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Kinesin family deregulation coordinated by bromodomain protein ANCCA and histone methyltransferase MLL for breast cancer cell growth, survival and tamoxifen resistance

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Abstract

Kinesins are a superfamily of motor proteins and often deregulated in different cancers. However, the mechanism of their deregulation has been poorly understood. Through examining kinesin gene family expression in estrogen receptor (ER)-positive breast cancer cells, we found that estrogen stimulation of cancer cell proliferation involves a concerted regulation of specific kinesins. Estrogen strongly induces expression of 19 kinesin genes such as Kif4A/4B, Kif5A/5B, Kif10, Kif11, Kif15, Kif18A/18B, Kif20A/20B, Kif21, Kif23, Kif24, Kif25 and KifC1 while suppresses the expression of 7 others including Kif1A, Kif1C, Kif7 and KifC3. Interestingly, the bromodomain protein ANCCA/ATAD2, previously shown to be an estrogen-induced chromatin regulator, plays a crucial role in the up- and down-regulation of kinesins by estrogen. Its overexpression drives estrogen-independent up-regulation of specific kinesins. Mechanistically, ANCCA mediates E2-dependent recruitment of E2F and MLL1 histone methyltransferase at kinesin gene promoters for gene activation associated H3K4me3 methylation. Importantly, elevated levels of Kif4A, Kif15, Kif20A and Kif23 correlate with that of ANCCA in the tumors and with poor relapse-free survival of ER-positive breast cancer patients. Their knockdown strongly impeded proliferation and induced apoptosis of both tamoxifen-sensitive and -resistant cancer cells. Together, the study reveals ANCCA as a key mediator of kinesin family deregulation in breast cancer and the crucial role of multiple kinesins in growth and survival of the tumor cells.

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No Conflicts.

Implications—These findings support the development of novel inhibitors of cancer-associated kinesins and their regulator ANCCA for effective treatment of cancers including tamoxifen-resistant breast cancers.

Keywords

kinesin family; estrogen; bromodomain; breast cancer; poor survival

Introduction

The kinesin superfamily of motor proteins is encoded by over 40 different genes in humans. They function by traveling unidirectionally along the microtubules for intracellular transport of molecules or organelles. Many of them such as Kif4A, Kif10/CENPE and Kif11/Eg5 play important roles in cell division particularly in different stages of mitosis and cytokinesis (1). Several kinesins including Kif1A, Kif7 and KifC3 function primarily in non-mitotic processes such as protein complex or organelle movement and cellular signaling (2-6). Overexpression of mitotic kinesins including KifC1, Kif10/CENPE and Kif18A was found in several human cancers including breast cancer (BCa) (7-11). However, it is unclear whether other kinesins are aberrantly expressed in BCa and whether they play roles in the cancer cell proliferation and survival. Moreover, little is known about the mechanisms how the kinesin gene expression is deregulated in cancer cells.

The mitogenic effect of estrogen 17β-estradiol or E2 is primarily through its receptor ERα to control gene expression. E2 through binding to estrogen receptors (ERs) plays pivotal roles in BCa development and progression. In cells, E2 treatment elicits changes in expression of hundreds of genes with the ones involved in cell cycle progression and DNA synthesis and replication being the most robustly up-regulated. Interestingly, estrogen also strongly represses many antiproliferative and proapoptotic genes. Although the majority of them are likely direct targets of ERα, many are regulated indirectly through estrogen control of other transcriptional regulators (12-18).

ER-mediated transcriptional regulation involves assembly at target gene loci of chromatin regulators such as the p160/SRC coactivators, histone modifying or de-modifying enzymes and the ATP-dependent chromatin remodeling proteins (15, 16, 19-21). Different ER target genes may utilize different sets of specific chromatin regulators, which often act through transient and dynamic protein-protein interactions (19, 22). ANCCA (for AAA nuclear co-regulator cancer associated), a bromodomain containing, ATPase protein, was identified by us as a novel ERα coregulator and shown to play a critical function in mediating ER activation of CCND1, MYC and E2F1 as well as E2-stimulated BCa cell proliferation (23-28). Importantly, ANCCA expression is strongly induced by E2 in ER-positive BCa cells. Its overexpression in tumors is highly associated with poor outcomes of BCa patients (26). Here, we examined the function of ANCCA in estrogen control of kinesin family gene expression. Our results revealed a pivotal role played by ANCCA in mediating both estrogen induction and repression of specific kinesins and demonstrated a critical function of ANCCA activated kinesins in BCa cell proliferation and survival.

Materials and Methods

Cell culture, hormone treatment and siRNA transfection

MCF-7 and T-47D cells were obtained from ATCC and cultured in DMEM or RPMI 1640 (Invitrogen) respectively with 10% fetal bovine serum (FBS, Gemini). For ligand treatment, cells were grown in phenol red-free medium supplemented with 10% charcoal-dextran-stripped (cds) FBS (JRS Scientific) for 3 days before treated with E2 (at 10^{-8}M), 4-hydroxytamoxifen (Tam, at 10^{-6}M) or Fulvestrant (at 10^{-7}M; all from Sigma-Aldrich) for indicated times. MCF-7 cells that ectopicall express ANCCA were generated by transfection with pcD-HCMV-ANCCA (23, 28) and stable transfectant cells were clonally isolated and expanded in the presence of G418 (300 ug/ml). Tamoxifen-resistant MCF-7 subline LCC2 cells (a kind gift from Dr. Robert Clarke at Georgetown University) were cultured in DMEM + 10% cds-FBS containing 10^{-6}M Tam. For siRNA transfection, cells were seeded at 2×10^5 cells per well in 6-well plates in phenol red-free DMEM supplemented with 5% cds-FBS and transfected using Dharmafect with siRNAs (Dharmacon) targeting ANCCA (29), MLL1 (30) or Kif 4A, Kif15, Kif20A and Kif23 (Supplementary materials).

qRT-PCR and immunoblot analysis

Total RNA was isolated and the cDNA was prepared, amplified and detected in the presence of SYBR as previously (29). The fluorescent values were collected and a melting curve analysis was performed. Fold difference was calculated as described previously (29). Cell lysates were analyzed by immunoblotting with antibodies against specific kinesins, ANCCA and other proteins. The PCR primers, details of cell lysates and immnoblotting and antibodies are listed in the Supplementary materials.

Apoptosis and cell growth assays

For apoptosis, cells were transfected with siRNA on glass chamber slides and processed for TUNEL assay using the In Situ Cell Death Detection kit (Roche) as previously described (26). Random fields (10fields/condition) of TUNEL-positive cells were counted and averaged. For cell growth, cells were seeded in 6-well plates at 1×10^5 per well and treated as indicated. Total cell numbers were counted using a Coulter cell counter. The assays were performed in triplicates and the entire experiments were repeated three times.

