated xenografts (Supplementary Data 4). These results suggest that the anti-tumour activity of volasertib in this tumour is ER independent and not associated with a decreased proliferation but rather with a cell cycle arrest in the G2 phase, consistent with its known mode of action.

To further define the subset of tumours susceptible to respond to PLK1 inhibition, we tested the anti-tumour activity of volasertib as compared to palbociclib in three additional PDX with amplification or CN gain of CCND1. PDX HBCx-124 was established from a young patient diagnosed with metastatic BC at...
| PDX ID  | Patient's primary tumour | Treatment before vertebroplasty | MFS (years) | Bone metast. and PDX IHC | Mutations | Gene amplification | Homologous deletion |
|---------|--------------------------|---------------------------------|-------------|--------------------------|-----------|-------------------|-------------------|
| HBCx-118| ER+ PR- HER2- K67 NA     | FEC, Vinorelbine, Tamoxifen, Letrozole | 7           | ER+ PR+ K67 NA           | BRCA2     | AKT1              |                   |
| HBCx-124| ER+ PR+ HER2- K67 50%   | Cyclophosphamide, AC, Paclitaxel | 10          | ER+ PR+ K67 35%          | FGFR1     | MTRM, CCNB1      |                   |
| HBCx-131| ER+ PR+ HER2- K67 NA    | Vinorelbine + Docetaxel, AC, Leukocyte, Letrozole | 1           | ER+ PR- K67 60%          | CCND1, FCR1, CDKN2A/B |                   |                   |
| HBCx-134| ER+ PR+ HER2- K67 50%   | Cyclophosphamide, Vinorelbine, AC, Paclitaxel | 0           | ER+ PR- K67 35%          | FGFR1     | CDKN2A/B         |                   |
| HBCx-137| ER+ PR+ HER2- K67 NA    | Cyclophosphamide, Letrozole, ER, Paclitaxel | 9           | ER+ PR- K67 60%          | CCND1, FCR1, CDKN2A/B |                   |                   |
| HBCx-139| ER+ PR+ HER2- K67 NA    | Docetaxel, Vinorelbine, Paclitaxel, Letrozole, Eribulin, Paclitaxel | 13          | ER+ PR- K67 35%          | FGFR1     | CDKN2A/B         |                   |
| HBCx-142| ER+ PR+ HER2- K67 NA    | Docetaxel, Vinorelbine, Paclitaxel, Letrozole, Eribulin, Paclitaxel | 14          | ER+ PR- K67 60%          | CCND1, FCR1, CDKN2A/B |                   |                   |
| HBCx-180| ER+ PR- HER2- K67 NA    | FEC as adjuvant followed by letrazole | 14          | ER+ PR- K67 35%          | FGFR1     | CDKN2A/B         |                   |
| HBCx-176| ER+ PR+ HER2- K67 NA    | FEC, Tamoxifen, Triptorelin, Tamoxifen, Paclitaxel, Letrozole, Capecitabine | 14          | ER+ PR- K67 60%          | CCND1, FCR1, CDKN2A/B |                   |                   |

Characteristics of patients and PDX: FEC: 5-FU epirubicine cyclophosphamide. AC: Adriamycin cyclophosphamide, FUN: 5FU Navelbine, MFS: metastasis-free survival.
The clinical history of patients was similar: both have been treated by HBCx-142 (Supplementary Fig. 2; Supplementary Data 3). The median). Mean ± SD. Volasertib treated xenografts were monitored after end of treatment to detect tumour recurrence. Two xenografts relapsed at day 70 fulv.). AKT1 and mTOR inhibitors in two PDX with hotspot mutation of PIK3CA, and with stable disease to volasertib, AZD5363 and palbociclib. Despite the clinical benefit of CDK4/6 inhibitors, tumour resistance develops in most patients in the metastatic setting and finding new therapeutic targets for these patients remains an unmet clinical need. We therefore developed two PDX models of acquired resistance to palbociclib: the first PDX was derived from the HBCx-134 PDX, established from the bone metastasis of a 71-year-old patient. The patient’s breast tumour Neo-adjuvant chemotherapy followed by tamoxifen (adjuvant setting), prior to vertebroplasty and PDX establishment. Both PDXs were resistant to fulvestrant. PDX HBCx-118 (BRC2 and AKT1 mutated) responded to the combination of AZD5363 plus fulvestrant, while PDX HBCx-142 (AKT1 and mTOR mutated) responded with tumour regression to everolimus and with stable disease to volasertib, AZD5363 and palbo + fulvestrant.

In summary, targeting PLK1 lead to a striking tumour regression in three out of four CCND1-driven PDX models, while AKT1 and mTOR mutated PDX preferably responded to AKT1 and mTOR inhibitors.

PLK1 is a therapeutic target in PDX with acquired palbociclib resistance. Despite the clinical benefit of CDK4/6 inhibitors, tumour resistance develops in most patients in the metastatic setting and finding new therapeutic targets for these patients remains an unmet clinical need. We therefore developed two PDX models of acquired resistance to palbociclib: the first PDX was derived from the HBCx-134 PDX, established from the bone metastasis of a 71-year-old patient. The patient’s breast tumour...
carried a PIK3CA mutation (also found in the PDX) and the patient was treated with a PI3Kα inhibitor combined with the AI letrozole in the neo-adjuvant setting (Fig. 3a). After pathological examination the residual tumour was classified as RCB-III (no response to neo-adjuvant treatment). Patients’ and PDX tumours both carried CCND1 amplification and a homozygous deletion of CDKN2A/B was identified in PDX (Fig. 3b). HBCx-134 xenografts which initially responded to palbociclib (Fig. 3c), were exposed long term to CDK4/6 inhibition in order to establish a palbo-resistant PDX. Xenograft Palbo R31 showed cross-resistance to alpelisib plus fulvestrant but was highly responsive to volasertib (tumour growth inhibition of 96%; p < 0.0001, Mann–Whitney test) (Fig. 3d). Genomic profiling and a NGS analysis of 571 cancer associated genes revealed no differences in CN (Supplementary Fig. 3) and mutation profile between the parental and the palbo-resistant PDX: with both carrying a mutation in PIK3CA (His1047Arg) and a TP53 mutation (Arg248Trp).

