FOXO factors and breast cancer: outfoxing endocrine resistance

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Abstract
The majority of metastatic breast cancers cannot be cured and present a major public health problem worldwide. Approximately 70% of breast cancers express the estrogen receptor, and endocrine-based therapies have significantly improved patient outcomes. However, the development of endocrine resistance is extremely common. Understanding the molecular pathways that regulate the hormone sensitivity of breast cancer cells is important to improving the efficacy of endocrine therapy. It is becoming clearer that the PI3K–AKT–forkhead box O (FOXO) signaling axis is a key player in the hormone-independent growth of many breast cancers. Constitutive PI3K–AKT pathway activation, a driver of breast cancer growth, causes down-regulation of FOXO tumor suppressor functions. This review will summarize what is currently known about the role of FOXOs in endocrine-resistance mechanisms. It will also suggest potential therapeutic strategies for the restoration of normal FOXO transcriptional activity.

Key Words
FOXOs, breast cancer, endocrine resistance, PI3K–AKT pathway, post-translational modifications

Introduction
Breast cancer is a leading cause of female death worldwide. Each year, almost 1.4 million women are diagnosed with breast cancer, and the disease will be responsible for 450,000 deaths (Siegel et al. 2011). This review will highlight the importance of forkhead box O (FOXO) activity in the development of endocrine resistant breast cancer. FOXO transcription factors are key regulators of gene expression in numerous physiological and pathological processes. FOXO nuclear exclusion is a key feature of breast cancer cells transformed by oncogenic PI3K–AKT signaling (Zhang et al. 2011, Fig. 1). It is also becoming clear that aberrant FOXO activity promotes a number of characteristic features of hormone-independent breast cancer growth, including altered estrogen receptor (ER) function. Although breast cancer cells express multiple FOXOs, most studies have focused on the FOXO3A isoform. FOXOs are subject to extensive post-translational regulation, and targeting these processes may provide therapeutic strategies to overcome resistance.

FOXO gene family
The FOXO genes encode for the O-subfamily of proteins that belong to the larger family of forkhead transcription factors. Forkhead transcription factors are named after the Drosophila melanogaster forkhead gene (fkh; Weigel et al. 1989), and to date, ~200 members have been identified; in species that range in complexity from Saccharomyces cerevisiae that has four members (Wijchers et al. 2006) to human with 43 members (Katoh & Katoh 2004). These proteins are characterized solely by the presence of a highly conserved DNA-binding domain (DBD) called the
FOX, forkhead (FKD) (Weigel & Jackle 1990), or alternatively the winged-helix domain (Gajiwala & Burley 2000). On the basis of minor sequence variation within the FOX-domain, FOX family members are divided into sub-families, that are each designated with a different letter (currently there are 17 subfamilies, ranging from A to Q). Outside of the FOX-domain, there is little shared homology, and the structure and function of FOX proteins is found to be incredibly diverse. As is the case for members of all large transcription factor families, FOX genes been implicated in the transcriptional regulation of an extensive range of biological processes. In the case of FOX genes, such reported functions include regulation of cell proliferation, differentiation, DNA-repair, apoptosis, metabolism, and also immune-regulation (Lam et al. 2006, Wijchers et al. 2006, Ziegler 2006, Laoukili et al. 2007).

In mammalian species, there are four members of the FOXO gene family. Three of the members exhibit a high degree of sequence homology: FOXO1 (previously called FKHR), FOXO3A (FKHRL1), and FOXO4 (AFX). The fourth member of the family, the FOXO6 gene, exhibits major structural differences compared with the other three family members.

In keeping with a high degree of sequence homology between DBDs shared by members of the same forkhead subfamily, all FOXO proteins share the same DNA-binding specificity, recognizing gene regulatory elements that share a central core DNA motif consisting of the nucleotides TTGTTTAC. Profiling of genome-wide FOXO DNA-binding has been mostly been performed in the study of the immune-system, with FOXO cistrome having been determined for B-cells and macrophages. (Fan et al. 2010, Lin et al. 2010, Litvak et al. 2012). These studies are somewhat limited in their ability to determine direct downstream effects of FOXO activity. However, Eijkelenboom et al. (2013) used an inducible cell-culture system (DLD1 colon carcinoma cells) to measure the direct effects of FOXO3A activation. Analysis of the FOXO3A cistrome reveals that the factor functions predominantly as a classical transcriptional activator. Furthermore, a significant part of FOXO3A gene-regulation is found to proceed through enhancer regulatory elements.

FOXOs are expressed throughout the human body, and there is broad overlap in their expression patterns. However, each factor is uniquely enriched within particular tissue types: FOXO1 in adipose and liver tissue, FOXO3A in the brain, and FOXO4 in skeletal muscle. Uniquely, the FOXO6 gene appears to be expressed almost exclusively in adult brain (Furuyama et al. 2000). Despite tissue specific enrichment, there clearly exists some level
of functional redundancy between FOXO factors, with the net activity of multiple co-expressed FOXOs having an accumulative effect on gene expression. This is highlighted by observations drawn from compound gene-knockout experiments. Initially, such studies that utilized single- or double-knockouts to disrupt one or two FOXO factors did not yield the expected tumor-prone phenotype (see the section covering ‘Tumor suppressor function of FOXOs’). However, broad somatic-cell deletion of all three FOXO factors yields the full tumor phenotypes characterized by thymic lymphomas and hemangiomas (Castrillon et al. 2003, Hosaka et al. 2004, Paik et al. 2007). Strikingly, despite widespread expression of FOXOs throughout the body, carcinogenesis is restricted to thymocytes and endothelial-derived cell lineages in this model. At present it is not clear why this is the case. Interestingly, hemangiomas are a characteristic feature of Cowden’s disease, which is caused by germline inactivation of the phosphatase and tensin homolog (PTEN) gene, leading to hyperactivity of the PI3K–AKT pathway. The mechanism by which constitutive PI3K–AKT pathway activation causes down-regulation of FOXO activity is discussed in more detail below.

