Holding the balance; the equilibrium between ERα-activation, epigenetic alterations and chromatin integrity

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Chapter 7

Discussion

ERα-positive breast cancer and endocrine resistance prediction

Breast cancer remains the most prevalent form of cancer in women, with approximately 1.7 million annual new diagnoses, while despite the improvement of breast cancer treatment over the years, still over half a million women die of this disease every year (1). The majority of these tumors (70-80%) express estrogen receptor α (ERα) and tumor cell proliferation is thought to be dependent on the activity of this hormone-mediated transcription factor (2, 3). Endocrine treatments options of ERα-positive breast cancer mainly consist of receptor-inhibition by anti-estrogens (e.g. tamoxifen) (4-6) or by inhibition of the estrogen biosynthesis (e.g. aromatase inhibitors) (7). Despite the fact that these treatment modalities have greatly aided in the improvement of patients survival, a large proportion of the patients do not respond, which can ultimately leading to a relapse with limited additional treatment options left due to the development of endocrine resistance (8, 9). As there are multiple ways tumors can become resistant to therapy, a better understanding of the mode of action of ERα and the development of resistance, coupled with the use of biomarkers that can predict the treatment response of a patients on an individual bases could further increase patient survival.

An example of how the discovery of a mechanism behind tamoxifen-resistance can lead to the discovery of a predictive biomarker, is the activation of the protein kinase A (PKA) pathway and the resulting phosphorylation of ERα at Serine residue 305 (ERαS305-P) (10) (**Chapter 2**). Whereas tamoxifen normally inhibits the recruitment of essential coregulators, S305-P induces a conformational change still enabling the composition of an ERα transcriptional complex even when bound by tamoxifen (Michalides et al., 2004, Zwart et al., 2007), affecting the ERα cistrome and transcriptome (Carascossa et al., 2010, Lupien et al., 2009). In **Chapter 2** we demonstrated that although a large proportion of ERαS305-P chromatin interactions overlapped with the cistrome of total ERα, a surprising significant increase in promoter deposition was be observed, resulting in differential gene expression and tamoxifen resistance. The recent finding that besides PKA-activation, pro-inflammatory cytokines are also capable of inducing tamoxifen resistance in MCF-7 cells by the induction of S305-P (11), further strengthens the role of ERαS305-P in tamoxifen resistance. Stender et al. demonstrated that cytokine activation of ERα is dependent on the S305-phosphorylation and is mediated by IKKβ and, similar to PKA-activation, leads to increased levels of MYC. Additionally they show that also this mode of S305-phosphorylation results in a cistrome that substantially overlaps with the conventional estradi-

ol induced ERα cistrome, although the resulting differential gene expression profile differs from the one we found. This difference might be best explained by the difference in stimulation (three hours of estradiol versus four hours of tamoxifen) and the additional exposure of MCF-7 cells to 10 ng/ml TNFα or 10 ng/ml IL1β. Nevertheless, the above findings further strengthen the key role S305-phosphorylation can play in tamoxifen resistance, induced not only by PKA-activation, but possibly also by the tumor microenvironment (11).

The altered gene expression profile as a result of PKA-activation we found in **Chapter 2** could be translated into a gene signature capable of predicting patients response to tamoxifen treatment, potentially allowing the selection of patients that would not benefit from adjuvant tamoxifen and directly providing an alternative treatment modality. This ERαS305-P derived classifier has been tested in a cohort of patients treated with tamoxifen in the adjuvant setting, but unfortunately this cohort lacks a randomized control arm of patients not having received tamoxifen. As this set-up does not allow one to distinguish whether a biomarker is prognostic (natural course of the disease) or predictive (tamoxifen treatment specific), it is crucial to make use of a cohort containing a non-tamoxifen treated group, thus providing an extra level of evidence in predictive biomarker discovery (12).

Compared to the ERαS305-P derived classifier, our findings on the potential of SRC3-pS543 prognostication (**Chapter 6**) provide an additional level of evidence that SRC3-pS543 could be a predictive biomarker with regards to tamoxifen treatment outcome. We demonstrated that SRC3-pS543 phospho-specific antibodies could identify patients with a functional ERα pathway, which is indicative of a favourable outcome in the absence of adjuvant therapy and where tamoxifen is likely not to induce any survival benefit. This additional level of evidence was derived from the Nottingham Tenovus Primary Breast Carcinoma Series we used, which in contrast to the ERαS305-P cohort, besides patients that did received adjuvant tamoxifen, also contains patients which did not receive any adjuvant therapy (13). Herein patients with a poor prognosis (based on tumor grade) received adjuvant tamoxifen and patients with a good prognosis did not. However, this discrepancy between the survival prognosis of the two patients arms isn't ideal as it now remains unclear whether SRC3-pS543 phospho-specific antibodies are genuinely predictive of tamoxifen treatment, or whether they are only associated with survival in patients with a poor prognosis. In order to rule out this type of bias and to achieve even more confidence that a certain biomarker or gene signature is truly predictive of any treatment effect, one could