Abundance of phospho-RB was also similar in the parental and palbociclib resistant xenografts indicating that palbociclib resistance was not due to RB loss in this model (Fig. 3e). The second model of palbociclib acquired resistance (Palbo-R25) was established from a HBCx-124 xenograft that was exposed to palbociclib treatment for 5 months (Fig. 3f): tumour growth was arrested for the first 2 months prior to tumour escape. HBCx-124 Palbo-R25, re-implanted in another set of mice, showed a striking response to volasertib (Fig. 3g) with tumour regression observed in all xenografts. By contrast to the parental HBCx-124 PDX, we did not observe any significant predilection for CDKN2A/B homozygous deletion in the Palbo-R25 xenografts (Fig. 3h).
and PDX establishment (Fig. 4a). The genomic and IHC analyses of palbociclib of adjuvant endocrine therapy. She received three cycles of bone metastases 14 years after breast surgery and 4 years after end treatment. Patient corresponding to PDX HBCx-180 recurred with established from patients progressing on palbociclib 6.

**Fig. 3 Response to volasertib in CCND1-driven PDX models with acquired resistance to palbociclib.** a Clinical history of the patient corresponding to HBCx-134 PDX: the patient’s tumour carries a PIK3CA mutation and the patient was treated by a PI3Kα inhibitor + an aromatase inhibitor in the neo-adjuvant setting. At mastectomy the pathological analysis classified the tumour as RCB-III (resistance to neo-adjuvant treatment). The patient was treated by adjuvant chemotherapy (FEC, 5-FU, epirubicin, cyclophosphamide and docetaxel) and letrozole before metastatic recurrence and PDX establishment. b Genomic profile (WES) of patient’s tumour and PDX model showing amplification of CCND1 and CDKN2A homoygous deletion in the PDX. c Establishment of a PDX with acquired resistance to palbociclib from the HBCx-134 model: 4 HBCx-134 xenografts were treated by palbociclib during 4 months: all xenografts responded. Treatment was stopped and mice were followed-up: xenograft #°31 relapsed 4 months after end of treatment and exhibited resistance when retreated by palbociclib. d The PDX was re-engrafted in another set of mice and treated by palbociclib to confirm resistance, by volasertib and by the PIK3CA inhibitor alpelisib (BYL-719) + fulvestrant, during 3 months. Results confirmed palbociclib resistance and revealed a cross-resistance to alipelisib + fulvestrant. RTV relative tumour volume. Mean ± SD, n = 4 xenografts/group. P ≤ 0.0001 (Mann-Whitney test, two-sided). e Immunohistochemical analysis of phospho-RB in HBCx-134 and HBCx-134 palbo-R PDX (70% of phospho-RB positive cells in both tumours). Scale bar is 50 μm. f Establishment of a PDX with acquired resistance to palbociclib from a HBCx-124 xenograft that was treated by palbociclib during 5 months: tumour growth of xenograft n°25 was stabilised during the first 2 months and increased on treatment until day 150. g Tumour response to palbociclib alone or combined with fulvestrant and volasertib in the HBCx-124 palbo-R25 PDX. RTV = relative tumour volume. Mean ± SD, n = 5 in control, palbo, palbo+fulv.; n = 8 in volasertib group. P ≤ 0.0001 (Mann-Whitney t test, two-sided). h Immunohistochemical analysis of phospho-RB in HBCx-124 and HBCx-124 palbo-R PDX (38% and 36% of phospho-RB positive cells, respectively).

Finally, we tested the efficacy of volasertib in two PDX models established from patients progressing on palbociclib + letrozole treatment. Patient corresponding to PDX HBCx-180 recurred with bone metastases 14 years after breast surgery and 4 years after end of adjuvant endocrine therapy. She received three cycles of palbociclib + letrozole followed by paclitaxel, before vertebroplasty and PDX establishment (Fig. 4a). The genomic and IHC analyses of the PDX revealed an amplification of CCND1 (Supplementary Data 2), a mutation of PIK3CA (Supplementary Data 1), high expression of Ki67 and PLK1 and positivity of phospho-RB (Fig. 4b). The PDX reproduced resistance to palbociclib ± fulvestrant and responded to volasertib with stable disease (Fig. 4c). PDX HBCx-176 was established from a patient who relapsed with metastatic disease 14 years breast surgery and was treated...
with palbociclib + letrozole during 3 months (Fig. 4d). Both bone metastasis and PDX tumours were ER negative. The PDX carried a frameshift insertion in the NF1 gene (Supplementary Data 1), was positive for P-RB and exhibited strong expression of P-ERK, a marker of ERK-MAPK signalling activation (Fig. 4e). HBCx-176 responded to the MEK inhibitor trametinib with tumour regression and was resistant to both palbociclib and volasertib (Fig. 4f).

Overall, these results demonstrate that targeting PLK1 is an effective treatment in CCND1-driven PDX models with acquired resistance to palbociclib, including PDX established from patients pre-exposed to palbociclib. In addition, we show that a NF1-mutated tumour is resistant to both palbociclib and volasertib but respond to the MEK inhibitor trametinib.

Response to volasertib is correlated with high expression of PLK1 and Ki67. To assess whether the expression of PLK1 and the proliferation status of the PDX are correlated to volasertib response, we analysed the expression levels of PLK1 and Ki67 genes in the control xenografts of the seven PDX, by real-time polymerase chain reaction (RT-PCR) analysis. The expression of both PLK1 and Ki67 genes was higher in PDX responding with complete response or tumour regression as compared to PDX with no response or partial response to volasertib (Fig. 5a). Next, we analysed Ki67 expression changes in volasertib-treated xenografts to determine whether inhibition of proliferation was associated to response. Ki67 expression was decreased in six PDX models: three in the group of responding tumours and three in the group of tumours with no response or partial response to volasertib (Fig. 5a). Next, we analysed Ki67 expression changes in volasertib-treated xenografts to determine whether inhibition of proliferation was associated to response. Ki67 expression was decreased in six PDX models: three in the group of responding tumours and three in the group of tumours with no response or partial response to volasertib (Fig. 5a).