**Tumor suppressor function of FOXOs**

Interestingly, FOXO1, 3, and 4 were implicated in cancer biology immediately upon their discovery. FOXO1 was discovered as a fusion protein (the fusion partner being either the PAX3 or PAX7 DBD), the product of a chromosomal translocation event within the cells of pediatric alveolar rhabdomyosarcoma (Galili et al. 1993, Sublett et al. 1995, Schmitt-Ney & Camussi 2015). FOXO3A and FOXO4 were discovered as fusion partners with the mixed-lineage leukemia gene in acute myeloid leukemias (Parry et al. 1994). However, experiments that attempted to recapitulate a tumor phenotype by overexpressing these fusion proteins were largely unsuccessful (Lagutina et al. 2002), suggesting instead that loss of FOXO function might be the crucial step in mediating carcinogenesis. It is now clear that FOXOs are tumor-suppressors, and suppression of their activity contributes to a number of malignant processes. For instance, FOXOS are well characterized as transcriptional regulators of a large number of genes implicated in carcinogenesis. These include: i) cell-cycle regulatory components such as p27kip1 (CDKN1B; Dijkers et al. 2000); ii) pro-apoptotic proteins like TRAIL (TNFSF10; Modur et al. 2002); iii) DNA repair enzymes such as GADD45 (Kobayashi et al. 2005); iv) detoxification pathway enzymes such as manganese superoxide dismutase 2 (Adachi et al. 2007); and v) genes involved in autophagy and the maintenance of cellular organelle and protein homeostasis (Webb & Brunet 2014).

**Breast cancer and hormone dependence**

There are four recognized major molecular subtypes of breast cancer, which are classed according to gene expression profiling (Perou et al. 2000). The vast majority of breast cancers (~70%) are ER positive (ER+). They belong to either the Luminal-A group (mostly ER+ and histologically low grade); or the luminal-B group (mostly ER+ and often higher grade). Endocrine therapy is the most efficacious treatment for these forms of breast cancer. The human epidermal growth factor receptor 2 (HER2, expressed by the ERBB gene)-amplified group, have been a great clinical success due to the development of effective HER2-targeting therapies (Bergamaschi et al. 2006, Chin et al. 2006). In contrast, the triple-negative breast cancer group (also known as basal-like breast cancers), so-called because they lack expression of ER, progesterone receptor (PR), and HER2, are a patient group who currently only have chemotherapy options (Perou 2011).

**Endocrine therapy**

The growth and development of normal breast tissue is governed by the signaling action of the ovarian hormones, 17β-estradiol (E2, the predominant estrogen) and progesterone, which signal through the nuclear receptors ER and PR respectively. Two major isoforms of ER exist, ERα and ERβ, which are encoded by separate genes, ESR1 and ESR2 (ER1 and ER2) respectively (reviewed by Jia et al. (2015)). The PR also exists as two isoforms, PR-A and PR-B, the products of transcription from two alternative promoters of the PR (PGR) gene (reviewed by Jacobsen & Horwitz (2012)). Immunohistochemical analysis of normal breast tissue, obtained from pre-menopausal women, shows that ERα, PR-A, and PR-B are expressed within the inner luminal epithelial layer of the acini and intralobular ducts, and also the myoepithelial layer of the interlobular ducts (Li et al. 2010). ERβ exhibits a more widespread expression pattern, being found within the epithelial, myoepithelial, and stromal cells of both acini and ducts. Normal growth of female breast tissue occurs during puberty, during the menstrual cycle and also during pregnancy; with E2 regulating the process of ductal elongation and branching (Deroo et al. 2009), and progesterone regulating side-branching, and lobular development (Conneely et al. 2007). The expression levels...
of ER and PR isoforms are also dynamically regulated during these processes (Arendt & Kuperwasser 2015). The classic function of ligand activated ERα is transcriptional regulation of hundreds of genes involved in processes such as proliferation, differentiation, survival, invasion, many of which are particularly relevant for cancer biology.

Seventy percent of all breast cancers exhibit detectable expression of the ERα and/or the PR (the role of PR is reviewed in depth by Braken (2013) and Seton-Rogers (2015)). A plethora of studies have demonstrated that ERα-signaling functions as a major driver of breast cancer tumorigenesis; promoting cancer-cell proliferation, survival, and invasive behavior (Osborne & Schiff 1990). In contrast ERβ is expressed in 50% of breast cancers (Borgquist et al. 2008), and it appears to possess ERα-independent function in breast cancer; but it remains much less well characterized, and studies have yielded conflicting results regarding its clinical significance (reviewed by Burns & Korach (2012)). In this review, ER will refer to the ERα isoform, unless otherwise stated.

Clinically, when compared with the behavior of ER-negative (−) cancers, affected patients are more likely to present with indolent disease, bone metastases, and late disease reoccurrence (Blanco et al. 2011). In contrast ERβ is expressed in 50% of breast cancers (Borgquist et al. 2008), and it appears to possess ERα-independent function in breast cancer; but it remains much less well characterized, and studies have yielded conflicting results regarding its clinical significance (reviewed by Burns & Korach (2012)). In this review, ER will refer to the ERα isoform, unless otherwise stated.

Currently, three broad classes of endocrine therapy are commonly used to treat ER+ breast cancers: i) selective ER modifiers, e.g., tamoxifen, which binds directly to the ER and inhibits its transcriptional activity; ii) selective ER down-regulators, e.g., fulvestrant, which bind to the ER and induce its degradation; and iii) aromatase inhibitors (AIs), e.g., letrozole, anastrozole (both non-steroidal/reversible) and exemestane (steroidal/irreversible), which act to inhibit aromatase enzymes expressed throughout peripheral tissues and the tumor itself, thus reducing the production, and therefore circulating levels of estrogen. Endocrine therapy has been successful, and the outcome for millions of breast cancer patients has been significantly improved over the past 30 years (Bliss et al. 2012).

**Endocrine resistance**

Although long-term remission is possible, a significant proportion of patients will develop some form of resistance to endocrine therapy. Four commonly occurring clinical scenarios are as follows: i) the breast cancer initially responds positively to endocrine therapy, but eventually acquires resistance. Often, in such cases, additional positive responses can be temporally gained from the use of alternative endocrine therapies; however, the cancer eventually evolves resistance to all therapy; ii) disease progression following an initial response to endocrine therapy, followed by a renewed response to the same therapy when administered years later; iii) de novo resistance to all endocrine therapies, or the acquisition of this resistance soon after the patient begins adjuvant treatment; and iv) de novo resistance to some, but not all therapies. There is pre-clinical data to suggest that de novo and acquired forms of resistance share common pathways (see section discussing 'Preclinical studies'). However, some clinical scenarios also support the existence of drug (or class of drug)-specific forms of resistance.