ChIP assay

ChIP was performed essentially as described previously (27, 28) with the following modifications. Cells were lysed and sonicated using Sonic Dismembrator 550 (Fisher). The crude chromatin solutions were first cleared with protein A beads (Invitrogen) that had been precoated with preimmune serum or normal IgG for 2 h at 4°C. Then, the precleared solutions were incubated at 4°C overnight with antibodies against ANCCA (26), E2F1 (1:1 mixture of C-20 and KH-95, Santa Cruz), MLL (clone 9-12, Millipore), H3K4me3 and H3K27ac (ab8580 and ab4729, ChIP grade, both from Abcam), prior to precipitation with protein A beads that had been preblocked with BSA and sonicated salmon sperm DNA. The precipitated DNA was analyzed by real-time PCR with SYBR green on an iCycler

instrument. Enrichment of genomic DNA was presented as the percent recovery relative to the input. The primers are listed in the Supplementary materials.

Analysis of kinesin expression in tumors for clinical outcome and ANCCA correlation and statistics

Microarray data from GSE9195 (31) were downloaded from the NCBI GEO website (http://www.ncbi.nlm.nih.gov/gds). The dataset consists of gene expression profiles of early stage, ER positive BCa tumors that had received tamoxifen only as adjuvant treatment. Normalized probe set expression intensities were obtained using robust multi-array average (RMA) for probe summarization and normalization as before (29). Correlations of ANCCA/atad2 and kinesin expression were assessed by computing the Pearson correlation coefficient (r) and a two-tailed *t*-test for significance. The Kaplan-Meier estimates were used to compute the survival curves. All above computations were conducted in R statistical package (http://www.r-project.org/). Kinesin expression and patient survival were also analyzed using an online survival analysis tool (32). For the assays, paired *t* test was performed as previously described (26).

Results

Estrogen stimulation of BCa cell proliferation involves a concerted up- and down-regulation of specific kinesin expression

We previously demonstrated that ANCCA is an estrogen responsive gene and controls the expression of cyclins and other genes important for cell proliferation and survival (23, 26). This prompted us to investigate whether ANCCA plays any function in control of mitotic kinesins in ER-positive BCa cells. Thus, we first identified kinesins with expression regulated by E2 in estrogen-sensitive MCF7 cells. Remarkably, among the 38 kinesin genes with mRNA expression detected in MCF7 cells, E2 strongly stimulated the expression of a large number (19 out of 38, over 2 fold in 12 hrs and/or 24 hrs of E2 treatment) of the detected kinesins, which include Kif2A, Kif3A, Kif3B, Kif4A, Kif4B, Kif5B, Kif10/CENPE, Kif11/EG5, Kif15, Kif16A, Kif18A, Kif18B, Kif20A, Kif20B, Kif21A, Kif23, Kif24, Kif25 and KifC1 (Fig. 1A). In most cases, the induction can be observed by 12hrs of E2 stimulation. Except Kif2A, Kif5B and Kif21A, most of them play important roles in mitosis and/or cytokinesis (1). Intriguingly, E2 also significantly repressed several kinesins including Kif1A, Kif1C, Kif3C, Kif7, Kif13B, Kif16B, and KifC3. Notably, most of the repressed kinesins have primary functions in non-mitotic processes such as synaptic vesicle transport in neurons (Kif1A), integrin transport for cell migration (Kif1C), control of the Hedgehog (Hh)-Gli signaling (Kif7) and Golgi positioning and integration with dynein (KifC3) (2, 5, 33).

Given the prominent function of E2-ER in promoting BCa cell proliferation, we focused our further analysis on mitotic kinesins. As shown before, ANCCA and its targets cyclin D1 and CDC6 are induced by E2 in MCF7 cells (Fig. 1B). Western blotting with available antibodies confirmed the E2 induction of mitotic kinesin proteins of Kif4A, Kif11, Kif15, Kif20A and Kif23 (Fig. 1B). To examine whether the E2 regulation is through ERα, cells were treated with ERα pure antagonist fulvestrant. Indeed, when cells were treated

simultaneously with E2 and fulvestrant, the kinesin induction by E2 was mostly suppressed (Fig. 1B, top panel), indicating that E2 induction of Kif4A, Kif11, Kif15, Kif20A and Kif23 is through ERα. Similar results were obtained from another estrogen-sensitive cell T-47D (Fig. 1B, bottom panel). Together, the results suggest that estrogen via ERα coordinately regulates kinesin family gene expression with up-regulation of mitotic kinesins and down-regulation of non-mitotic kinesins.

ANCCA plays a crucial role in mediating E2 regulation of kinesins

To determine whether ANCCA mediates E2 control of the kinesins, we measured their expression in MCF7 cells with ANCCA suppressed by siRNA. As shown in Fig. 2A, ANCCA suppression markedly diminished E2 induction of most (18 out of 19) of the kinesins, which include Kif2A, Kif3A, Kif3B, Kif4A, Kif4B, Kif5B, Kif10/CENPE, Kif11/EG5, Kif15, Kif16A, Kif18A, Kif18B, Kif20A, Kif20B, Kif21A, Kif23, Kif25 and KifC1. More strikingly, for the seven kinesins that are repressed by E2, upon ANCCA silencing by siRNA, their repression by E2 was largely lost (except for Kif16B). Kif14 is the only one that were not significantly regulated by E2 but decreased by ANCCA suppression. Suppression of ANCCA by a different siRNA yielded essentially the same effects on E2 regulated kinesin mRNAs (data not shown). As expected, ANCCA suppression by the two siRNAs resulted in strong reduction of protein levels of mitotic kinesin Kif4A, Kif11, Kif 15, Kif20A and Kif23 in the cancer cells (Fig. 2B).

To further examine the function of ANCCA in control of kinesin expression, we analyzed whether elevated ANCCA promotes the kinesin expression. Strikingly, in the absence of E2, ectopic ANCCA was able to stimulate the mRNA expression of Kif4A, Kif11, Kif15, Kif20A and Kif23 to the level almost equivalent to that induced by E2 in the vector control cells. Except Kif4A, E2 further stimulated their expression in the ANCCA-overexpressing cells (Fig. 3A), suggesting that part of the ANCCA enhancement of kinesin expression is through ERa. Moreover, the ANCCA stimulatory effects can be observed on the kinesin proteins (Fig. 3B). Thus, these results strongly suggest that chromatin-associated protein ANCCA is a key mediator of estrogen coordinated regulation of kinesin gene family in BCa cells.