make use of a cohort containing a matched non-tamoxifen treated group, as was used to assess the predictive capacity of FEN1 (**Chapter 5**). Herein a cohort was used containing tissue blocks from postmenopausal breast cancer patients randomized between tamoxifen and no adjuvant therapy (14), allowing us to directly assess FEN1's tamoxifen specific predictive potential. However it must be noted that during patient accrual in this cohort, it became clear that lymph node positive patients show a great survival benefit from tamoxifen, so after 1989, these patients skipped the first randomization and all received 1 year of tamoxifen, meaning all analyses had to be stratified for nodal status (negative versus positive). Previous research had already shown that FEN1 levels could be indicative of patient outcome but limited its investigation merely to the total population of breast cancer patients, thus analysing both ERα-negative and ERα-positive patients together (15). By analysing the different hormone receptor status of breast cancer patients separately, we were able to demonstrate that FEN1 levels are not indicative of outcome in ERα-negative breast cancers but are in ERα-positive patients, suggesting an ERα-specific biomarker in breast cancer. More importantly, we demonstrated that only in ERα-positive patients receiving adjuvant tamoxifen FEN1 levels were associated with outcome, allowing the use of FEN1 as a predictive marker for tamoxifen treatment response. In line with these findings, we provided evidence that in breast cancer cell lines FEN1 levels are able to dictate ERα-driven cell proliferation in the presence of tamoxifen. By combining successful biomarker-driven patient stratification with matching cell line experiments and biological insights, one can increase the body of evidence that a certain biomarker is truly predictive by incorporating causal cell line data.

The final step to determine with the highest level of confidence whether FEN1 is a predictive marker of tamoxifen efficacy would be to step away from the use of retrospective patients cohorts and move to a prospective trial where patients are randomized between adjuvant tamoxifen or no tamoxifen on the basis of their FEN1 levels. Recently the results from such a trial were reported for a 70-gene signature (MammaPrint) (16, 17), demonstrating the clinical utility of this signature. When drawing the parallel between our FEN1 biomarker and this 70-gene signature, which was first reported in 2002, it becomes clear that still a lot of work has to be done in order to fully demonstrate the clinical functionality of FEN1 as a predictive marker for tamoxifen resistance. In order for this type of prospective trial to be worthwhile for FEN1, it will first be important to validate our findings in additional cohorts, preferably in patient cohorts containing more contemporary hormonal thera-

pies (e.g. five years of an aromatase inhibitor (AI) alone, two-three years of tamoxifen followed by five years of an AI, or less common 5 years of TAM). Although these types of cohorts would be extremely valuable, they are unfortunately extremely rare. In the patient cohort that was utilized for FEN1 explorations, patients were treated with adjuvant tamoxifen only, while currently most postmenopausal breast cancer patients receive an aromatase inhibitor alone, or preceding/following tamoxifen treatment. In part we addressed this difference by investigating FEN1 levels in the non-randomized METABRIC (18), in which FEN1 levels were predictive of treatment outcome in patients treated with more contemporary hormonal therapy strategies. Additionally our cell proliferation data show that in the absence of estradiol FEN1 was able to stimulate cell proliferation, a setting close to the aromatase inhibitor setting.

ERα-cistromics and posttranslational modifications

As described above, in **Chapter 2** we demonstrated that the PKA-induced post-translational modification of ERα (S305-P) can have a major impact on its cistrome, transcriptome and cellular phenotypic behaviour. Besides this well-known and characterized ERα-phosphorylation, other phosphorylation sites are also know to alter ERα-activity and/or correlate with patient outcome (e.g. serine residues 104/106 (19), 118 (20) and 167 (21)). In **Chapter 3**, we provided experimental evidence for a previously unknown ERα-phosphorylation (T594P) and demonstrate that 14-3-3 proteins can interact directly with ERα, which is the basis of the regulatory role of T594P in ERα-regulation. We were able to increase the levels of T594P in cell lines by shielding the phosphorylation site with fusicoccin (FC) (22), the most-likely mechanism of action being a blocked access of phosphatases to the phosphorylated Threonine (23, 24). Induction of T594P greatly decreased ER-chromatin interactions, E2-driven gene transcription and ultimately blocked cell growth. What makes T594P different from most other ERα-phosphorylations is that it is a relatively short-lived intermediate and it regulates ERα-activity in such a different way than other well characterized phosphorylation; our data indicates a model wherein T594P at the ERα C-terminal tip negatively affects receptor dimerization and transactivation through its interaction with 14-3-3 proteins, which could be enhanced by stabilizing the T594P with FC. Perhaps this role of T594P could be part of a regulatory mechanism to keep the levels of ERα-activity within normal physiological boundaries. A first step to determine how prominent this role is would be to compare T594P levels between

normal breast tissue and breast cancer tissue by IHC. Higher levels of T594P levels could indicate that during breast cancer development, the inhibitory mechanism of T594P gets down-regulated.