**FOXOs in breast cancer**

FOXO3A appears to be a key isoform in mediating hormone-independent breast cancer growth. FOXO3A overexpression significantly inhibits the growth of breast tumors in vitro and in animal models (Hu et al. 2004, Yang et al. 2008, Zou et al. 2008), and also negatively impacts upon angiogenesis; a process required for malignant tumor growth, invasion, and metastasis (Potente et al. 2005). Hu et al. (2004) observed that cytoplasmic FOXO3A staining correlated with the expression of inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ), raised phospho-AKT and overall poorer patient survival. Gargini et al. (2015) showed that FOXO proteins, in concert with Bim (a member of the BCL2 protein family), mediate the PI3K–AKT driven cancer stem-cell phenotype within three commonly used breast cancer cell-lines (MDA-MB-231, MCF7-Ras, and MCF10A) (Gargini et al. 2015). Furthermore, Lv et al. (2013) found that C-terminus of Hsc70-interacting protein induced apoptosis resistance to chemotherapeutics in normal and breast cancer cells, specifically by modulation of the PI3K–AKT–FOXO3A–Bim signaling axis (Lv et al. 2013).

**Interaction between FOXOs and ER signaling**

The function of FOXO3A as a tumor repressor appears to be dependent on the co-expression of ER. Sisci et al. (2013) observed that overexpression of FOXO3A induces a decrease in the malignant behavior (motility, invasiveness, and anchorage-independent growth) of an in vitro ER+ cancer cell model, while eliciting the opposite effect in ER− (or ER-silenced) cell-lines. Immunohistochemical analysis performed in the same study revealed that on the
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Interestingly, ER has also shown to interact with a context, exhibit co-activator or co-repressor functions on estrogen-responsive DNA regulatory elements (Schuur et al. 2001, Zhao et al. 2001, Zou et al. 2008). Interestingly, ER has also shown to interact with a diverse number of FOX proteins including FOXE1 (Park et al. 2012), FOXP1 (Halacli & Dogan 2015), and FOXA1 (Wright et al. 2014); suggestive of a conserved mechanism of association between the receptor and the wider forkhead family. The significance of the ER–FOX interaction is highlighted by the role of FOXA1 as a pioneer factor, which facilitates ER DNA-binding at cis-regulatory elements within heterochromatin. Indeed, FOXA1 expression is positively correlated with a good prognosis of ER+ breast cancers, probably because FOXA1 expression is indicative of a functional ER (reviewed in depth by Tokunaga et al. (2014)).

Currently, no data exists to whether the potential pioneer activity FOXOs (reviewed by Lalmansingh et al. (2012)) can also modulate ER DNA-binding in breast cancer. However, Morelli et al. (2010) demonstrated that FOXO3A, functioning as a classical co-repressor of ER, could exert a protective role in ER+ breast tumors. In agreement with this observation, targeted inhibition of AKT isoform 2 activity in these cells inhibited ER-directed transcription via FOXO3A activation.

Other molecular mechanisms of endocrine resistance

HER2 amplification is a well-established marker for endocrine resistance (Arpino et al. 2004). A meta-analysis showed that HER2+ metastatic breast cancers are less responsive to all forms of endocrine therapy (De Laurentits et al. 2005). The molecular mechanisms of HER2 mediated hormone resistance are becoming clearer. HER2-containing heterodimers, particularly HER2–HER3 heterodimers, induce PI3K–AKT pathway activation (Tzahar et al. 1996), and HER2 amplification is positively correlated with AKT activity in breast carcinomas (Stal et al. 2003, Zhou et al. 2004, Tokunaga et al. 2006). However, <10% of ER+ breast cancers are also HER2+. Thus, the underlying resistance mechanism(s) for the majority of ER+ breast cancers remains to be elucidated.

Pre-clinical studies have implicated growth factor signaling pathways in the estrogen-independent activation of ER. In addition to HER2, tyrosine receptor kinases implicated in the development of endocrine resistance include: epidermal growth factor receptor (EGFR), insulin-like growth factor receptor 1 (IGF1R), insulin receptor, receptor originated from nantes, and fibroblast growth factor receptor 1 (Frogne et al. 2009, McClaine et al. 2010, Turner et al. 2010, Fox et al. 2011). All of these receptors converge on the PI3K–AKT signaling pathway, hyper-activation of which promotes the acquired estrogen-independent growth of ER+ breast cancer cells (Miller et al. 2010). They also activate the rat sarcoma viral oncogene homolog (RAS)/ERK signaling pathway, contributing to estrogen-independent ER activation; even in cultured breast cancer cells that are grown in the presence of tamoxifen. Under these conditions, ERK phosphorylates ER at serine 118 (Ser118), a site that is normally phosphorylated in response to estrogen-stimulation; itself an ERK-independent mechanism (Kato et al. 1995, Bunone et al. 1996).

In addition to perturbed growth factor signaling, another mechanism of endocrine resistance involves the inhibition of transcriptional co-activators, such as steroid receptor co-activator 1, that interacts with ER (Shang & Brown 2002). Decreased interactions between ER and the nuclear receptor co-repressor can also lead to tamoxifen resistance (Lavinsky et al. 1998). Indeed, Shang et al. (2000) showed that tamoxifen recruits co-repressors to ER-responsive gene promoters, but not co-activators.

Autophagy, the process by which a cell degrades and recycles damaged or unrequired organelles, has also recently been revealed to play a central role in endocrine resistance. The unfolded protein response (UPR) is an evolutionary conserved stress-responsive pathway, activated when unfolded or misfolded proteins accumulate within the cells endoplasmic reticulum. The master regulator of ubiquitin specific peptidase (USP) signaling is the protein chaperone glucose-regulated protein 78 (GRP78), which integrates signals from several pathways to concurrently inhibit stress-induced apoptosis and stimulate a prosurvival autophagic response (Cook et al. 2012, Clarke & Cook 2015). Elevated GRP78 expression has been observed within all breast cancer molecular subtypes, as compared with the levels of expression in normal breast tissue (Cook et al. 2012). Within breast cancer cells GRP78 controls the autophagic response via...
signaling through the 5'-AMP-activated protein kinase and mammalian target of rapamycin (mTOR) signaling pathways (Cook et al. 2012). Cook et al. (2014) observed that treatments such as tamoxifen and fulvestrant can stimulate the prosurvival UPR and autophagy signaling in breast cancer cells.