E2 induction of kinesins involves the assembly and function of ANCCA, E2F and their associated histone methyltransferase MLL

To examine the mechanism of ANCCA function in estrogen control of kinesin expression, we first performed ChIP assays for potential direct involvement of ANCCA. Indeed, marked ANCCA occupancy was observed at promoters of Kif4A, Kif15, Kif20A and Kif23 upon E2 treatment for 1 or 3 hrs (Fig. 4A, 4B and Supplementary Fig. S1). Inspection of the local sequences with ANCCA occupancy suggests binding sites for transcription factors E2Fs and c-Myc at the kinesin promoters. ChIP with anti-E2F1 antibody demonstrated a significant E2 induction of recruitment at the same locations, in support of our previous finding that ANCCA interacts with E2Fs and acts as a potent coactivator of E2Fs (27). However, no consistent, E2-induced c-Myc binding was observed with a c-Myc antibody (data not shown). Notably, the ANCCA recruitment was associated with a significant increase of gene activation-linked histone marks such as H3K4me3 and H3K27ac, with H3K4me3 being

highly elevated by E2. Since H3K4me3 is primarily deposited by histone methyltransferases such as MLL1, we next examined whether MLL1 is involved. Indeed, strong E2 induced recruitment of MLL1 at the kinesin gene promoters was observed. To determine the role of ANCCA in the E2-induced chromatin events, we performed ChIP with si-ANCCA knockdown cells. Remarkably, suppression of ANCCA strongly diminished E2-dependent recruitment of E2F1 and MLL1 at the kinesin promoters. Expectedly, MLL1-associated H3K4me3 level was also largely reduced (Fig. 4C, 4D and Supplementary Fig. S1). Knockdown of MLL1 strongly mitigated E2 induction of the kinesin expression (Fig. 4E and 4F). Together, these results suggest that ANCCA plays an important role in mediating the assembly of E2F and MLL complexes and H3K4me3 mark elevation at the kinesin gene promoters for E2 induction of their expression.

Kif4A, Kif15, Kif20A and Kif23 are important for proliferation and survival of tamoxifen-sensitive and -resistant BCa cells

Several kinesins such as Kif11, Kif14, and Kif18A have recently been shown to be important for BCa cell proliferation. We thus focused on our analysis on the role of ANCCA controlled Kif4A, Kif15, Kif20A and Kif23 in cell growth and survival. Each of the four kinesins was knocked down by two different siRNAs. Each knockdown of Kif4A, Kif15, Kif20A and Kif23 resulted in strong inhibition of E2-stimulated proliferation of tamoxifen-sensitive MCF7 cells (Fig. 5A). Consistent with the marked reduction of viable cells, depletion of each of the kinesins caused a pronounced apoptotic cell death as indicated by the number of TUNEL positive cells and the induction of cleaved caspase 7 and PARP1 proteins (Fig. 5B and 5C).

To further examine the function of ANCCA in control of kinesins, we performed the knockdowns in a tamoxifen-resistant subline (LCC2) of MCF-7 cells (34-37). Comparing to the MCF-7 cells where the kinesin and ANCCA expression was strongly inhibited by tamoxifen, LCC2 cells were essentially unresponsive to the inhibitory effect of tamoxifen on Kif4A, Kif15, Kif20A and Kif23 (Fig. 6A). Interestingly, ANCCA level was significantly higher in LCC2 cells than MCF-7 cells, especially when the cells were treated with tamoxifen. As expected, tamoxifen treatment alone did not alter LCC2 cell growth. Silencing each kinesin in LCC2 cells, however, markedly inhibited their hormone-independent proliferation (Fig. 6B and Supplementary Fig. S2, compare siCont-Tam and siKif-Tam). Importantly, the kinesin silencing also dramatically increased LCC2 cell sensitivity to tamoxifen inhibition (compare si- Cont+Tam and siKif+Tam). Altogether, the results suggest that ANCCA regulated kinesins play crucial roles in proliferation and survival of both tamoxifen-sensitive and -resistant BCa cells.

High level of kinesins correlates with that of ANCCA and with poor outcome of ER-positive tumors

The above results that ANCCA regulated kinesins play an important role in BCa cell growth and survival prompted us to examine the clinical significance of our experimental findings. In a dataset of gene expression profiles obtained from early stage, ER positive BCa tumors that had received tamoxifen only as adjuvant treatment, high expression of Kif4A, Kif15, Kif20A and Kif23 strongly correlated with poor relapse-free survival. Importantly, their

expression also correlated significantly with the expression of ANCCA in the tumors (Fig. 7A and 7B and Supplementary Fig. S3). To test further the impact of high specific kinesin expression, we used an online survival analysis tool with a large database of BCa (1413 ER-positive tumors) and found that high levels of each of the four kinesins tend to associate with poor prognosis (relapse free survival) (Fig. 7C and Supplementary Fig. S3). Together, these clinical data support the role of ANCCA as a key regulator of specific kinesins and indicate that their overexpression may drive BCa progression such as resistance to tamoxifen therapy.

Discussion

Little is known about the mechanism of kinesin family deregulation in human cancers. In this regard, this study made several unique and unexpected findings. First, a large number of kinesins are regulated by estrogen in BCa cells. Mammalian kinesin family has 45 members and many of them are expressed in tissue or cell specific manner (1). Remarkably, among the 38 kinesins with expression detected in BCa MCF-7 cells, 26 of them are regulated by E2, therefore likely making it one of the most closely regulated gene families in estrogen signaling. Second, estrogen regulation of kinesins is bidirectional. Although many of the kinesins are up-regulated by E2, at least seven are strongly down-regulated by E2. Third, estrogen regulated kinesins appear to play distinct and non-redundant function. Although many are mitotic regulators (Kif4A, Kif10, Kif11, Kif15, Kif18A, Kif20A, and Kif23), Kif3B is primarily involved in cell migration and Kif24 is a centriolar kinesin and functions to remodel the local microtubules for cilia assembly (38). Knockdown of individual mitotic kinesins such as Kif 4A, Kif15, Kif20A and Kif23 demonstrated that each of them is critical for cell proliferation or survival. Interestingly, kinesins repressed by E2 which include Kif1C, Kif3C, Kif7, Kif16B, and KifC3, are mostly non-mitotic kinesins (1) and they likely play different functions too. For instance, Kif1C is involved in stabilization of cellular trailing adhesions (33). Kif1C gene is found mutated in a subset of metastatic, ERa-positive BCa (39). Interestingly, Kif7 plays an important role in regulation of Hedgehog (Hh)-Gli signaling and its deletion contributes to the development of skin basal cell carcinoma (40). One unexpected finding is that both E2-dependent induction and repression of kinesins involves the function of ANCCA. We reported previously that ANCCA acts as a novel coactivator of ERα (23). The fact that ANCCA depletion results in de-repression of specific kinesins strongly suggests that ANCCA also plays an important role in E2-dependent transcriptional repression. Although future study is needed to understand the underlying mechanism, it is conceivable that ANCCA, through its direct interaction with ERα, is recruited to the ER-repressed target genes and that recruited ANCCA, via its AAA type ATPase activity, may facilitate the assembly of repressive protein complexes such as the corepressor-HDAC, NRIP1/RIP140 or p300-CtBP (41, 42).