Besides ERα itself, the phosphorylation of ERα-coactivators can also redirect the cistromic repertoire of ERα, as exemplified by the activating S543-phosphorylation of SRC3 (**Chapter 6**). SRC3 upregulation is, in combination with increased ERBB2 expression, known to correlate with a poor tamoxifen response (25-28). SRC3 is normally predominately found together with ERα at distal enhancers and introns. SRC3-pS543 however, showed a striking increase in promoter deposition, both in cell lines and tumor material, although for now it remains unclear which event is first; the S543-phosphorylation or the deposition of an SRC3/ERα complex near a gene promoter triggering subsequent SRC3-phosphorylation. The use of SRC3 knock-out/down cells with a reconstituted dominant active S543-pointmutant in combination with SRC3 ChIP-seq could, by investigating the potential enrichment of binding events at the SRC3-pS543 enriched promoters, aid in the understanding of this order of events. The altered cistromic profile, together with our findings that SRC3-pS543 expression was associated with a poor response to tamoxifen treatment, makes it very likely that pS543 alters the gene expression profile of breast cancer and thus ultimately its phenotype. A possible explanation for this could come from differential cofactor recruitment by ERα when bound by SRC3-pS543 when compared to an un-phosphorylated SRC3. Investigating the composition of the ERα-transcriptional complex by making use of RIME (rapid immunoprecipitation mass spectrometry of endogenous proteins) (29) in SRC3 knock-out/down cells with an inactive S543-pointmutant (thus disabling phosphorylation) could shed a light on any altered cofactor recruitment. Recent publications have not only shown promising results in SRC3-targeted therapies (30-32), but also demonstrated that ERα-bound SRC3 results in the recruitment of CARM1 which enables methyltransferase activity of the ERα-complex and induces a conformational change of p300 which increases its HAT-activity and ultimately enhanced transcriptional activity of the ERα-complex (33). Additionally, the posttranslational modifications of ERα-coactivators such as SRC2-S736-phosphorylation (facilitating SRC2 recruitment to the ERα complex) (34) and sumolation of the CREB-binding protein (CBP) at lys 999, 1034, and 1057 (repressing its transcriptional activity) (35), further demonstrate the impact of coactivators and their posttranslational modifications can have on the functionality and behaviour of the ERα-complex. It is likely that the ERα-transcriptional com-

plex functions within a fine balance of activating and inhibiting modifications which together determine ERα's cistromic profile and functionality.

Novel drug targets in ERα-biology

Minimization of predictive gene-profiles, as found in **Chapter 2**, can make a classifier more easily implementable in clinical practice. Especially when only protein levels of one or a few proteins are sufficient this can be added with relative ease to existing pathological (IHC) assessment. Besides their use in patient prognostication, predictive gene-profiles can also be useful for identification of novel drug targets or biological insights in ERα biology. An example of this is the identification of FEN1 as a crucial ERα-regulator by the computational refinement of a previously reported predictive gene profile consisting of 111-genes (36) (**Chapter 5**). We were able to minimize the number of genes needed from this classifier towards a minimum of 4 genes (FEN1, HBP1, MCM2 and STARD13). Individual assessment of these genes as predictive biomarkers in both ERα-positive and negative patients demonstrated the exclusive ERα-positive predictive potential of FEN1. Where FEN1 previously has been reported to be a prognostic biomarker in breast cancer patients (containing both ERα-positive and negative patients) (15), we demonstrated that this is most likely due to the ERα-positive patient population. We found that FEN1 is an ERα-cofactor and modifying its activity by knockdown or overexpression altered ERα-activity, implying FEN1 might be a promising drug target in ERα-positive breast cancer. The small compound screen we performed for FEN1 inhibition, ultimately led to the discovery of a FEN1-specific and potent inhibitor, active in the nanomolar range. To demonstrate its potential as novel drug target we assessed its efficacy in cancer cell lines, where the inhibitor showed a clear sensitivity of ERα-positive breast cancer cell lines when compared to ERα-negative cell lines. Additionally, tamoxifen resistant derivatives of ERα-positive cell lines showed an increased sensitivity for the FEN1 inhibitor, suggesting FEN1 inhibition might be useful in tamoxifen resistant breast cancer patients as an alternative therapy. Although tamoxifen-resistant cell lines (37) and tumors (38) still require ERα function and are thereby targetable by FEN1 inhibition, the exact reason behind this increased sensitivity remains unknown. A possible explanation could be the role FEN1 plays in ERα-mediated DNA-demethylation as in the tamoxifen resistant cell line MCF-T, activation of growth-promoting genes by promotor hypomethylation was observed more frequently than in sensitive cells (39). It's possible FEN1 plays a crucial role in this promoter demethyla-

tion, but further research is needed to validate this hypothesis.

As we tested our inhibitor in cell lines that have been around for a long time and were cultured in the artificial setting of a petridish and might therefor not resemble the primary tumor situation anymore, we next turned to the use of clinical specimens. Primary tumor explants of ERα-positive breast cancer patients were cultured in the presence or absence of our FEN1 inhibitor, demonstrating a clear decrease in tumor proliferation (as assessed by Ki76 staining) upon FEN1 inhibition, performing equally well as tamoxifen in this setting. FEN1 inhibitors have previously been described to be effective as chemo-sensitizers (40, 41) or as a part of synthetic lethal interactions (42, 43), but were not considered to be an effective therapy strategy on their own (44). We however demonstrate that by specifically targeting ERα-positive breast cancer, FEN1 inhibition might be an effective single-agent application. Although promising, it is still far too early to state whether FEN1 inhibition is a realistic therapeutic option on its own.