Moreover, as response to PLK1 inhibitors is associated with mitotic arrest of treated tumours8,14, we performed a pharmacodynamics study with two responder (HBCx-124 and HBCx-137) and two resistant PDX (HBCx-118 and HBCx-131) to analyse phospho-histone H3 (P-HisH3) expression (a marker of mitosis) at baseline and 24 h after a single drug administration. Results show an increase of P-HisH3 staining up to 10% of tumour cells in the two responder PDX and in 3% of tumour cell in the HBCx-131 model (Fig. 5b). In summary, these results indicate that tumour response to volasertib is greater in highly proliferating tumours and is associated with post-treatment mitotic arrest.

PLK1 expression is a strong predictor of a shorter metastasis free survival. In order to determine the prognostic significance of
PLK1 expression pattern in human breast tumours, we analysed PLK1 mRNA levels in a large series of 441 primary breast tumours from patients with known clinical/pathological status and long-term outcome (Supplementary Table 2). Results in the global population showed that metastasis-free survival (MFS) of patients with high PLK1-expressing tumours (5-year MFS 68.4%; 10-year MFS 58.5%; 15-year MFS 49.8%) was shorter than that of patients with low PLK1-expressing tumours (5-year MFS 88.7%; 10-year MFS 77.4%; 15-year MFS 74.4%) (P < 0.0001; Fig. 5c).

This prognostic significance of PLK1 expression status in the global population was restricted to the hormonal receptor (HR)-positive (HR+HER2+ and HR+HER2−) sub-group (P < 0.0001), and was not observed in the triple negative sub-group, as well as in the HR-HER2+ sub-group.

The prognostic significance of parameters identified in univariate analysis and PLK1 expression status persisted, except for Scarff Bloom Richardson (SBR) histopathological grade and progesterone receptor (PR) status, in the Cox multivariate regression analysis of MFS (Supplemental Table 3).

As PLK1 promotes cell proliferation of various cancers, we tested possible correlation between PLK1 and various genes involved in cell cycle signalling pathway, i.e., AURKA, MK67 and NEK2, quantified by RT-PCR analysis in the same cohort (Supplemental Fig. 4). We observed high positive association between PLK1 and these three genes (r = +0.828, P < 0.0001 for AURKA; r = +0.749, P < 0.0001 for MK67; r = +0.782, P < 0.0001 for NEK2; Spearman rank correlation test).

PLK1 does not interact with ER in vitro. Previous studies suggest PLK1 may influence ER transcriptional activity. However, expression of ERGs was not decreased in volasertib-treated tumours of HBCx-137 PDx (Fig. 1g). In order to analyse PLK1 and ER interaction in vitro, a panel of cell lines resistant to long-term oestrogen-deprivation (LTED), which harboured naturally occurring ESR1Y537C, ESR1Y537S or ESR1 wild-type (wt)17, were subjected to escalating concentrations of volasertib. In keeping with our DPD models, the drug caused a dose-dependent decreased in the proliferation of all the cell lines tested.
MCF7-LTED<sup>Y537C</sup> were the most sensitive with an IC<sub>50</sub> of 5 nM compared with MCF7-LTED<sup>wt</sup>, HCC1428-LTED and SUM44-LTED which had IC<sub>50</sub> values between 10 and 16 nM. Expression of PLK1 was not a determinant of sensitivity either at the RNA or protein level (Fig. 6b, c).

In order to assess the interaction of ER with PLK1, we carried out comparative RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins) and dimethyl-labelling in wt-MCF7 and wt-SUM44 cells in the presence of oestrogen and MCF7-LTED and SUM44-LTED in the absence of oestrogen. (Fig. 6d, Supplementary Fig. 5a and Supplementary Data 5). As expected, the most abundant interaction in all models was with ER. Furthermore, the main ER-interactors were NCOA3, GREB1, TRIM28 and HDAC1. No interaction with PLK1 was evident in any of the models and interrogation of publicly available data similarly showed no interaction<sup>18–20</sup>. In order to test this further, targeted co-IPs were performed in MCF7-LTED cells confirming the lack of interaction (Supplementary Fig. 5b).
As ER-trancriptional activity may be affected by PLK1 indirectly\textsuperscript{11}, we used siRNA targeting PLK1 and treatment with volasertib to assess ER-mediated transactivation and the impact of PLK1 perturbation on the expression of specific oestrogen regulated genes (ERGs). Inhibition of PLK1 with volasertib had minimal or no impact on ER/ERE-mediated transactivation in wt-MCF7, wt-HCCI1428 or wt-SUM44 in the presence or absence of oestrogen (Fig. 6e). Furthermore, no significant impact was evident in the LTED models with the exception of SUM44-LTED where volasertib caused a significant rise in ER/ERE reporter activity, an observation in contrast to previous studies\textsuperscript{11}. In addition, siPLK1 caused no significant decrease in the expression of prototype ERGs (TFF1, GREB1 and PGR) (Supplementary Fig. 6).

In order to address the potential impact of time on the ability of PLK1 to influence ER-mediated transactivation, we assessed the effect of volasertib treatment on ER/ERE-luciferase activity over 16 h in MCF7-LTED cells. No impact on hormone independent transcriptional activity was evident (Fig. 6f). In order to address the potential that PLK1 influences ER at different points during cell cycle, we synchronised MCF7-LTED cells using a CDK1 inhibitor (RO3306) followed by treatment with volasertib to block PLK1 activity or volasertib plus the MPS1 inhibitor (BOS172722). Fulvestrant, which down-regulates ER activity was used as a positive control (Fig. 6g, h). As expected, inhibition of PLK1 had no impact on the abundance of TFF1, GREB1 or ER. However, a time dependent decrease in MPS1 was evident in the presence of volasertib and BOS172722 indicative of cell cycle arrest. Contrastingly, fulvestrant caused a reduction in ER, TFF1 and GREB1 which appeared cyclical. Noteworthily, fulvestrant caused a reduction in MPS1 and PLK1, 4 h post-treatment, concordant with cell cycle arrest (Fig. 6g) and our previous observations.