We focused our mechanistic analysis on the mitotic kinesins Kif4A, Kif15, Kif20A and Kif23. Our ChIP analysis clearly showed that ANCCA directly occupies their promoter in the absence of hormone and that its occupancy is strongly increased upon E2 treatment. Thus, ANCCA may be involved in both estrogen-dependent and –independent activation of the kinesins. This is consistent with recent findings that these mitotic kinesins are overexpressed in many types of cancers such as lung cancer, pancreatic cancer, and glioma

that generally do not involve strong estrogen stimulation but also show ANCCA overexpression (41-45). Interestingly, we showed here that ANCCA is required for E2F1 recruitment to the kinesin genes. We also found that ANCCA is required for E2-induced recruitment of MLL1 histone H3K4 methylase and the E2 induction of H3K4me3 mark at the kinesin gene promoter, as we demonstrated for androgen induction of EZH2 (46). H3K4me3 mark is necessary for the RNA polymerase II pre-initiation complex assembly (47). Thus, together our results suggest that upon estrogen stimulation, ANCCA occupancy at the kinesin genes is enhanced to facilitate the recruitment of E2F1 and MLL-mediated H3K4 methylation, which results in the assembly of Pol-II complex for transcriptional activation. How E2 stimulates the function of ANCCA at kinesin promoters is currently unknown. Although we were able to detect E2-induced robust recruitment of ERα to cyclin D1 enhancer and promoter, we were unable to detect any significant ERα recruitment to the ANCCA occupied promoter regions of the kinesin genes examined (data not shown). It is possible that ERα acts through tethering to other transcription factor(s) (14, 21) or is recruited to an uncharacterized distal enhancer of the kinesin to facilitate ANCCA loading to the promoter through chromosomal looping. Future studies will be needed to determine the functional mechanism of ERα in the induction of specific kinesins.

Importantly, this study revealed that multiple kinesins including Kif4A, Kif15, Kif20A and Kif23 are crucial for growth and survival of both tamoxifen-sensitive and -resistant BCa cells. We also found that among the patients treated with tamoxifen, high levels of the four kinesins are strongly associated with poor recurrence-free survival. Thus, the results strongly suggest that kinesins such as Kif4A, Kif15, Kif20A and Kif23 can have important values as both prognostic factors and new therapeutic targets for endocrine therapy-resistant BCa. However, kinesins may play similar cellular functions (1), which would necessitate simultaneous targeting of multiple kinesins for effective therapy. One alternative can be blocking MLL-mediated histone methylation which is required for the kinesin gene activation. However, members of the MLL family are found mutated or deleted in multiple human cancers (48, 49). Given ANCCA being a common activator of these kinesins, targeting ANCCA can be a more attractive option. With the small molecules targeting bromodomain proteins such as BRD4 are near clinical trial, development of inhibitors specifically targeting ANCCA bromodomain or its ATPase is highly desirable for effective treatment of aggressive BCa.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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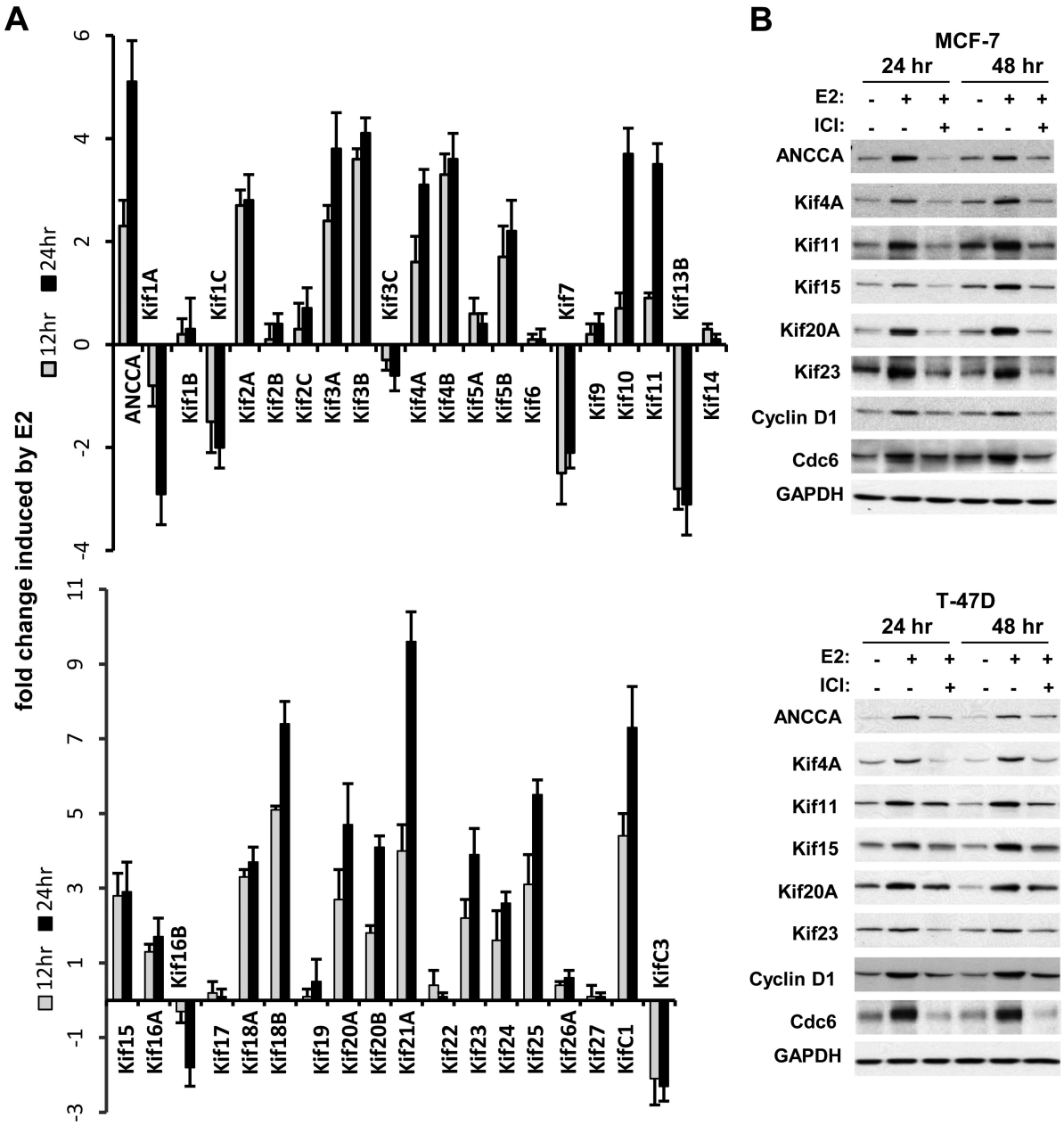