Multiple rounds of validation, drug optimization and cytotoxicity assessments would have to be performed, before a phase 1 clinical trial could be considered. We did already perform some exploratory cytotoxicity experiments in mice, demonstrating mice were coping well with levels of up to 10mg/kg of FEN1 inhibitor, administered bi-daily. Measurements of FEN1 inhibitor levels in the blood of these mice, showed that there was on average 400-500 nM of compound left four hours after injection. Unfortunately a rapid decrease in inhibitor levels (estimated half-time 2 hours) was observed, which could possibly limit drug efficacy on tumor growth. We have tested the compound in 20 mice, but our screen hit was not sufficiently effective to block ERα-driven tumor cells in these animals. An explanation for the lack of efficacy could be found in the fact that due to the high half-time of 2 hours, there might not have been high enough inhibitor levels to achieve a (relative) continues blockage of FEN1 function, enabling recovery of ERα-function during times of FEN1 inhibitor absence. This explanation is substantiated by cell lines experiments where a continuous exposure of FEN1 inhibitor yielded the best results with regards to inhibition, and bi-daily drug treatment with extensive washing after four hours was inferior to the continues exposure with regards to the degree of inhibition of cell proliferation (data not shown). As the inhibitor we have now used has not gone through a medicinal chemistry compound optimization pipeline, and thus is not expected to have optimal AdMe/Tox properties, it would be advisable to see whether drug optimization can increase the bioavailability and stability of the compound. Additionally,

investigating previously reported compounds might provide better in vivo bioavailability/stability and thereby greater drug efficacy, although tumor cell growth inhibition by the previously reported FEN1 inhibitor PTPD (45) was inferior to our novel FEN1 inhibitor (data not shown). At the moment the therapeutic option of FEN1 inhibition in ERα-positive breast cancer is far from clinically applicable, nevertheless our findings do show the potential minimization of predictive gene-profiles can have in identifying the causal genes in prognostication and the discovery of novel drug targets.

An additional novel therapeutic option may lay in the use of compounds hindering ERα dimerization such as fusicoccin (FC) (22). We demonstrated that the endogenous interaction between 14-3-3 proteins and ERα, can be stabilized by administration of FC, resulting in an inhibition of ERα chromatin interactions, diminished transactivation and subsequent block of cell proliferation (**Chapter 3**). An additional benefit of FC might be that its specificity for ERα still enables the anti-proliferative role of ERβ in breast cancer (46-48), although the occurrence of ERβ in breast cancer has recently been questioned (49). Despite the fact that we have demonstrated FC is small molecule ligand that has potential as a drug target in ERα-positive breast cancer, the relatively low affinity of the compound might pose as a problem for further pre-clinical development. As FC is a member of the group of fusicoccanes (50), investigation of these members and their specific potential to stabilize the ERα and 14-3-3 interaction might yield more potent hits which could possibly be chemically modified to increase their efficacy in stabilizing this interaction and subsequent T594-Phosphorylation even further. Additionally the recent discovery of secondary sites on 14-3-3 proteins bound by small molecule ligands by applying fragment-based screening methods (51) might further facilitate the discovery of compounds with a higher affinity (52).

Novel mechanistic insights in ERα action

As described above, there are multiple ways tumors can become resistant to therapy, making it clear that a better understanding of the mode of action of ERα is required. As discussed in **Chapter 1**, ERα-function is not only influenced by its coregulators, but also by other nuclear receptors. An example of this is liver receptor homolog-1 (LRH-1) (**Chapter 4**), which canonically has a role in the regulation of bile acid and cholesterol homeostasis (53) as well as dictating inflammatory responses in the liver and gut (54). In the ERα-positive setting however, LRH-1 but has been found to be an ERα-regulated gene

capable of directly regulating cell proliferation (55), although the exact mechanism behind this regulation remained unclear. However, we identified a subset of 222 genes that were differentially expressed when LRH-1 levels were knocked down in breast cancer cells. As these genes were known for their response to estrogen, this suggested that LRH-1 can regulate ERα-responsive genes. We tested this hypothesis by mapping LRH-1 chromatin binding events using ChIP-seq, where we found a large proportion of LRH-1 sites shared with ERα. At these shared regions both nuclear receptors promoted each other's recruitment, resulting in increased recruitment of ERα co-regulators such as p300, CBP and SRC3 and subsequently altered ERα-responsive gene expression. As the above findings are suggestive for direct nuclear receptor interactions, as previously described for Retinoic acid receptor-alpha (RAR alpha) (56), and the Progesterone Receptor (PR) (57) and Glucocorticoid Receptor (GR) (58, 59), it is seems plausible the same holds true for ERα and LRH-1. However, our efforts to demonstrate such direct physical interactions between ERα and LRH-1 failed as we were unable to detect binding between them, using either ChIP-reChIP or co-immunoprecipitation. An alternative explanation for the shared binding sites between ERα and LRH-1 could be found in the possibility of "assisted loading" (60); A mode of action wherein one nuclear receptor is able to induce binding of a second receptor by promoting e.g. chromatin accessibility, resulting in increased co-occupancy at the same region in a population of cells. This hypothesis would be consistent with our findings that knockdown of LRH-1 altered chromatin remodelling at ERα binding sites and that E2 promotes LRH-1 recruitment, but not in the presence of ERα-degrading agent fulvestrant. Besides the previously mentioned interactions between ERα and PR/GR, the Androgen Receptor (AR) is also able to bind shared regions in ERα-positive breast cancer cells resulting in inhibition of ERα-activity, most likely by direct competition between ERα and AR binding the same genomic regions (61), although inhibition by cross-interference, or "squelching", might also play a role (**Chapter 1**) (62, 63). To add even more complexity to this interaction, preclinical studies have demonstrated AR may have both proliferative (64) as well as anti-proliferative properties (61, 65, 66). Altogether it becomes clear that a complex interplay exists between not only different steroid hormone receptor family members, but also nuclear receptors as LRH-1, where it is likely that multiple receptors together can influence the direction of ERα-activation.