De-regulation of PI3K–AKT signaling in breast cancer

Constitutive activation of PI3K–AKT signaling is recognized as a major driver of cancer progression. Dysregulation of the pathway has been implicated in an extensive range of human malignancies; including breast, ovarian, head and neck, and colorectal cancer. It is also a central player in the mechanisms leading to endocrine resistance of breast cancer (Tokunaga et al. 2014). Several causal mechanisms have been reported; all consequent to DNA mutations in genes encoding principal components of the pathway (see Fig. 1). The exact nature and frequency of mutation is unique to each type of cancer (Thorpe et al. 2015).

The PI3K–AKT pathway is extremely well characterized and has been reviewed in considerable detail by others (Fruman & Rommel 2014, Martini et al. 2014, Thorpe et al. 2015). Briefly, the PI3K protein/lipid kinases are grouped into classes I (which itself is further divided into IA and IB), II, or III according to their structure and specific substrate specificity. The best characterized of these are class IA PI3Ks, which are heterodimeric; each composed of a catalytic subunit (p110) and a regulatory adaptor protein (p85). They function to transduce input signals from a wide variety of upstream sources, such as growth factor/hormone stimulated-receptor tyrosine kinases (RTKs), G-protein coupled receptors, and also oncogenic signaling molecules such as RAS.

The p110 catalytic subunit has three isoforms: p110α, p110β, and p110δ that are encoded for by three individual genes; PIK3CA, PIK3CB, and PIK3CD respectively. The p85 regulatory subunit also has three isoforms: p85α, p55α, and p110α which are also transcribed from three different genes; PIK3R1, PIK3R2, and PIK3R3 respectively.

Following growth factor/hormone activation of a RTK, the p85 subunit binds via its SH2-domain to the phosphorylated-protein motifs of the RTK. This recruitment has the effect of lifting p85-mediated inhibition of the p110 subunit, allowing p85 to catalyze conversion of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3). The tumor suppressor gene PTEN encodes a phosphatase that removes phosphate from PIP3; and thus functions as a negative regulator of the PI3K–AKT pathway. Another phosphatase with tumor-suppressor function is inositol polyphosphate-4-phosphatase, type II (INPP4B), the gene product of which also negatively regulates the pathway, by depleting cellular levels of PIP2. Upon generation, PIP3 functions as a secondary messenger, attaching to the pleckstrin-homology-domain of proteins such as v-akt murine thymoma viral oncogene homolog 1 (AKT) and pyruvate dehydrogenase kinase isoform 1. PIP3 binding to AKT targets this Ser/threonine (Thr) kinase to the cellmembrane where it can be activated by phosphorylation at Ser473 by mTOR, and then at Thr308 by phosphoinositol dependent kinase 1. AKT activation initiates cascades of downstream phosphorylation events that impact on several aspects of cellular behavior that are of relevance to cancer; including cell cycle progression, enhanced chemotherapeutic resistance, elevated cell metabolism, increased resistance to hypoxia, and tumor metastasis.

According to The Cancer Genome Atlas PIK3CA is frequently mutated gene in breast cancer, present at a frequency of 49 and 32% in ER+ luminal A and B subtypes; 42% of HER2-enriched, and 7% of basal-like breast cancer subtypes. Other mutations impacting upon PI3K–AKT pathway activity, such as PTEN mutation/loss and INPP4B loss were also commonly found in all four subtypes (Cancer Genome Atlas N 2012). Recent studies have reported that alterations to the AKT3 gene (e.g. the MAGI–AKT3 gene fusion), leading to constitutive AKT activation, also occur in some ER+ and triple-negative breast cancers (Kirkegaard et al. 2010, Banerji et al. 2012, O’Hurley et al. 2014).

The PI3K–AKT pathway can also be activated via signaling crosstalk with the ER. The ER binds to the p85 regulatory subunit of PI3K, in an estrogen-dependent manner, resulting in downstream activation of AKT (Simoncini et al. 2000). The treatment of breast cancer cells with E2 stimulates cellular proliferation via PI3K–AKT pathway activation (Lee et al. 2005). Interestingly, this activity was found to be ERβ-dependent, while ERα appears not to play a role.

The role of PI3K–AKT–FOXO signaling in breast cancer

Mechanisms of FOXO regulation Cancer associated FOXO dysregulation activity can arise from genetic mutations (chromosomal translocation; described above) or through disruption of their transcriptional activity. In-keeping with the pleiotropic functioning of FOXOs, their activity is subjected to a variety of different regulatory mechanisms depending upon the cellular
context. These can be broadly divided into those which are exerted at the transcriptional level, and those which involve post-translational modifications.

Transcriptional regulation of FOXOs Recent studies have suggested that the expression of FOXO1 and FOXO3A is subjected to modulation at the gene-promoter level by the transcription factor E2F1. Nowak et al. (2007) found that E2F1, in a glioblastoma in-vitro model, induced the transcription of both of FOXO1 and FOXO3A. Both gene promoters contained evolutionary conserved E2F1 binding-sites, and therefore, they are highly likely to be direct targets of E2F1. Interestingly, the ER regulated E2F1 expression has been associated with tamoxifen resistance in ER+ breast cancer cells (Montenegro et al. 2014).

FOXO gene transcription is regulated by nutrient intake and insulin signaling in an in vivo insulin resistance model. Reduced nutrient availability in an artificially induced insulin-resistance state, stimulated transcription of FOXO1, FOXO3A, and FOXO4 in rat hepatocytes (Imae et al. 2003). Recently, observations of an in vivo model of fasted blood glucose revealed the CAMP–protein kinase A (PKA) stimulated transcriptional co-activator p300 induces transcription of FOXO1 (Wondisford et al. 2014).