Finally, we assessed the association of PLK1 with expression of ERG in a cohort of 69 postmenopausal women with stage I to IIIB ER+ early BC who had received single agent neoadjuvant anastrozole\textsuperscript{21} (Fig. 7a). Global gene expression data from paired samples taken at baseline and 2-week post-treatment were available. PLK1 expression did not correlate with ERGs (rho = 0.103, p = 0.328) or ESR1 (rho = 0.041, p = 0.695) (Fig. 7a). Furthermore, correlations of PLK1 with individual ERGs similarly showed no association (TFF1, PGR, GREB1, PDZ2K1, MYC and RET) (Supplementary Fig. 7). Contrastingly, PLK1 expression strongly correlated with cell cycle regulated genes including CCND1 (Supplementary Fig. 8). On the same cohort of patients we found that high on-treatment expression of PLK1 associates to a poor response to anastrozole (Fig. 7b). Finally, we looked at PLK1 expression in CCND1-amplified as compared to CCND1-diploid tumours in the TCGA and Metabric breast cancer datasets (Fig. 7c). In both cohorts, PLK1 expression was higher in the group of CCND1-amplified breast tumours.

In conclusion, these data suggest PLK1 does not influence ER-activity in either a ligand-dependent or independent manner and that high on-treatment expression of PLK1 correlated with poor response to ET.

**Discussion**

In this study we developed PDX from bone metastasis biopsies of patients progressing on ET to identify new therapeutic targets in a context of hormone-resistance. The transcriptomic analysis of bone metastasis-derived PDX as compared to patients’ early breast tumours, showed an enrichment in gene sets associated with G2/M checkpoint and mitosis. Molecular profiling on matched bone metastasis and primary tumours are limited due to the challenge of obtaining DNA and RNA from decalcified samples. However one study performed a RNA sequencing of 11 patient-matched primary BC and decalcified bone metastasis and reported G2/M checkpoint as one of the three most significantly enriched gene sets in bone metastases\textsuperscript{22}. The same study found downregulation of EMT, NF-KB/TNF and different stem cell gene sets in bone metastases, that were also downregulated in our PDX. The striking similarity of our results with those reported by Priedigikeit et al. suggests that the up-regulation of mitotic genes in PDX is not an artefact of tumour engraftment but rather a characteristic of the bone metastasis. Furthermore, a RNAseq analysis of matched primary breast tumours and tamoxifen-treated liver metastases identified cell cycle and DNA replication as up-regulated pathways in metastases\textsuperscript{23}.

At the genomic level, five out of seven PDX carried amplification or CN gains of CCND1 gene with additional alterations in cell cycle genes CCNE2, CDK7, AURKA or CCNB1. For six patients, we could match the copy number profile of patient’s primary breast tumour and PDX (with the bone met. biopsy in two cases) and found a high concordance of CNA. Common oncogenic drivers such as CCND1 and FGFR1 or FGFR2 were amplified in the primary breast tumours and can be considered early events in these patients’ tumours. Amplification of CCND1 is one of the major genetic determinant of hyperactivity of the cyclin D–CDK4/6 pathway that occurs frequently in ER positive BC\textsuperscript{24,25}. The CCND1–CDK4/6 axis promotes G1 exit through RB phosphorylation which results in activation of a proliferation transcriptional programme and phosphorylation cascades that
required for G2/M progression. These data suggest that in CCNB1, mitosis driving tumour cells through the S phase, as well as CN gains and CCND1. If CCND1 amplification or CN gain of CCND1 in chromosome 11: one at 11q13.3 (HBCx-131) or in the PDX (HBCx-134), in patients who recurred 10 years and 1 year, respectively, after treatment with an aromatase inhibitor. CDKN2A loss, that promotes cell cycle progression by increasing CDK4/6 activity, has been reported in therapy exposed BC metastases in different studies, suggesting that this genetic event might contribute to endocrine resistance or tumour progression of metastatic BC.

Different targetable kinases associated to mitosis were up-regulated in bone metastases, including PLK1, AURKA, AURKB, CDK1 and MPS1. Importantly, we found that four out of seven PDX models responded dramatically (with complete response or tumour regression) to the PLK1 inhibitor volasertib. The efficacy of PLK1 inhibitors in BC has been reported in triple-negative BCs or ER+ BC cell line derived xenografts. Our results show that PLK1 is a therapeutic target in PDX models of advanced ER-positive BC. Those models showing the greatest sensitivity to volasertib carried concomitant amplification or CN gains of CCND1 and CCNE2 both of which are involved in RB inactivation driving tumour cells through the S phase, as well as CN gains in CCNB1 and CDK7 (HBCx-139) or AURKA (HBCx-124), genes required for G2/M progression. These data suggest that drive cancer cells into the S phase, DNA replication and mitosis.

If CCND1 amplification was present in the primary tumours, CDKN2A homozygous deletion appeared in the bone metastases (HBCx-131) or in the PDX (HBCx-134), in patients who recurred 10 years and 1 year, respectively, after treatment with an aromatase inhibitor. CDKN2A loss, that promotes cell cycle progression by increasing CDK4/6 activity, has been reported in therapy exposed BC metastases in different studies, suggesting that this genetic event might contribute to endocrine resistance or tumour progression of metastatic BC.

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responder to volasertib, indicating that PLK1 inhibition and response to volasertib are not associated with an impaired ER-dependent transcription. These results are not in line with previous data reported in cell line models of endocrine resistance showing that PLK1 targeting with siRNA or volasertib inhibits ER-dependent transcription. To further explore the role of PLK1 in ER-mediated transcription, we used different cell lines models of endocrine resistance and confirmed that PLK1 does not influence ER-activity in either a ligand-dependent or -independent manner. 

In clinical practice, the combination of CDK4/6 inhibitors and ET is now standard of care for metastatic ER + BC. Our results show that the three tumours responding to volasertib are partially responsive to palbociclib and PDX with primary resistance to palbociclib were also resistant to volasertib, suggesting that cell cycle is the common determinant of response to CDK4/6 and PLK1 inhibitors. Moreover, our data show that PDX models driven by AKT1, mTOR or NF1 mutations preferably respond to AKT1, mTOR and MEK inhibitors and this finding might have important clinical impact for the choice of first-line treatment in this group of patients. Everolimus is approved for the treatment of metastatic BC and the AKT1 inhibitor Capivasertib has demonstrated anti-tumour activity in most patients with metastatic AKT1-mutant tumours enroled in the NCI-MATCH trial.