Another way breast cancer can become tamoxifen resistant is by the overexpression of FEN1 (**Chapter 5**). Besides the previously discussed link

between high levels of the FEN1 protein and the correlation with poor patient outcome and decreased sensitivity of cell lines for tamoxifen, we also revealed that FEN1 is an ERα-coregulator that is capable of dictating the transcriptional activity of ERα by regulating the formation and base excision repair of hormone-induced DNA damage. Others have previously proposed that the reason for this link between ERα-action and DNA-damage is the fact that nicking of the DNA could relieve the torsional stress resulting from DNA supercoiling at ERα-bound promotors and enhancers (67), although it remains unclear what the exact role of DNA supercoiling is as it has also been reported to aid enhancer-promotor looping and induce DNA conformational changes that can regulate transcription (68-70). We however propose an additional reason for this damage induction, as we identified FEN1 plays a key role during the excision of APOBEC3B mediated methylated-Cytosine-to-Uracil modifications (71) which, after inducing DNA-damage, can be replaced by an un-methylated Cytosine, alleviating some local epigenetic repression. Besides regions of hypomethylation, we also find E2 stimulation yielded regions of hypermethylation. A possible explanation for this might be that DNA methyltransferases have been reported to co-occupy ERα-interacting regions near the TFF1 and FOXA1 promoter (72, 73). It's possible that the balance between methylating (e.g. DNMT's) and demethylating (e.g. FEN1's role in BER) proteins at ERα-binding sites drives the directionality of DNA methylation alterations at these sites.

Additionally the FEN1-regulated ERα-mediated DNA-damage results in increased levels of yH2AX, which is known to promote chromatin remodelling by recruitment of the catalytic subunit of the SWI/SNF chromatin remodelling complex BRG1 (74). This link between chromatin remodelling and ERα-induced yH2AX was recently further illustrated by the impairment of APOBEC3B mediated C-to-U modifications (and thereby the need for BER), which resulted in decreased induction of activating histone marks H3K9ac and H3K4me3 (71). This reduction in APOBEC3B activity also led to reduced BRG1 recruitment, similar to the reduced BRG1 chromatin interactions at ERα-bindings sites we find upon FEN1 inhibition. Altogether, this is suggestive of a mode-of-action where FEN1 can, at least partly, decrease ERα-activity by altering its epigenetic landscape.

Besides these roles in epigenetic regulation, FEN1 can also modulate ERα-activity by stabilizing its chromatin interactions after activation. As we found that pre-treatment with proteasome inhibitor MG132 prevented the FEN1 inhibition-induced reduction of ERα-chromatin interactions

and ERα-chromatin interactions were not affected by FEN1 inhibition until the point of damage induction, we hypothesise that FEN1 can also dictate ERα-activity by regulating the stability of its chromatin binding after activation by E2 by altering its proteasome degradation. It seems plausible that FEN1 is being part of a fail-safe mechanism, only allowing the induction of relevant damage intermediates when FEN1 is part of the ERα complex. A similar mode-of-action has been reported for DNA-dependent protein kinase (DNA-PK), where DNA-PK inhibition by NU7441 resulted in the absence of γH2AX formation upon ERα-activation (71). Consequently, this favours a model wherein FEN1 blockade reduces ERα-responsive gene expression and ERα-driven cell proliferation by deregulating ERα-chromatin interactions after activation by E2, most likely due to improper induction/processing of DNA damage, which result in proteasome-mediated degradation of ERα.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer. 2015;136(5):E359-86.

2. Hayashi SI, Eguchi H, Tanimoto K, Yoshida T, Omoto Y, Inoue A, et al. The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application. Endocr Relat Cancer. 2003;10(2):193-202.

3. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, Korach KS, et al. International Union of Pharmacology. LXIV. Estrogen receptors. Pharmacol Rev. 2006;58(4):773-81.

4. Jordan VC, Murphy CS. Endocrine pharmacology of antiestrogens as antitumor agents. Endocrine reviews. 1990;11(4):578-610.

5. Katzenellenbogen BS, Miller MA, Mullick A, Sheen YY. Antiestrogen action in breast cancer cells: modulation of proliferation and protein synthesis, and interaction with estrogen receptors and additional antiestrogen binding sites. Breast cancer research and treatment. 1985;5(3):231-43.