FIGURE 1.

Coordinated regulation of kinesin family expression in BCa cells by estrogen-ERα. A. MCF-7 cells were hormone depleted for three days and then treated with 17beta-estrodial (E2) at 10^{-8}M for indicated hours before harvested for real-time RT-PCR analysis. Fold change was obtained by comparing the normalized qPCR value from E2-treated cells with the value from cells without E2 harvested at the same time point. The data are expressed as the mean ± SD from three independent experiments. B. MCF-7 and T-47D cells were treated with E2 as in A, or fulvestran/ICI182,780 (ICI) at 10^{-7}M for indicated hours before harvested for immunoblotting with indicated antibodies.

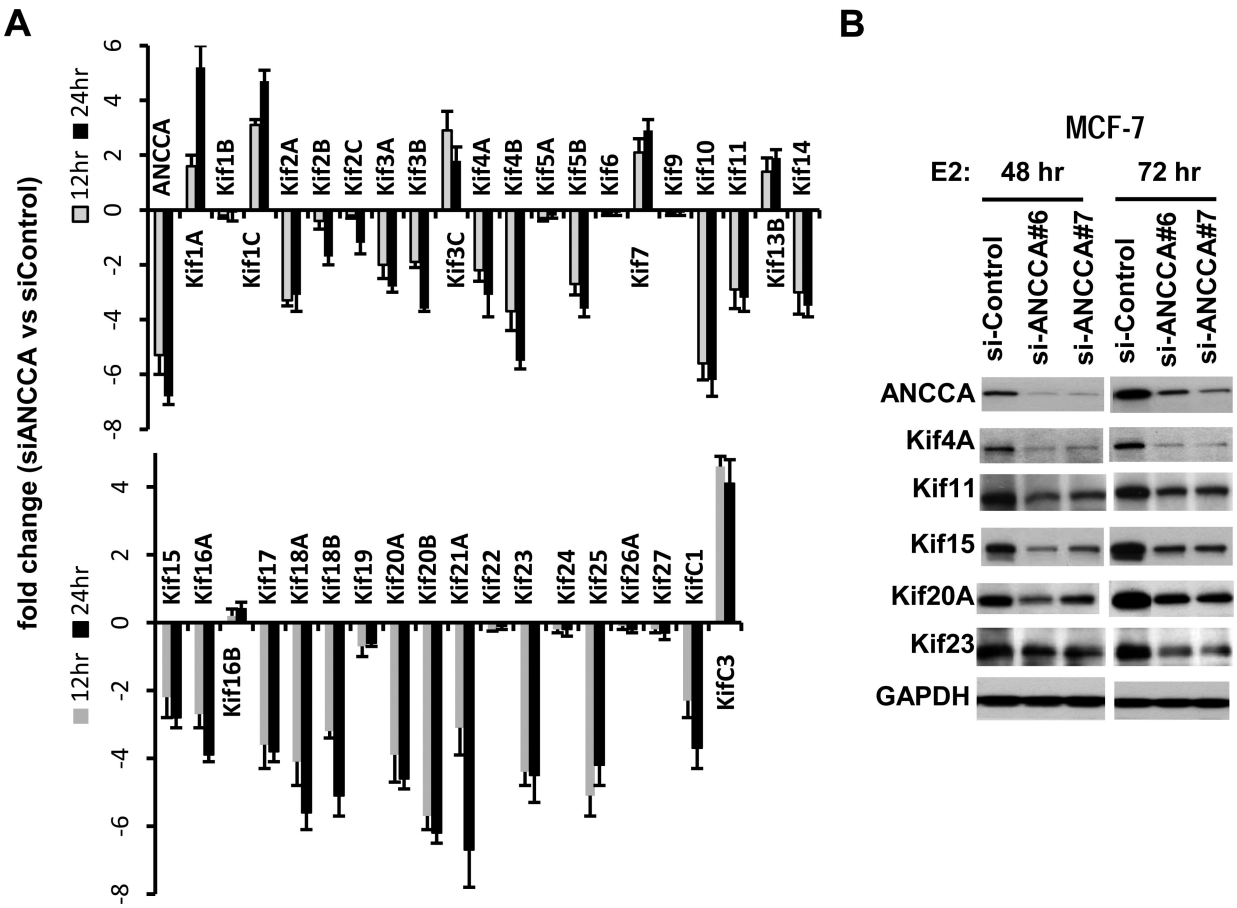

FIGURE 2.

ANCCA plays a crucial role in mediating E2-ER regulation of kinesin expression. A. MCF-7 cells were in hormone depleted medium for 24 hrs, transfected with control or ANCCA siRNA, and 48 hrs post-transfection treated with 17beta-estrodial (E2) at 10^{-8}M for 12 or 24 hrs before harvested for real-time RT-PCR analysis. Fold change was obtained by comparing the E2-induced qPCR value change from ANCCA siRNA transfected cells with the value change from control siRNA transfected cells harvested at the same time point of E2 treatment. The data are expressed as the mean ± SD of three independent experiments. B. MCF-7 cells were siRNA transfected and treated with E2 as in A for indicated hours before harvested for immunoblotting with indicated antibodies.