Post-translational regulation of FOXO activity Post-translational protein modification of FOXOs has been extensively studied, and it is apparent the function of these factors is subjected to multiple layers of regulation. Although, apparently complex, FOXO post-translational regulation appears to operate by modulating two complementary mechanisms: i) altered FOXO subcellular localization and ii) modulation of FOXO DNA-binding and/or association with other DNA-binding proteins. The net action of these processes can profoundly impact upon FOXO-regulated gene transcription. Outlined below are the most extensively characterized FOXO protein modifications:

Phosphorylation Brunet et al. (1999) discovered that FOXOs were subject to regulation by the PI3K–AKT signaling pathway. They demonstrated that AKT possesses high binding affinity for the FOXO3A protein motif RXRXXS/T, and upon binding to the FOXO factor, catalyzes the phosphorylation of three specific amino-acids: Thr32, Ser253, and Ser315. All FOXO proteins, with the exception of FOXO6, contain evolutionary conserved variants of these phosphorylation sites (see Fig. 2). The phosphorylated-FOXO3A isoform is subsequently translocated out of the nucleus and sequestered in the cytoplasm. Each of the three phosphorylation events contributes to this translocation by a different mechanism. Phosphorylated Thr32 is directly involved in regulating the binding of 14-3-3 chaperone proteins (Brunet et al. 2002). The residue Ser253 resides within a nuclear localization sequence (NLS), and the addition of a negatively charged phospho-group to this basic sequence physically disrupts the signal, and inhibits the re-entry of cytoplasmic FOXOs into the nucleus. In contrast, Ser315 phosphorylation results in the unmasking of a nuclear export sequence (NES), which mediates physical association with the exportin chromosome region maintenance 1 (CRM1; also known as exportin-1 or Xpo1 protein). Thus, cultured cells treated with the CRM1 inhibitor leptomycin exhibit predominant FOXO nuclear localization and this inhibition of nuclear export is independent of FOXO phosphorylation status (Brunet et al. 2002). The importance of the NES is also highlighted by the behavior of FOXO6, which is the only FOXO to lacks the NES, and unlike the other three subfamily members; it is predominantly a nuclear protein (Jacobs et al. 2003).

Other inhibitory kinases The conserved RXRXXS/T protein motif also serves as a binding-substrate for several other kinases such as PKA, PKC, and serum and glucocorticoid-induced kinase (SGK; Pearce et al. 2010). Thus, several growth factors signaling converge on the FOXOs to negatively regulate their function. Interestingly, in vitro experiments utilizing dominant-negative mutant forms of AKT or SGK, inhibiting their respective pathways, both lead to nuclear accumulation of FOXOs; suggesting a non-redundant role for at least some kinases in targeting FOXOs to the cytoplasm (Brunet et al. 2001). It remains unclear why such redundancy exists, but it may be the case that the activity of each kinase is specific to a particular physiological/cellular context.

ERK phosphorylates FOXO3A on the amino-acid residues: Ser294, Ser344, and Ser425, and similarly to AKT-mediated phosphorylation, these promote the cytoplasmic sequestration of the factor. However, an additional consequence of these modifications is they target the FOXO3A for proteasomal degradation. It has been proposed that this may occur due to increased interaction with the E3-ligase, MDM2 (Yang et al. 2008; see ‘Ubiquitination’ section).

The IκB kinase (IKK), a component of the NFκB pathway, phosphorylating Ser644, appears to inhibit FOXO3A activity in a similar manner (Hu et al. 2004). This may be of particular relevance to breast tumorigenesis, as constitutive activation of the kinase has been linked to the disease. Furthermore, Chen et al. (2015a)
have recently shown that activated-IKKβ is required to sustain the 'stemness' of breast cancer stem cells.

Casein kinase 1 (CK1) plays a role in cell differentiation and its de-regulation has been shown to contribute to cancer development. CK1 phosphorylates the amino acid residues Ser318 and Ser321 of the FOXO protein, but only following the prior phosphorylation of Ser315 by SGK. *In vitro* experiments have shown that these phosphorylation events occur in a specific hierarchical fashion: phosphorylation by SGK creates the consensus recognition sequence motif for CK1 phosphorylation (S/T(P)XXS/T) at Ser318, which in turn creates a second consensus sequence for the CK1 catalysed phosphorylation of Ser321. These modifications are generally believed to influence the rate of nuclear export of FOXOs, potentially through differential interaction with components of the nuclear export complex, though this remains to be proven (Rena et al. 2002).

**Dual specificity tyrosine phosphorylated and regulated kinase 1A (DYRK1A)**

Dual specificity tyrosine phosphorylated and regulated kinase 1A (DYRK1A), a Ser/Thr kinase, catalyses the phosphorylation of Ser325, in a manner that appears to be independent of SGK-and-CK1 kinase activity, and this acts to induce the nuclear accumulation of FOXOs. DYRK1A mediated modifications also appears to occur independently of PI3K–AKT activity. However, phosphorylation of Ser325 does seem to exhibit a synergistic effect when occurring in concert with SGK-and-CK1-mediated phosphorylation. This also may occur as the result of further enhancement of the association of FOXOs with the nuclear export complex; though this also needs to experimentally verified (Gao et al. 2012).

**Co-factor interactions** The phosphorylation-directed binding of 14-3-3 proteins can also negatively impact the ability of FOXOs to interact with other transcriptional regulatory proteins. FOXOs participate in several known transcriptional complexes, such as: i) ER (Schuur et al. 2001, Zhao et al. 2001); ii) p53 (Wang et al. 2008); and iii) CREB binding protein (CBP)/p300. In the case of CBP/p300, the phospho-Thr32/Ser253-isoform has been shown to have lost the ability to physically associate with these transcriptional co-activator proteins (Wang et al. 2009, 2012a), and thus, this modification negates the transcriptionally favorable chromatin remodeling that this protein association generally promotes. CBP/p300 also functions to acetylate the lysine residues within the FOX-domain, thus...
repressing FOXO DNA-binding activity (see ‘FOXO acetylation’ section).