6. Arpino G, De Angelis C, Giuliano M, Giordano A, Falato C, De Laurentiis M, et al. Molecular mechanism and clinical implications of endocrine therapy resistance in breast cancer. Oncology. 2009;77 Suppl 1:23-37.

7. Fabian CJ. The what, why and how of aromatase inhibitors: hormonal agents for treatment and prevention of breast cancer. International journal of clinical practice. 2007;61(12):2051-63.

8. Ali S, Coombes RC. Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer. 2002;2(2):101-12.

9. Osborne CK, Schiff R. Mechanisms of endocrine resistance in breast cancer. Annu Rev Med. 2011;62:233-47.

10. Michalides R, Griekspoor A, Balkenende A, Verwoerd D, Janssen L, Jalink K, et al. Tamoxifen resistance by a conformational arrest of the estrogen receptor alpha after PKA activation in breast cancer. Cancer Cell. 2004;5(6):597-605.

11. Stender JD, Nwachukwu JC, Kastrati I, Kim Y, Strid T, Yakir M, et al. Structural and Molecular Mechanisms of Cytokine-Mediated Endocrine Resistance in Human Breast Cancer Cells. Mol Cell. 2017;65(6):1122-35 e5.

12. Beelen K, Zwart W, Linn SC. Can predictive biomarkers in breast cancer guide adjuvant endocrine therapy? Nat Rev Clin Oncol. 2012;9(9):529-41.

13. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology. 1991;19(5):403-10.

14. Michalides R, van Tinteren H, Balkenende A, Vermorken JB, Benraadt J, Huldij J, et al. Cyclin A is a prognostic indicator in early stage breast cancer with and without tamoxifen treatment. Br J Cancer. 2002;86(3):402-8.

15. Abdel-Fatah TM, Russell R, Albarakati N, Maloney DJ, Dorjsuren D, Rueda OM, et al. Genomic and protein expression analysis reveals flap endonuclease 1 (FEN1) as a key biomarker in breast and ovarian cancer. Mol Oncol. 2014.

16. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature. 2002;415(6871):530-6.

17. Cardoso F, van't Veer LJ, Bogaerts J, Slaets L, Viale G, Delaloge S, et al. 70-Gene Signature as an Aid to Treatment Decisions in Early-Stage Breast Cancer. N Engl J Med. 2016;375(8):717-29.

18. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature. 2012;486(7403):346-52.

19. Thomas RS, Sarwar N, Phoenix F, Coombes RC, Ali S. Phosphorylation at serines 104 and 106 by Erk1/2 MAPK is important for estrogen receptor-alpha activity. J Mol Endocrinol. 2008;40(4):173-84.

20. Kok M, Holm-Wigerup C, Hauptmann M, Michalides R, Stal O, Linn S, et al. Estrogen receptor-alpha phosphorylation at serine-118 and tamoxifen response in breast cancer. J Natl Cancer Inst. 2009;101(24):1725-9.

21. Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, et al. Phosphorylation of estrogen receptor alpha serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast Cancer Res. 2005;7(5):R753-64.

22. Ballio A, Chain EB, De Leo P, Erlanger BF, Mauri M, Tonolo A. Fusicoccin: a New Wilting Toxin produced by Fusicoccum amygdali Del. 1964;203:297.
23. Olsson A, Svennelid F, Ek B, Sommarin M, Larsson C. A phosphothreonine residue at the C-terminal end of the plasma membrane H+-ATPase is protected by fusicoccin-induced 14-3-3 binding. Plant Physiol. 1998;118(2):551-5.
24. Kinoshita T, Shimazaki K. Analysis of the phosphorylation level in guard-cell plasma membrane H+-ATPase in response to fusicoccin. Plant Cell Physiol. 2001;42(4):424-32.
25. Osborne CK, Schiff R. Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. Breast. 2003;12(6):362-7.
26. Hurtado A, Holmes KA, Geistlinger TR, Hutcheson IR, Nicholson RI, Brown M, et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. Nature. 2008;456(7222):663-6.
27. Shou J, Massarweh S, Osborne CK, Wakeling AE, Ali S, Weiss H, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst. 2004;96(12):926-35.
28. Zhao W, Zhang Q, Kang X, Jin S, Lou C. AIB1 is required for the acquisition of epithelial growth factor receptor-mediated tamoxifen resistance in breast cancer cells. Biochem Biophys Res Commun. 2009;380(3):699-704.
29. Mohammed H, D'Santos C, Serandour AA, Ali HR, Brown GD, Atkins A, et al. Endogenous purification reveals GREB1 as a key estrogen receptor regulatory factor. Cell Rep. 2013;3(2):342-9.
30. Wang L, Yu Y, Chow DC, Yan F, Hsu CC, Stossi F, et al. Characterization of a Steroid Receptor Coactivator Small Molecule Stimulator that Overstimulates Cancer Cells and Leads to Cell Stress and Death. Cancer Cell. 2015;28(2):240-52.
31. Wang Y, Lonard DM, Yu Y, Chow DC, Palzkill TG, O'Malley BW. Small molecule inhibition of the steroid receptor coactivators, SRC-3 and SRC-1. Mol Endocrinol. 2011;25(12):2041-53.
32. Yan F, Yu Y, Chow DC, Palzkill T, Madoux F, Hodder P, et al. Identification of verrucarin a as a potent and selective steroid receptor coactivator-3 small molecule inhibitor. PLoS One. 2014;9(4):e95243.
33. Yi P, Wang Z, Feng Q, Chou CK, Pintilie GD, Shen H, et al. Structural and Functional Impacts of ER Coactivator Sequential Recruitment. Mol Cell. 2017;67(5):733-43 e4.
34. Lopez GN, Turck CW, Schaufele F, Stallcup MR, Kushner PJ. Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. The Journal of biological chemistry. 2001;276(25):22177-