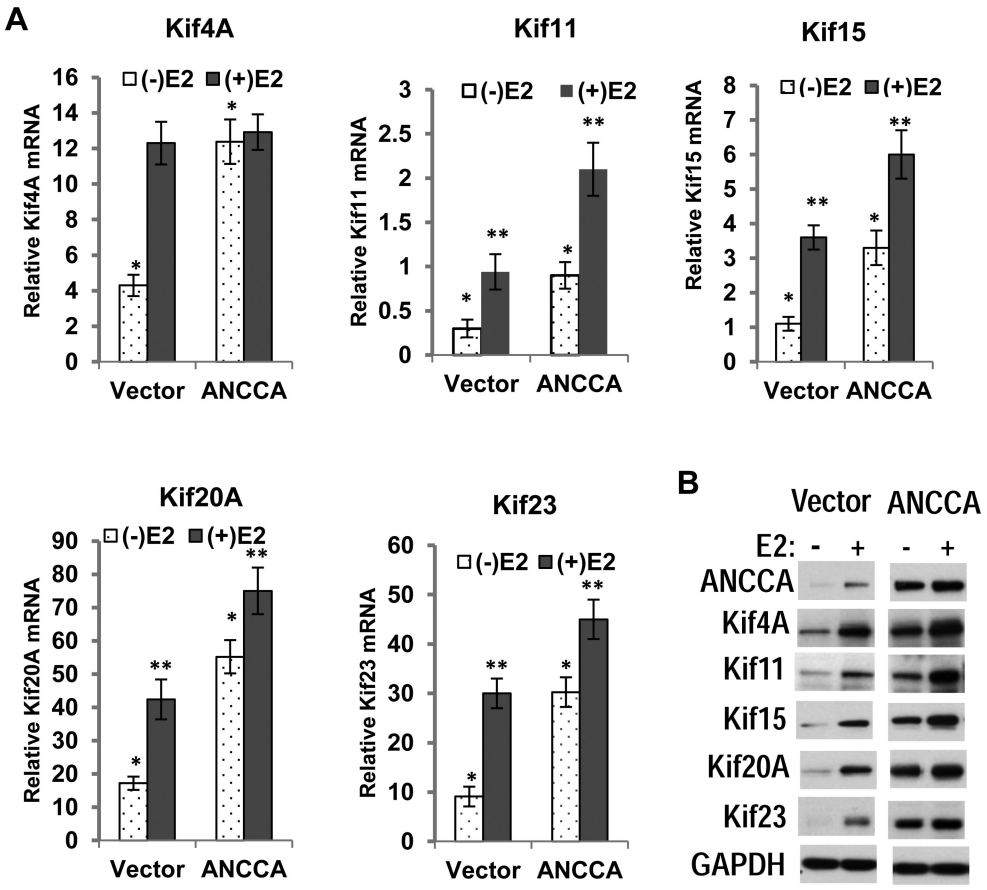

FIGURE 3.

ANCCA overexpression enhances estrogen-dependent and –independent expression of kinesins. A and B. MCF-7 cells without (Vector) or with (ANCCA) ectopic expression were hormone depleted and then treated with 17b-estrodial (E2) at 10^{-8}M for 24 hours before harvested for real-time RTPCR analysis (A) or immunobloting (B). Data represent the mean ± SD of three independent experiments (* $p < 0.01$ for groups without E2 for each Kif gene; ** $p < 0.05$ for groups with E2 for each Kif gene; paired *t* test).

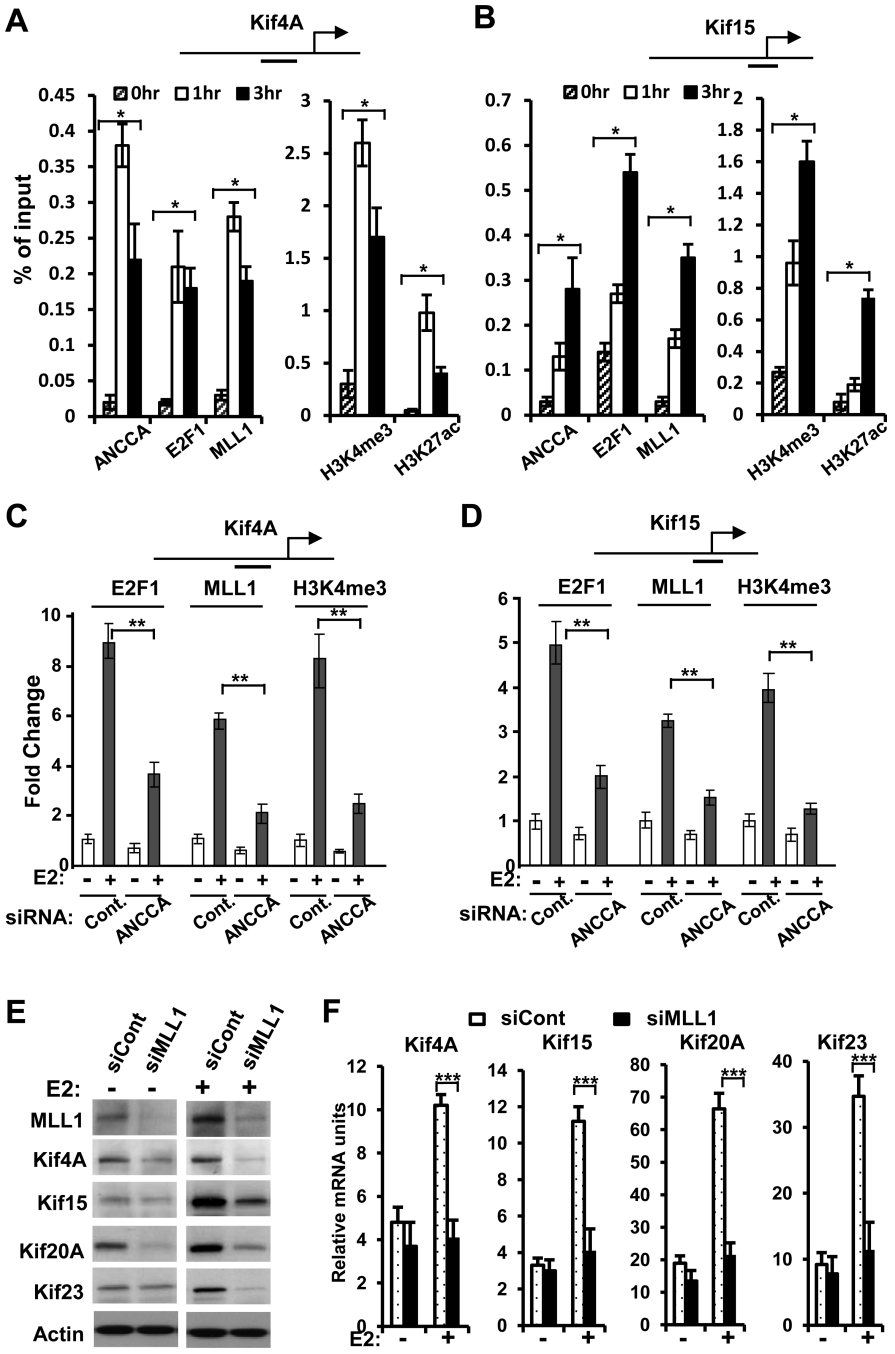

FIGURE 4.