In the clinic, acquired resistance to CDK4/6 inhibitors occurs frequently and represents the next major clinical challenge for the treatment of advanced ER + BC. Therefore, we established 2 PDX with acquired resistance to palbociclib, both of which conserved RB phosphorylation. The genomic analysis of palbociclib-resistant PDX did not reveal any change in CN level nor emergence of mutations in genes associated to cell cycle. Further analysis are ongoing to identify potential mechanisms of resistance in these PDX. The striking response to PLK1 inhibition in these PDX indicate that loss of G1/S dependency can be targeted with inhibitors of the G2/M cell cycle transition. In addition, we showed that targeting PLK1 might be a relevant therapeutic strategy in a context of secondary resistance to palbociclib and cross-resistance to PI3Kα inhibitor.

PLK1 inhibitors, including volasertib, have been shown to arrest tumour cells in mitosis, with an increase in the polyploid cell population that lead cancer cells to mitotic death. Accordingly, the increase of histone H3 phosphorylation, which occurs specifically in mitosis, and of CENPE expression, a gene specifically expressed during the G2/M phase, indicates an increase of mitotic cells in volasertib treated xenografts. The RT-PCR analysis of treated xenografts also shows that Ki67 expression was decreased by volasertib treatment in six out ten PDX models. However Ki67 expression decrease was not always associated with tumour regression, suggesting that inhibition of cell proliferation per se can not be considered a marker of response.

Finally, we found that high PLK1 expression was a strong predictor of worse survival in a large cohort of ER positive BC patients. This is consistent with previous data showing that PLK1 expression is strongly correlated with a lower MFS. More-predictor of worse survival in a large cohort of ER positive BC patients always associated with tumour regression, suggesting that inhibition of PLK1 targeting agent would be significant.

In a second cohort of early stage BC treated by anastrozole in the pre-surgical setting, we found that on treatment expression of PLK1 was associated to a poor response to anastrozole.

In summary our results strongly support the development of PLK1 inhibitors in patients with endocrine-resistant metastatic ER + BC including patients with acquired resistance to the CDK4/6 inhibitor palbociclib. A highly Ki67 index could represent a valuable biomarker to identify patients who could benefit from PLK1 targeting.

Methods

Patient-derived xenografts (PDX) establishment. PDX models of ER+ metastatic BC were obtained by engrafting biopsies from spinal bone metastases of ER-positive BC patients progressing under ET and treated with vertebralprolotherapy to restore biomechanical vertebral properties (stabilisation) and reduce back pain. The protocol was approved by the Institut Curie Hospital committee (CRE Comité de Revue Institutionnel). Bone metastasis biopsies were engrafted with informed consent from the patient into the interscapular fat pad of female Swiss nude mice (Charles River Laboratories), which were maintained under specific pathogen-free conditions. Their care and housing were in accordance with institutional guidelines and the rules of the French Ethics Committee: CEEA-IC (Comité d’Éthique en matièr de expérimentation animale de l’Institut Curie, National registration number: #118). The project authorisation no. is 02163.02. The housing facility was kept at 22 °C (±2 °C) with a relative humidity of 30–70%. The light/dark cycle was 12 h light/12 h dark.

In vivo efficacy studies. Volasertib (BI 6727), AZD4547 (FGFR1,2,3 inhibitor), AZD5343 (AKT1 inhibitor), Trametinib (MEK inhibitor), Alpelisib (BYL-719) and palbociclib (CDK4/6 inhibitor) were purchased from MedChemExpress. Volasertib was administered orally 4 days per week at a dose of 10 mg/kg. AZD4547, AZD5343, Trametinib, Alpelisib and palbociclib were administrated orally 5 days per week at 12, 100, 1, 35 and 75 mg/kg, respectively. Everolimus (Ceritan, Novartis Pharma) was administered orally at a dose of 2.5 mg/kg 3 times per week. Fulvestrant (Faslodex, AstraZeneca, Macclesfield, UK) was administered by intramuscular injection at a dose of 50 mg/kg once a week.

For efficacy studies, tumour fragments were transplanted into female 8-week-old Swiss nude mice. Xenografts were randomly assigned to the different treatment groups when tumours reached a volume comprised between 100 and 200 mm³. Tumour size was measured with a manual calliper twice per week. Tumour volumes were calculated as V=(LxW²)/2, being the longest diameter, L, the smallest. Tumour volumes were then reported to the initial volume as relative tumour volume (RTV). Means (and SD of RTV) in the same treatment group were calculated, and growth curves were established as a function of time. For each tumour the percent change in volume was calculated as (V₀ − V)/V₀×100, V₀ being the beginning of treatment and V the final volume (at the end of treatment). A decrease in tumour volume of at least 50% was classified as regression, an increase in tumour volume of at least a 35% identified progressive disease and volumes changes between +35% and −50% were considered as stable disease. The statistical analysis of tumour growth inhibition was performed with the Mann-Whitney test or the Dunn’s multiple comparisons test.

Gene expression analysis. Gene expression microarrays were performed at the Genomics platform of Institut Curie. GeneChip Human 1.1 ST arrays were hybridised according to Affymetrix recommendations, using the Ambion WT Expression Kit protocol (Life Technologies) and Affymetrix labelling and hybridisation kits. Affymetrix CEL files were imported into the Gene Expression Worklow in Partek® Genomics Suite version 7.0 (Partek Inc., St. Louis, MO, USA, www.partek.com). Background correction, quantile normalisation, log2 transformation, and probe-set annotation were performed using default settings for the Robust Multichip Average (RMA) procedure.

Gene set enrichment analysis (GSEA, v4.0.3) software and MsigDB database (v7.0) were used to identify overrepresented biological functions for differentially expressed genes between PDX and primary breast tumours.

Enriched pathways were then represented as an interactive network, using EnrichmentMap® and Cytoscape® application that determine relationships between pathways. Pathways are represented by nodes and connected by edge if they shared common genes. Highly interconnected nodes are clustered in order to identify major biological processes. Affymetrix CEL files and normalised log2 RNA data are available at the GEO database (accession No. GSE146661; [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146661]).