**Activation by kinases** There are some reported phosphorylation events that have a positive impact on FOXO activity. For instance, protein kinases such as MST1 (Lehtinen et al. 2006), JNK (Essers et al. 2004), and CDK1 (Yuan et al. 2008), promote nuclear accumulation of FOXOs, generally in response to signals arising from cellular stress (e.g. oxidative stress).

**FOXO acetylation** The acetylation of FOXOs changes their transcriptional activity. The acetylation-status of a given FOXO is the product of an equilibrium between the action of histone deacetylases (HDACs), such as silent information regulator 1 (SIRT1) and SIRT2; and histone acetyltransferases (HATs), such as CBP/p300 (Daitoku et al. 2011).

Acetylation by HATs deactivates FOXOs through two sequential steps. First, when FOXO are acetylated at three conserved lysine residues (corresponding to Lys242, Lys245, and Lys262 of FOXO1; see Fig. 2), their DNA binding capacity is dramatically reduced (van der Heide & Smidt 2005, Matsuzaki et al. 2005). Secondly, the acetylated isoform of FOXO has increased potential as an AKT substrate, and it is thus ‘sensitized’ to PI3K–AKT induced translocation out of the nucleus.

The deacetylation of FOXOs is performed by both class I HDACs and the class III, NAD-dependent histone HDACs (members of the Sirtuin protein family). The effect of SIRT1 activity on the transcription regulatory function of FOXOs varies for different subsets of FOXO-regulated genes. It appears that some FOXO-regulated genes, especially genes related to cell-cycle control and senescence, are up-regulated; while pro-apoptotic genes are down-regulated. For instance, SIRT1-mediated acetylation increases the ability of FOXOs to induce cell-cycle arrest and promote resistance to oxidative stress (e.g. up-regulation of p27<sup>KIP1</sup> and GADD45; Daitoku et al. 2004, Kobayashi et al. 2005). In contrast, SIRT1 negatively regulates the FOXO-induced expression of pro-apoptotic genes. Wang et al. (2015), investigating the role of SIRT1 in an in vitro oxidative stress-induced apoptosis model, observed that a decrease in FOXO3A-acetylation accompanied a corresponding increase in ubiquitination (see ‘Ubiquitination’ section). The subsequent degradation of the protein leads to down-regulation of FOXO3A-regulated pro-apoptotic genes. Also, in an in vitro mouse myoblast oxidative stress model, the expression of FOXO1, FOXO3A, and FOXO4 were all found to be indispensable for anti-apoptotic effects of SIRT1 activity (Hori et al. 2013). The underlying mechanisms responsible for this selective effect of SIRT1 on FOXO3A-regulated genes are not understood.

Other members of SIRT family like SIRT2 and SIRT3 can also interact and deacetylate FOXOs (Jacobs et al. 2008, Sundaresan et al. 2009, Wang et al. 2012b). Also, class I HDAC4/5 has been observed to recruit the deacetylase activity of HDAC3 to FOXO, which results in activation of FOXO-regulated genes (Mihaylova et al. 2011).

**Ubiquitination** Expression levels of FOXOs are also governed by ubiquitin-regulated proteolysis. The PI3K–AKT, NFκB, and ERK signaling pathways have all been implicated in regulating this process. The E3-ubiquitin ligase S-phase kinase-associated protein 2 (SKP2) is recruited by the AKT-phosphorylated Ser256 of cytoplasmic FOXO1 and proteasomal-mediated degradation of FOXO1 can be rapidly induced in response to PI3K–AKT pathway stimulation (Huang et al. 2005). In contrast, the NFκB appears to be the relevant signaling pathway for targeting FOXO3A to the proteasome. Hu et al. (2004) found that the IKKβ mediated phosphorylation of FOXO3A at Ser664, targeted the protein for ubiquitin-dependent proteolysis; however, as or yet, the ubiquitin responsible remains to be identified.

Another E3-protein ligase MDM2 is activated by ERK-pathway activity, to induce both mono- and poly-ubiquitination of FOXOs. Interestingly, these two modifications of FOXO have very different effects on their function. The monoubiquitinated isoform of FOXO cannot be acetylated, thus the modification maintains the factor in an in-active, but ‘transcriptionally poised’ state (van der Horst et al. 2006, Brenkman et al. 2008). It is thought that this form of the protein can be rapidly activated, in response to certain stress stimuli (e.g. oxidative stress), most likely through the action of the deubiquitinating enzyme USP7/herpesvirus-associated ubiquitin-specific protease (van der Horst et al. 2006). In contrast, polyubiquitination targets FOXO proteins for proteasome-mediated degradation, and so, long-term inhibition of FOXO transcriptional activity (Fu et al. 2009). Activation of the PI3K–AKT pathway has been implicated in stimulating FOXO proteolysis in several different cellular contexts (Matsuzaki et al. 2003, Plas & Thompson 2003, Aoki et al. 2004). In addition to AKT, other kinases such as IKK and ERK have been observed to promote the proteolysis of FOXO3A (Hu et al. 2004, Yang et al. 2008).
It has been proposed, that in response to certain cellular conditions (e.g. high expression levels of MDM2), monoubiquitination of FOXO4 can promote its subsequent polyubiquitination by SKP2.

**Therapeutics targeting the PI3K–AKT–FOXO signaling axis**

Clinical interventions that are able to restore FOXO tumor suppressor function, could potentially be used as therapeutic strategies to overcome endocrine resistance (Hill *et al*. 2014a). To this end, potential drug targets that warrant future investigation include: i) growth factor signaling pathways that converge on, and negatively regulate FOXO; ii) the cellular transportation machinery responsible for the nuclear/cytoplasmic shuttling of FOXOs; iii) proteasomes that target FOXOs for degradation; and iv) the DNA-binding activity of the FOXO proteins themselves (see Fig. 3).

**PI3K–AKT pathway inhibition**

**Preclinical studies** As has been described, numerous studies have implicated PI3K–AKT–FOXO dysregulation as possessing a significant contributory role in the development of endocrine resistance. This has spurred the trial of endocrine/PI3K-inhibition combination therapies, and initial pre-clinical studies have generally yielded encouraging results in terms of the potential utility of these strategies to prevent or overcome resistance.

Unfortunately, the examination of FOXO activity was beyond the scope of the majority of these studies; though it is likely that future experiments will demonstrate that FOXOs have a contributory role in many of the positive effects observed.