Estrogen induction of kinesins involves assembly and function of ANCCA, E2F and MLL at the kinesin promoters. A and B. MCF-7 cells were treated with E2 for 1 or 3 hours and harvested for ChIP with indicated antibodies. ChIP and input DNA was analyzed by real-time PCR with primers amplifying a region in the promoter (presented by the long line for about 0.8 kb). ChIP data represent the mean ± SD of three independent experiments (*$p <$ 0.006 for groups between 0 hr and 3 hr of E2). C and D. MCF-7 cells were transfected with indicated siRNAs and treated with E2 for 3 hours before harvested for ChIP with antibodies

for E2F1, MLL1 and H3K4me3. ChIP data were analyzed as above and the normalized qPCR values of ChIP DNA from cells treated with control siRNA and without E2 were set as 1. ChIP data represent the mean ± SD of three independent experiments (** $p < 0.01$ for groups between siCont and siANCCA for each Ab). E and F. MCF-7 cells were treated with E2 and siRNA and analyzed, as in Fig. 2 for protein (E) and mRNA (F), except that mRNA analysis was performed with 24 hr E2 treatment (*** $p < 0.01$ for groups between siCont and siMLL1 for each Kif expression).

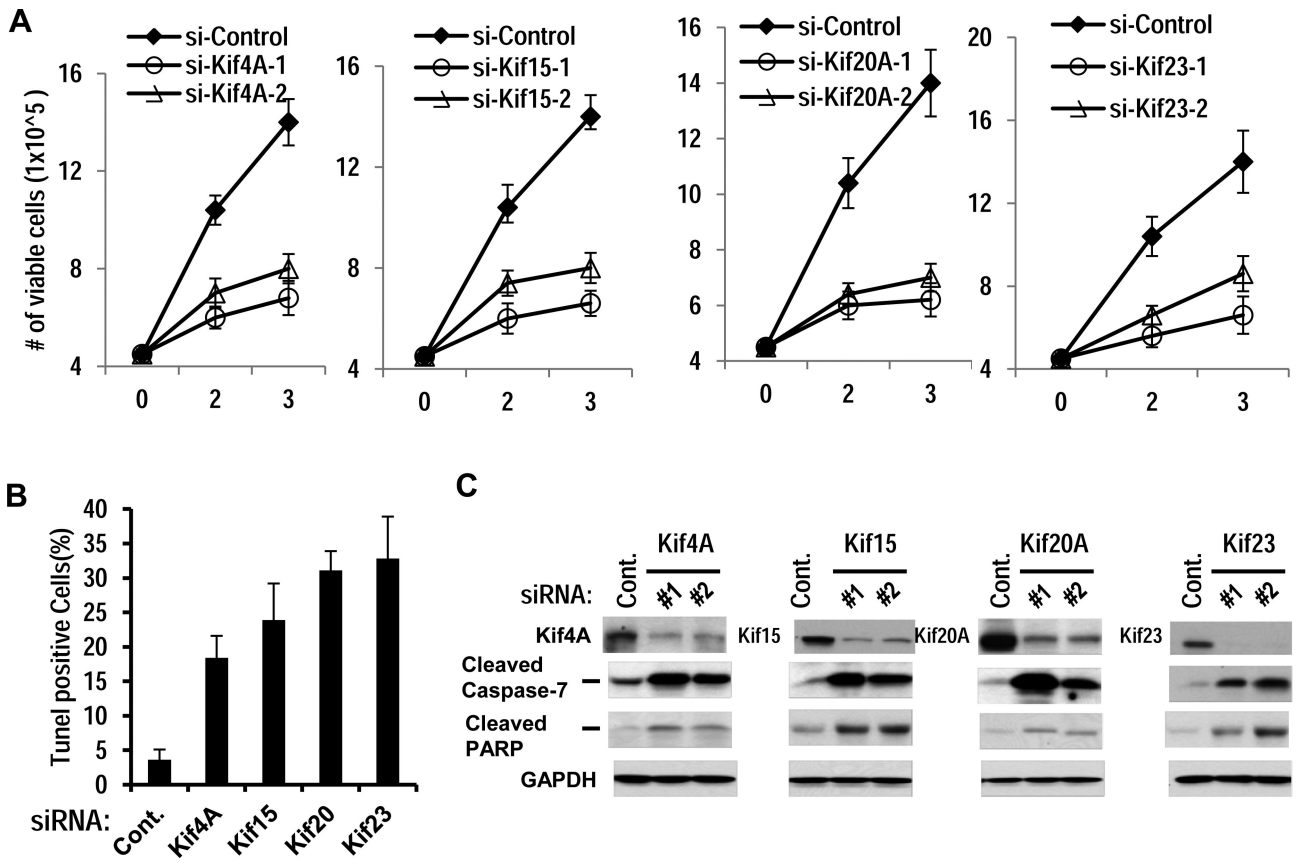

FIGURE 5.

Knockdown of kinesins decreases proliferation and survival of tamoxifen-sensitive BCa cells. A. MCF-7 cells were hormone depleted, siRNA transfected, and treated with E2 at 10^{-8}M for indicated days before harvested for counting of viable cells. B and C. MCF-7 cells were treated with siRNA and E2 as in A for 3 days before harvested for TUNEL assay and for immunoblotting with indicated antibodies. The experiments were repeated three times. The *p* values for differences between cells treated with control siRNA and kinesin siRNA were < 0.001.