RNA extraction and RT-PCR analysis of PDX. RNA extraction was performed by using acid-phenol guanidium method and Electroetherscope through agarose gel electrophoresis and performed with dedicated RNA quality 18S and 28S RNA bands were visualised under ultraviolet light. RNA was reverse transcribed in a final volume of 20 µl containing 1× RT buffer [500 mm each dNTP, 3 mm MgCl₂, 75 mm KCl, and 50 mm Tris-HCl (pH 8.3)], 10 units of RNasin® RNase inhibitor (Promega, Madison, WI), 10 mm DTT, 50 units of Superscript II RTase (Life Technologies, Inc., Gaithersburg, MD), 1.5 mm random hexamers (Pharmacia, Uppsala, Sweden), and 1 µg of total RNA. The samples were incubated at 20 °C for 10 min and 42 °C for 30 min, and
reverse transcriptase was inactivated by heating at 99 °C for 5 min and cooling at 5 °C for 5 min.

We used protocols for PCR amplification described in detail elsewhere. Briefly, we obtained quantitative values from the Ct value (cycle number) at which the increase in the fluorescence signal associated with exponential growth of PCR products was detected by the laser detector of the ABI Prism 7900 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA), using according to the manufacturer’s manuals (SDS Software v2.3).

The human TATA box-binding protein (TBP, GenBank accession no. NM_003194) was used as gene normalisation. Results, expressed as N-fold differences in target gene expression relative to the TBP gene and termed “Ntarget”, were calculated as Ntarget = 2−ΔΔCt, where the ΔCt value was determined by subtracting the average Ct value of target gene from the average TBP Ct value.

The target gene values of the patients’ breast tumour samples were subsequently normalised such that the median of the target gene values for the ten normal breast tissues was 1.

The primer pairs used were: TBPRD1, 5′-CTCAGAGGAGGAAAGTGTTGA-3′ (upper) and 5′-CACATCATGTGTGACACTCT-3′ (lower); PLK1, 5′-GGGAGCAT ATT-TGCAGAGAACAGGTTTGT-3′ (upper) and 5′-CTCTCTCCCAATACATCGGC-3′ (lower); AURKA, 5′-GGATCGCGGCCAGAGAGCTGA-3′ (upper) and 5′-GCTTCGCTTGATGTGAATCG-3′ (lower); TBP, 5′-CTCCGTTTCTTCTGCCTCAAT-3′ (lower); ESR1, 5′-CCACCAACACTGGCACATT-3′ (upper) and 5′-GGTTCTTTGTATGATCCTCCCT-3′ (lower); PGR, 5′-GCGGCTCTCACTGCACTGC-3′ (upper) and 5′-TGATACGCGCCCTTCTGGTA-3′ (lower); TFF1, 5′-CATGACGCTCTCAGCAGAGG-3′ (upper) and 5′-CTCTGCCCTCCTGTCATGCT-3′ (lower); CTGAG, 5′-CTTCCCTGATCTGCTCAAAC-3′ (upper) and 5′-GGCTAGTGTGTGTTTGTA-3′ (lower); CENPE, 5′-TGCCATTACAAGGCTAATGTAC-3′ (upper) and 5′-ATGATCTTCTGAACATCTGTTCACTGC-3′ (lower); PNA, 5′-TCTGAAAGGAGGAAAGGCTC-3′ (upper) and 5′-CCAGCATACTGCTTGCTCAGTGT-3′ (lower).

In N-fold differences for each xenograft were normalised to yield a median value of 1 for the control xenograft group.

Targeted NGS of PDX. Patient-derived xenografts genomes, with the exception of HBCx-180, were analysed by targeted NGS of 95 genes (Supplementary Data 1), chosen among the most frequently mutated genes in breast cancer (>1%). Targeted sequencing was performed for variants confined to exons. Prime PCR amplification was performed on Illumina HiSeq2500 sequencer and the genomic variants were annotated with COSMIC and 1000 Genome databases. Reads were aligned using Burrows-Wheeler Aligner (BWA) software, allowing up to 4% of mismatches with the reference. Variants with low allelic frequency (<5%) or low coverage (<100x) were excluded from the analysis.

The parental and palmo-resistant HBCx-134 and HBCx-124, as well as the HBCx-180 PDX, were also sequenced with a targeted NGS panel (called “DRAAG”) that has been recently developed in the genetics department of our Institute. It is composed of 571 genes of interest in oncology from diagnosis, prognosis and theranostics (Supplementary Data 1). NGS primers were selected based on their specificity on the human genome.

Sequencing for both panels were performed on an Illumina HiSeq2500 sequencer and raw data were processed using the GAP methodology to obtain absolute copy number profiles. Copy number less than average ploidy-1 were defined as losses, and modal copy number 0 were considered as homogenous deletions.

Affymetrix cytoscan HD array. Patients corresponding to HBCx-139, HBCx-176 and HBCx-180 did not provide the specific consent to perform NGS on their wild type tissues. In absence of this consent, we performed cytoscape analysis in order to derive copy number data from copy number data generated in this PDX. Tumour samples were profiled using Affymetrix Cytoscan HD array according to the manufacturer’s instructions.

Raw data was processed using the GAP methodology to obtain absolute copy number profiles. Cytoscan raw data are available in the GEO database (GSE149038).

Immunohistochemistry. For immunohistochemistry studies, 3μm thick sections from formalin-fixed paraffin-embedded tissue were de-paraffinised, hydrated and unmasked in target retrieval solution pH 9 for 15 at 95 °C.

Five μL of PLK1 (20845) was provided by Cell Signalling, the detection was performed by immunoperoxidase technique and 3,3’-Diaminobenzidine tetrahydrochloride hydrate (DAB) chromogenic substrate revelation on the Bond RX using the Bond Polymer Refine Detection Kit (Leica) according to the manufacturer’s protocols. Following blocking of endogenous peroxidase activity and incubation of non-specific staining, the diluted antibody (1/50) was applied to slides for 60 min at room temperature. After washing with PBS the slides were incubated with polymeric horseradish peroxidase (HRP)-linker antibody conjugate system for 8 min. A DAB substrate solution was used to detect immunoreactive areas. The sections were counterstained with Mayer’s Hematoxylin.