The simultaneous treatment of ER+/HER2+ breast cancer cells (the BT-474 cell-line) with the anti-HER2 antibody trastuzumab (Herceptin) and tamoxifen, synergistically inhibits their growth *in vitro*, and also within a xenograft mouse model (Argiris *et al*. 2004, Wang *et al*. 2005). Targeting HER2 was found to potently inhibit both the PI3K–AKT and ERK signaling pathway in these experiments. Another pre-clinical study found that treating tamoxifen-resistant breast cancer cells (MCF7 cells expressing constitutively active AKT) with the mTOR inhibitors CCI-779 (temsirolimus) or rapamycin, restored the sensitivity of these cells to tamoxifen-induced growth inhibition (deGraffenried *et al*. 2004). The mTOR1 complex is a downstream target of PI3K–AKT signaling; directing cellular metabolism and growth, and its dysregulation is frequently implicated in human malignancy (Dibble & Cantley 2015).

Miller *et al*. (2010) observed that their *in vitro* acquired resistance model is heavily reliant on PI3K–AKT–mTOR signaling. They created several long-term estrogen deprivation (LTED) breast cancer cell-lines to study the mechanisms of escape from E2 dependent growth. Molecular characterization of the LTEDs revealed significantly increased AKT and mTOR activity compared with...
the corresponding parental cell-lines. Furthermore, the PI3K/mTOR dual inhibitor, BEZ235, was extremely effective at inducing growth arrest and apoptosis of LTEDs, and also effectively prevented the emergence of new resistant cancer cell populations (Miller et al. 2010). In a subsequent study, Miller et al. (2011) observed an E2-independent ER transcriptional activity; that directs the cell-cycle progression of both LTEDs and primary cultures of AI treated breast tumors. Combined down-regulation of ER (fulvestrant) and PI3K inhibition with BKM120 (Buparlisib) induces a regression of tumors comprising these cells (Miller et al. 2011).

To date, only a small number of studies have directly examined FOXO activity in response to PI3K inhibition within breast cancer cells. Recently, Hill et al. (2014a,b) reported that the compound ETP-45658, a pyrazolo-pyrimidine derivative that is a PI3K inhibitor, potently inhibited the growth of breast cancer cells via cell-cycle arrest (observed in MCF7 and MDA-MB231 breast cancer cell-line models). The authors observed treatment of MCF7 cells inhibited and increased phosphorylation (Ser253) and nuclear accumulation of FOXO3A, respectively, and specifically induced a FOXO-dependent transcriptional response enriched for cell-cycle related genes (Hill et al. 2014b). Chu et al. (2015) found that treatment of human breast cancer cells with a 2-aryl benzimidazole compound, that likely targets EGFR and HER2 signaling activity, inhibited the phosphorylation of FOXO1 (Ser319 and Ser256) and FOXO3A (Thr32 and Thr24), promoting the translocation of these FOXO proteins from the cytoplasm into the nucleus.

Although there is little data in the context of breast cancer, restoration of FOXO activity by PI3K–AKT inhibition has also been observed in the treatment of a variety of other human cancers. For instance, treatment of chronic myeloid leukaemia with the pan tyrosine kinase inhibitor imatinib (Gleevec), inhibited PI3K–AKT signaling, and effectively restored FOXO3A transcriptional activity (Fernandez de Mattos et al. 2004, Essafi et al. 2005). A similar effect is also observed in osteosarcoma cells treated with the PI3K–AKT inhibitor Grifolin (Jin et al. 2007).

**Clinical trials** Clinical trials have also shown the efficacy of PI3K–AKT pathway inhibition as a strategy to overcome endocrine resistance. Two trials have investigated the utility of treating ER+/HER2+ metastatic breast cancers with combination of anastrozole (AI) treatment and HER2 inhibition; with either trastuzumab or latatinib. In both trials, progression-free survival (PFS) was found to be superior in the combination arms (Johnston et al. 2009, Kaufman et al. 2009).

More recently, some large clinical trials have explored whether targeting mTORC1 is of benefit to patients with ER+ breast tumors; that have relapsed following previous AI treatment. In the tamoxifen plus RAD001 phase-II trial, metastatic breast cancer patients were randomized to tamoxifen combined with the mTOR inhibitor everolimus (formerly known as RAD001) or tamoxifen alone (Bachelot et al. 2012). Statistically significant improvements in PFS and clinical benefit were seen in the combination arm (8.6 months vs 4.6 months with tamoxifen alone, hazard ratio (HR) 0.54). Of note, subgroup analysis revealed that the benefit received from combination therapy is only seen in patients with acquired resistance.

In the breast cancer trials of oral everolimus 2 (BOLERO-2) phase-III trial, post-menopausal with ER+ HER2− advanced breast cancer were randomized to groups receiving everolimus or placebo, combined with the AI exemestane (Baselga et al. 2012, Yardley et al. 2013). The final study results (median follow-up of 18 months) showed that everolimus combined with the AI improves PFS in patients with ER+ HER− cancers that had been previously treated with non-steroidal AIs (letrozole or anastrozole). The primary end-point PFS was more than doubled in the combination treatment arm (7.8 months vs 3.2 months in the combination and exemestane alone arms, respectively; HR 0.89; 95% CI 0.38–0.54; P < 0.0001; Yardley et al. 2013). Furthermore, subgroup analysis showed that clear improvement in PFS in elderly patients (improved median PFS by 2.82 months in patients >65 years of age, HR 0.59; and by 5.26 months in patients ≥70 years of age, HR 0.45; in the combination and exemestane alone arms respectively), an outcome of particular clinical importance considering the typical age distribution of women with ER+ advanced breast cancer (Pritchard et al. 2013).

However, despite being generally well-tolerated, there are still some significant toxicity issues caused by everolimus treatment. Commonly reported symptoms include fatigue, stomatitis, rashes, hyperglycemia, hypolipidemia, myelosuppression, and diarrhea. Also, 3% of patients have reported non-infectious pneumonia which appears to be immunologically mediated. These are typical class-effects associated with mTOR inhibitors. Fortunately most of these adverse effects are not life-threatening and can be managed by proper prevention and management strategies (reviewed by Rugo (2015)).