82.
35. Kuo HY, Chang CC, Jeng JC, Hu HM, Lin DY, Maul GG, et al. SUMO modification negatively modulates the transcriptional activity of CREB-binding protein via the recruitment of Daxx. Proc Natl Acad Sci U S A. 2005;102(47):16973-8.
36. Zwart W, Theodorou V, Kok M, Canisius S, Linn S, Carroll JS. Oestrogen receptor-co-factor-chromatin specificity in the transcriptional regulation of breast cancer. EMBO J. 2011;30(23):4764-76.
37. Hurtado A, Holmes KA, Ross-Innes CS, Schmidt D, Carroll JS. FOXA1 is a key determinant of estrogen receptor function and endocrine response. Nature genetics. 2011;43(1):27-33.
38. Robinson DR, Wu YM, Vats P, Su F, Lonigro RJ, Cao X, et al. Activating ESR1 mutations in hormone-resistant metastatic breast cancer. Nature genetics. 2013;45(12):1446-51.
39. Fan M, Yan PS, Hartman-Frey C, Chen L, Paik H, Oyer SL, et al. Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. Cancer Res. 2006;66(24):11954-66.
40. He L, Zhang Y, Sun H, Jiang F, Yang H, Wu H, et al. Targeting DNA Flap Endonuclease 1 to Impede Breast Cancer Progression. EBioMedicine. 2016.
41. Panda H, Jaiswal AS, Corsino PE, Armas ML, Law BK, Narayan S. Amino acid Asp181 of 5'-flap endonuclease 1 is a useful target for chemotherapeutic development. Biochemistry. 2009;48(42):9952-8.
42. van Pel DM, Barrett IJ, Shimizu Y, Sajesh BV, Guppy BJ, Pfeifer T, et al. An evolutionarily conserved synthetic lethal interaction network identifies FEN1 as a broad-spectrum target for anticancer therapeutic development. PLoS genetics. 2013;9(1):e1003254.
43. McManus KJ, Barrett IJ, Nouhi Y, Hieter P. Specific synthetic lethal killing of RAD54B-deficient human colorectal cancer cells by FEN1 silencing. Proc Natl Acad Sci U S A. 2009;106(9):3276-81.
44. Exell JC, Thompson MJ, Finger LD, Shaw SJ, Debreczeni J, Ward TA, et al. Cellularly active N-hydroxyurea FEN1 inhibitors block substrate entry to the active site. Nature chemical biology. 2016;12(10):815-21.
45. Dorjsuren D, Kim D, Maloney DJ, Wilson DM, 3rd, Simeonov A. Complementary non-radioactive assays for investigation of human flap endonuclease 1 activity. Nucleic acids research. 2011;39(2):e11.
46. Bartella V, Rizza P, Barone I, Zito D, Giordano F, Giordano C, et al. Estrogen receptor beta binds Sp1 and recruits a corepressor complex to the estrogen receptor alpha gene promoter. Breast cancer research and treatment. 2012;134(2):569-81.