Anti Phospho-Rb (Ser807/811) (D02B12) and anti FGFR1 (D4E4) were purchased from Cell Signalling (Rabbit Monoclonal #8161 and #9740) and respectively used at 1:2000 and 1:1000. Anti-PLK1 (20845) was used at 1:200. Anti-phospho-Rb and in EDTA buffer pH 8 for Anti FGFR1. Phospho-p44/p42 MAPK (Erk1/2) (Thr202/Tyr204) (D1314.4E) rabbit monoclonal antibody (Cell Signalling #4373) was used at 1:140. Phospho-histone H3 (Ser10) rabbit polyclonal antibody (Cell Signalling #9701) was used at 1:1000. Ki67 monoclonal antibody (clone SP6, ThermoFisher # RM9100651) was used at 1:100. All steps were performed in the Discovery XT autostainer (VENTANA Medical Systems, Roche) using an UltraMap HRP XT Kit according to the manufacturer protocol.

A semi-quantitative histologic score (H-score: intensity (0–3)× frequency (0–100%)) was performed (score 0: negative staining, score 1: weak staining, score 2: moderate staining, score 3: strong staining). Ki67 was scored as the percentage of nuclei-stained cells out of all cancer cells in the invasive front of the tumour regardless of the intensity in 100 high-power fields, 500–1000 tumour cells were counted in each case.

Immunohistochemically phospho-HisH3 stained slides were photographed with medium magnification using an Imager Z1 zeiss Microscope and a CamHMR Zeiss Camera and analysed in a blinded manner. Homogenous nuclear staining and nuclei with at least four or more stained foci were considered positive for expression. Positive- and negative-nuclear staining were quantified manually using four fields of approximately 1000 cells for each tumour, and the percentage of P-His3 staining was evaluated in each fields.

Cell culture. Human BC cell lines were obtained from the ATCC and Asterand, banked in aliquots to reduce phenotypic drift and identity confirmed using short tandem repeat analysis. Cells were also routinely screened for mycoplasma contamination. Wild-type cells were cultured in phenol red-free RPMI1640 containing 10% foetal bovine serum (FBS) and 1 nM estradiol (E2) and their respective LTED, represented pre-vascular, pre-coated by 100nM and phenol red-free RPMI1640 containing 10% charcoal-dextran stripped FBS (DCD). All cell lines were stripped of steroids for 48-72h prior to each targeted sequence were conserved. Duplicate reads were identified and discarded using Picard Tools (v1.130, https://broadinstitute.github.io/picard/). Finally, local realignment of reads around small insertions and deletions (and insertions) were re-aligned using GATK (v3.5)53. For tumour xenograft sequence data, a computational deconvolution of mouse and human reads was performed using XenoffDlg R package (0.99.0)34. Whole exome sequencing data are available at the European Genome-phenome Archive (EGA) (No. EGAS00001004268) [https://ega-archive.org/studies/EGAS00001004268].
qRT-PCR analysis of cell lines. RNA was extracted using the RNeasy kit (Qiagen), quantified and reverse-transcribed with SuperScriptIII First Strand Synthesis System (Invitrogen). Ubiquitin gene expression assays (Applied Biosystems) were used to quantify TFF1 (Hs00970239_m1), GREB1 (Hs00356409_m1), CBN1 (Hs00999188_m1), PLKI (Hs00983277_m1), ESRI (Hs00174860_m1) and the house-keeping genes FKBP13 (Hs00314840_m1). The relative quantity was determined using ΔΔCt, according to the manufacturer’s instructions (Applied Biosystems).

Transcriptional assays. Cell lines were seeded in 24-well plates in 10% DCC medium and left to acclimatise overnight. Transfection was performed with Eugene 6 at a ratio of 6:1 (Promega) with 0.1 µg of oestrogen response element linked luciferase (EREIIluc) and 0.1 µg of β-galactosidase (pCH110) reporter constructs, as previously detailed.

Twenty-four hour, cells were treated with the drugs combinations and left for 24 h. A luminometer was used to measure Luciferase (Promega) and β-galactosidase (GalactoStar, Applied Biosystems) activities. Each experiment was performed three times with three to four replicates per treatment.

Immunoblotting. Whole cell extracts were generated from cells cultured under basal conditions or after the treatments specified. Equal amounts of protein resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis. Antigen–antibody interactions were detected with ECL reagent (Amersham, UK) using the following antibodies: total-ER (Santa Cruz Bio-technology: sc-8002, clone F-10, 1:200), TFF1 (AbCam: ab92377, clone EPR3972 1:2000), β-catenin (AbCam: ab7191, 1:1000), MIB1 (Dako: 05-0900; clone 3-472-1: 1:1000), H3 (abCam: Ab1791; 1:1000), PLKI (Millipore: 05-04: 04-04; clone 35-206; 1:1000) and tubulin (Sigma-Aldrich; T-9026, clone D61A, 1:10000).

Immunoprecipitation. Cell lysates were pre-cleaned, incubated in primary anti-bodies IgG (Dako); ER, (Santa Cruz Biotechnology, sc-543); PLKI (Millipore: 05-044) at 4°C overnight. Immuno-Complexes were recovered using protein G, washed six times in extraction buffer and resolved by SDS-PAGE, as specified above.

RIME and dimethyl-labelling. RIME and stable isotope dimethyl-labelling were performed, as previously described. Briefly, cells were labelled with the medium and light isotope reagent. Labelled samples were pooled at an approximate 1:1 ratio, dried down and fractionated using 12 cm IPG strip pH 3-10. RIME and dimethyl-label fractions were desalted (SUM SSBV, The Nest Group Inc) and run through LC-MS/MS using LTQ Velos Orbitrap MS. Raw data for RIME and dimethyl-labelling were analysed using MaxQuant V1.5.1.1. Statistical analysis was performed using Perseus software (version 1.6.1.3)28. Raw data have been deposited to the ProteomeXChange Consortium via PRIDE partner repository with the dataset identifier PXD004648 (MC7) and PXD004807 (SUM44).

Institut Curie clinical cohort. Samples of 441 invasive primary breast cancers excised from patients treated at Institut Curie—Hôpital René Huguenin have been analysed. All patients cared in our institution before 2007 were informed that their tumour samples might be used for scientific purposes and had the opportunity to decline. After 2007, patients gave their approval by signed informed consent. This study protocol was approved by the local ethical committee (Breast Group of René Huguenin Hospital). Samples were stored in liquid nitrogen until RNA extraction. Tumour samples were included in the study with a proportion of tumour cells of at least 70%. All patients (mean age 61.8 years, range: 31–91 years) met the following criteria: primary unilateral nonmetastatic breast carcinoma with clinical, histological and biological data available; no radiotherapy or chemotherapy before surgery; and full follow-up at Institut Curie—Hôpital René Huguenin.