These data are generally encouraging that strategies that employ simultaneous targeting of PI3K–AKT and ER...
pathways may be of benefit to breast cancer patients with acquired resistance. Indeed, on the basis of the BOLERO-2 study, FDA approval was granted for the use of everolimus in combination with exemestane for the treatment of ER+ HER2 advanced breast cancer that have progressed during treatment with either letrozole or anastrozole. However, since then, analysis of the overall survival data has revealed that for this secondary end-point of the study, there was no significant difference between the treatment arms (Piccart et al. 2014). This lack of a robust clinical response could be, as is often the case for PI3K–AKT targeting therapeutics, the result of cellular resistance mechanisms to PI3K–AKT inhibition.

Resistance mechanisms to PI3K–AKT inhibition Both clinical and pre-clinical studies have identified several different mechanisms by which resistance to PI3K–AKT–mTOR inhibition can arise. These include feedback activation of AKT, increased expression of RTKs and cross-talk with other growth-promoting pathways (reviewed in detail by Thorpe et al. (2015)).

Of particular relevance to the subject of this review, restored FOXO activity, following AKT inhibition, directly up-regulates the expression of HER3, IGF1R, and insulin RTK gene expression (Chandarlapaty et al. 2011).

Recently, Bihani et al. (2015), investigated the mechanisms of acquired resistance of breast cancer cells to everolimus. The authors demonstrated that breast cancer cell-lines, having acquired everolimus resistance, exhibit bromo domain containing protein 4 (BRD4)-mediated up-regulation of the MYC gene. Up-regulation of MYC expression in ER+ antiestrogen resistance breast cancer cells triggers a pro-survival autophagic adaptation in glucose deprived culture conditions (Shajahan-Haq et al. 2014). As has already been discussed, autophagy appears to be a central player in mechanisms of endocrine resistance. Promisingly, it is possible to pharmacologically inhibit BRD4, and doing so has been shown to overcome the resistance of the cancer cells to mTOR1 inhibition (Bihani et al. 2015).

Concerning the feedback activation of AKT, the Src/c-Abl, multi-kinase-inhibitor dasatinib, has been shown to effectively block AKT activation following mTOR inhibition in several breast cancer cell lines (Yori et al. 2014). Furthermore, the combination treatment of dasatinib and rapamycin has a synergistic effect upon tumor regression in mouse models, accompanied by a reduction in pulmonary metastasis as well as an increase in time to tumor recurrence (Yori et al. 2014).

Other potential therapeutic targets Although the targeting of PI3K–AKT still presents the most promising strategy to restore FOXO activity, acquired resistance to PI3K–AKT inhibition currently presents a significant barrier that needs to be overcome. The current trend of trialling combination therapies, whereby PI3K–AKT is inhibited in conjunction with additional molecular targets, may prove effective in maximizing the clinical efficacy of the PI3K inhibitors. Fortunately, the complex cellular regulation of FOXO factors summarized in this review, presents a number of additional molecular targets that could be potentially investigated.

Nuclear export machinery Nuclear export of FOXOs can be blocked by inhibitors of the 14-3-3 chaperone protein family and related exportins. Dong et al. (2007) demonstrated that treatment of an in vitro leukemia cell-model with the peptide R18, down-regulated 14-3-3 protein expression, which in turn leads to increased nuclear accumulation of FOXO3A; and restored transcription of its anti-proliferative targets p27Kip1 and Bim. More recently, Mori et al. (2014) have developed novel compounds that also interact and inhibit the 14-3-3 chaperone proteins, increasing the possibility that eventually some compounds may be suitable for clinical use.

Proteasome Inhibiting the proteasome-mediated degradation of FOXOs could represent an interesting strategy to restore their cellular levels. However, to date, there are no studies on this topic available in the literature.

FOXOs Transcription factors are predominantly located in the nucleus and they do not possess enzymatic activity. For these reasons they have historically been considered to be ‘undruggable’ molecular targets. However, a growing number of studies have challenged this assumption. Improved understanding of transcription factor biology, coupled with improved drug design and delivery, make transcription factors attractive and realistic alternative drug targets. This may be particularly useful in clinical scenarios where targeting kinase signaling pathways in cancer cells have met with limited efficacy (Rodon et al. 2013).

Although no examples of directly targeting FOXOs exist, attempts at targeting other members of the FOX family have been reported. Notably, Bhat et al. (2009)
identified two thiazole antibiotic compounds, siomycin-A and thiostrepton, that selectively bind to and inhibit the transcriptional activity of the oncogenic FOXM1 protein in in vitro cancer cell-line models. Although the poor water solubility of these compounds (Zhang & Kelly 2012) make them of limited clinical use; nevertheless these observations have propelled a search for other small molecule inhibitors of FOXM1 that can be used safely (Chen et al. 2015b).

**A need for caution**

It is important to consider that FOXOs are regulators of cellular stress resistance mechanisms, and in at least some circumstances, such treatments may also increase resistance to chemotherapeutics. Therefore, it is crucial to fully characterize FOXO-regulated transcriptional programmes in specific disease-states; to identify those which would be most suitable for targeted reactivation of FOXO tumor suppressor function.

**Conclusion**

In conclusion, the dysregulation of FOXO factors has emerged as a key molecular feature of endocrine resistance mechanisms. Both pre-clinical and clinical research has shown the promising potential of targeting the PI3K–AKT pathway; as a therapeutic strategy to restore hormone-sensitivity to resistant breast tumors. Furthermore, restoration of FOXO tumor suppressor appears to be a key player in this process. However, PI3K–AKT inhibition may not turn out to be the best approach. As has been discussed, cancer cells can utilize a multitude of different resistance mechanisms to acquire resistance to PI3K–AKT inhibition. Although, preclinical experiments show promising data with regard to blocking PI3K–AKT resistance pathways, it remains to be ascertained whether such strategies will translate into superior clinical efficacy. Therefore, one of the aims of this review was to provide a generalized overview of the multiple interconnected regulatory layers that impact upon FOXO transcriptional activity. Further understanding these processes may identify future molecular targets that can allow clinicians to manipulate FOXOs in a more therapeutically targeted manner.

**Declaration of interest**

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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