47. Fox EM, Davis RJ, Shupnik MA. ERbeta in breast cancer--onlooker, passive player, or active protector? Steroids. 2008;73(11):1039-51.
48. Palmieri C, Cheng GJ, Saji S, Zelada-Hedman M, Warri A, Weihua Z, et al. Estrogen receptor beta in breast cancer. Endocr Relat Cancer. 2002;9(1):1-13.
49. Andersson S, Sundberg M, Pristovsek N, Ibrahim A, Jonsson P, Katona B, et al. Insufficient antibody validation challenges oestrogen receptor beta research. Nature communications. 2017;8:15840.
50. de Boer AH, de Vries-van Leeuwen IJ. Fusicoccanes: diterpenes with surprising biological functions. Trends Plant Sci. 2012;17(6):360-8.
51. Sijbesma E, Skora L, Leysen S, Brunsveld L, Koch U, Nussbaumer P, et al. Identification of Two Secondary Ligand Binding Sites in 14-3-3 Proteins Using Fragment Screening. Biochemistry. 2017;56(30):3972-82.
52. Stevers LM, Sijbesma E, Botta M, MacKintosh C, Obsil T, Landrieu I, et al. Modulators of 14-3-3 Protein-Protein Interactions. Journal of medicinal chemistry. 2017.
53. Fayard E, Auwerx J, Schoonjans K. LRH-1: an orphan nuclear receptor involved in development, metabolism and steroidogenesis. Trends Cell Biol. 2004;14(5):250-60.
54. Venteclef N, Jakobsson T, Ehrlund A, Damdimopoulos A, Mikkonen L, Ellis E, et al. GPS2-dependent corepressor/SUMO pathways govern anti-inflammatory actions of LRH-1 and LXRbeta in the hepatic acute phase response. Genes Dev. 2010;24(4):381-95.
55. Annicotte JS, Chavey C, Servant N, Teyssier J, Bardin A, Licznar A, et al. The nuclear receptor liver receptor homolog-1 is an estrogen receptor target gene. Oncogene. 2005;24(55):8167-75.
56. Ross-Innes CS, Stark R, Holmes KA, Schmidt D, Spyrou C, Russell R, et al. Cooperative interaction between retinoic acid receptor-alpha and estrogen receptor in breast cancer. Genes Dev. 2010;24(2):171-82.
57. Mohammed H, Russell IA, Stark R, Rueda OM, Hickey TE, Tarulli GA, et al. Progesterone receptor modulates ERalpha action in breast cancer. Nature. 2015;523(7560):313-7.
58. Karmakar S, Jin Y, Nagaich AK. Interaction of glucocorticoid receptor (GR) with estrogen receptor (ER) alpha and activator protein 1 (AP1) in dexamethasone-mediated interference of ERalpha activity. The Journal of biological chemistry. 2013;288(33):24020-34.
59. Yang F, Ma Q, Liu Z, Li W, Tan Y, Jin C, et al. Glucocorticoid Receptor:MegaTrans Switching Mediates the Repression of an ERalpha-Regulated Transcriptional Program. Mol Cell. 2017;66(3):321-31 e6.

60. Voss TC, Schiltz RL, Sung MH, Yen PM, Stamatoyannopoulos JA, Biddie SC, et al. Dynamic exchange at regulatory elements during chromatin remodeling underlies assisted loading mechanism. Cell. 2011;146(4):544-54.

61. Peters AA, Buchanan G, Ricciardelli C, Bianco-Miotto T, Centenera MM, Harris JM, et al. Androgen receptor inhibits estrogen receptor-alpha activity and is prognostic in breast cancer. Cancer Res. 2009;69(15):6131-40.

62. Cahill MA, Ernst WH, Janknecht R, Nordheim A. Regulatory squelching. FEBS letters. 1994;344(2-3):105-8.

63. Lopez GN, Webb P, Shinsako JH, Baxter JD, Greene GL, Kushner PJ. Titration by estrogen receptor activation function-2 of targets that are downstream from coactivators. Mol Endocrinol. 1999;13(6):897-909.

64. De Amicis F, Thirugnansampanthan J, Cui Y, Selever J, Beyer A, Parra I, et al. Androgen receptor overexpression induces tamoxifen resistance in human breast cancer cells. Breast cancer research and treatment. 2010;121(1):1-11.

65. Szelei J, Jimenez J, Soto AM, Luizzi MF, Sonnenschein C. Androgen-induced inhibition of proliferation in human breast cancer MCF7 cells transfected with androgen receptor. Endocrinology. 1997;138(4):1406-12.

66. Cops EJ, Bianco-Miotto T, Moore NL, Clarke CL, Birrell SN, Butler LM, et al. Antiproliferative actions of the synthetic androgen, mibolerone, in breast cancer cells are mediated by both androgen and progesterone receptors. The Journal of steroid biochemistry and molecular biology. 2008;110(3-5):236-43.

67. Puc J, Aggarwal AK, Rosenfeld MG. Physiological functions of programmed DNA breaks in signal-induced transcription. Nat Rev Mol Cell Biol. 2017;18(8):471-6.

68. Luchnik AN. DNA conformational transitions induced by supercoiling control transcription in chromatin. Gene Regul Syst Bio. 2014;8:89-96.

69. Gilbert N, Allan J. Supercoiling in DNA and chromatin. Curr Opin Genet Dev. 2014;25:15-21.

70. Corless S, Gilbert N. Effects of DNA supercoiling on chromatin architecture. Biophys Rev. 2016;8(Suppl 1):51-64.

71. Periyasamy M, Patel H, Lai CF, Nguyen VT, Nevedomskaya E, Harrod A, et al. APOBEC3B-Mediated Cytidine Deamination Is Required for Estrogen Receptor Action in Breast Cancer. Cell Rep. 2015;13(1):108-21.

72. Kangaspeska S, Stride B, Metivier R, Polycarpou-Schwarz M, Ibberson D, Carmouche RP, et al. Transient cyclical methylation of promoter DNA. Nature. 2008;452(7183):112-5.

73. Gong C, Fujino K, Monteiro LJ, Gomes AR, Drost R, Davidson-Smith H, et al. FOXA1 repression is associated with loss of BRCA1 and increased promoter

methylation and chromatin silencing in breast cancer. Oncogene. 2015;34(39):5012-24.

74. Lee HS, Park JH, Kim SJ, Kwon SJ, Kwon J. A cooperative activation loop among SWI/SNF, gamma-H2AX and H3 acetylation for DNA double-strand break repair. EMBO J. 2010;29(8):1434-45.