Modified radical mastectomy was performed in 275 cases (63.5%) and breast-conserving surgery plus locoregional radiotherapy in 158 cases (36.5%). The patients had a physical examination and routine chest radiotherapy every 3 months for 2 years. Patients with an annual hybridisation for adenovirus DNA. In patients with hormone therapy was administered to 170 patients and both treatments to 101 patients. The histological classification and the number of positive axillary lymph nodes were determined at surgery. The SBR histoprognostic system was used to score the malignancy of infiltrating carcinomas. The status of oestrogen receptor alpha (ERα), PR and human epidermal growth factor receptor 2 (ERBB2) was determined at the protein level by using biochemical methods (dextran-coated charcoal method, enzyme immunoassay or immunohistochemistry) and confirmed by real-time quantitative RT-PCR assays. The population was divided into four groups according to HR (ERα and PR) and HER2 status, as follows: two luminal subtypes [HR+/HER2− (n = 501); and HR+/HER2+ (n = 283)]; an HER2 subtype [HR−/HER2+ (n = 41) and a triple-negative subtype [HR−/HER2− (n = 67)]. Standard prognostic factors of this tumour set are presented in Supplemental Table 2. During a median follow-up of 9.1 years (range 4.3 months to 33.2 years), 173 patients developed metastasis. Ten samples of adjacent normal breast tissue from breast cancer patients and normal breast tissue from women undergoing cosmetic breast surgery were used as sources of normal RNA.

Gene expression microarray analysis of breast tumours. Global gene expression and KEGG data were available from core-cut biopsies belonging to 69 post-neoadjuvant anastrozole and 2-week post-treatment of single agent neoadjuvant anastrozole. Detailed methodology for this work and patient demographics are published elsewhere31 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE153470). The Pearson correlation method was used to analyse correlation between PLK1 and the other genes. Data were extracted, transformed, normalized and filtered using the same methods as performed for the cell lines27. This study received approval from an institutional review board at each site and was conducted in accordance with the 1964 Declaration of Helsinki and International Conference on Harmonisation/Good Clinical Practice guidelines. Written informed consent was obtained from each patient before participation.

Statistical analysis. Median values and ranges of mRNA levels were used to analyse the distributions of target gene levels. Different nonparametric tests, namely the chi-square test (relation between two qualitative parameters), the Mann–Whitney U test (relation between one qualitative parameter and one quantitative parameter) and the Spearman rank correlation test (relation between two quantitative parameters) were used to analyse the relationship between different target genes and between mRNA levels and clinical parameters. Differences were considered significant at confidence levels greater than 95% (p < 0.05).

To analyse the efficacy of PLK1 level to discriminate two populations (patients that developed did not develop metastases) in the absence of an arbitrary cut-off value, data were summarised in an ROC (receiver operating characteristic) curve. The AUC (area under curve) was calculated as a single measure for discriminate efficacy. MFS was determined as the interval between initial diagnosis and detection of the first metastasis. Survival distributions were estimated by the Kaplan–Meier method, and the significance of differences between survival rates was determined with the log-rank test. The Cox proportional hazards regression model was used to assess prognostic significance and the results are presented as hazard ratios and 95% confidence intervals.

Data availability. Affymetrix raw data files and normalised log2 RNA data are available at the Gene Expression Omnibus (GEO) database (accession No. GSE146661) [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE146661]. Data from targeted NGS panels are available at the European Genome-phenome Archive (EGA) under the No. EGAS00001004268 [https://ega-archive.org/studies/EGAS00001004268]. Whole-exome sequencing data are available at the European Genome-phenome Archive (EGA) (No. EGAS00001004268) [https://ega-archive.org/studies/EGAS00001004268]. The source data for Figs. 1c, 5a–c; 6a–h, Supplementary Figs. 5b and 6 are available as a Source Data file. All other data that support the findings of this study are available from the corresponding author upon reasonable requests.

Code availability. The GAP methodology was previously described in detail27. An implementation of the GAP pattern recognition and detection of copy numbers and genotypes based on segmented profiles is available at the GAP download site of Institut Curie Bioinformatics: http://bioinfo-out.curie.fr/projects/snp-gap/.

Received: 6 December 2019; Accepted: 16 July 2020; Published online: 13 August 2020

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**Acknowledgements**

We thank the patients for participating in this study. We thank Sergio Roman–Roman for critical reading of the manuscript. We thank Odette Mariani and the CRB (Centre de ressources biologiques) of Institut Curie for their support in processing patients’ samples. We thank Institut Carnot and SIRIC2 (INCa-DGOS-Inserm_12554) for funding. High-throughput sequencing was performed at the Institut Curie ICGex NGS platform, which is supported by the ANR-10-EQPX-01 (Equipe) and ANR-10-INBS-09-08 (France Genomique Consortium) grants from the Agence Nationale de la Recherche (“Investissements d’Avenir” programme). We thank the animal platform of the Institut Curie. We thank Jean-Luc Servely for taking IHC pictures. The mouse icon was made by Freepik from www.fat-icon.com.

**Author contributions**

E.M., L.S., L.H., A.D., P.P. and F.N. established the PDX models and performed in vivo experiments. J.N.-B., C.R., N.S., S.P. and R.R. were involved in acquisition and analysis of in vitro data. S.V., W.C., R.E. and I.B. were involved in acquisition and analysis of RT-PCR data. J.M.-P., V.B., K.D., M.F. and Z.T. were involved in interpretation of NGS and cytoscan arrays. J.M.-P., V.B., K.D., M.F. and Z.T. were involved in interpretation of NGS and WES data and bioinformatics analysis of gene expression data. S.C.-J., A.N., D.M., A.V.-S. and T.L. were involved in acquisition and analysis of IHC data. D.D., F.C. and P.C. performed vertebroplasty and bone biopsies for PDX establishment. E. Ma. and L.-A.M. were involved in the study design and wrote the paper. I.B., P.C. and K.D. were involved in critical review of the paper.

**Competing interests**

The authors declare no competing interests.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-17697-1.

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**Peer review information** Nature Communications thanks Nihal Ahmad, Oscar Rueda and the other anonymous reviewer(s) for their contribution to the peer review of this work.

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