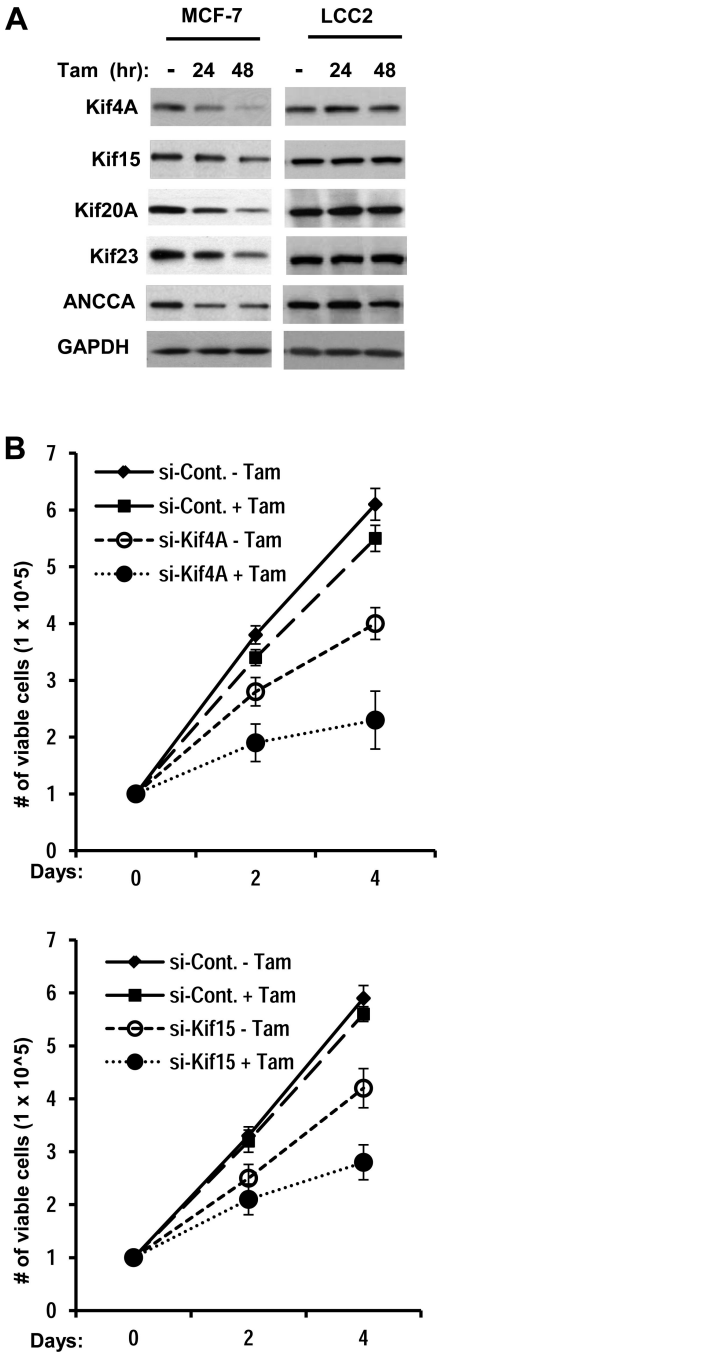

FIGURE 6.

Kinesins are important for proliferation and survival of tamoxifen- resistant BCa cells. A. MCF-7 and its tamoxifen-resistant subline LCC2 cells were treated with 4-hydroxytamoxifen (Tam) at 10^{-6}M for indicated hrs before harvested for immunoblotting with indicated antibodies. B. LCC2 cells were siRNA transfected and treated with 4-hydroxytamoxifen (Tam) at 10^{-6}M for indicated days before harvested for counting of viable cells. Cell numbers are presented as the mean ± SD of three independent experiments.

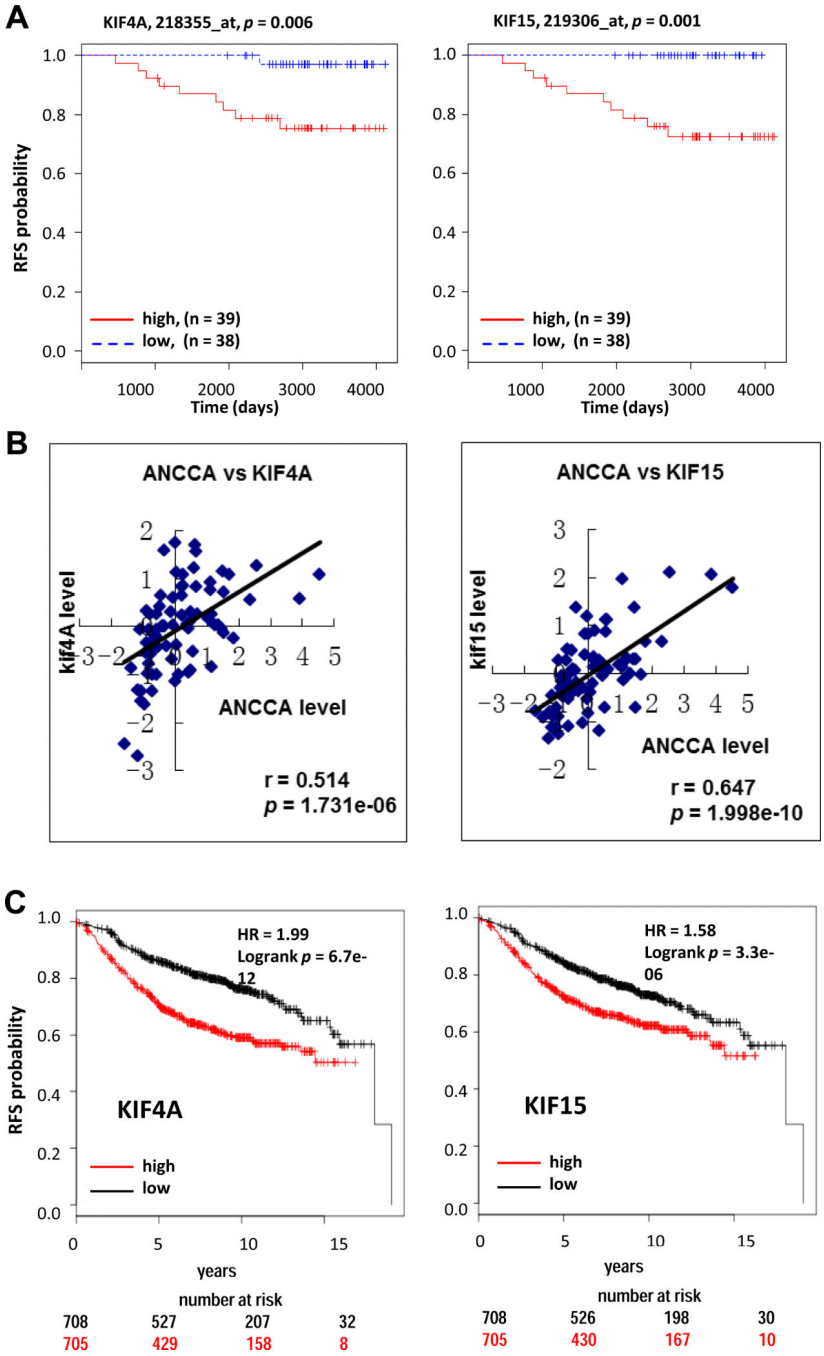

FIGURE 7.

Expression of ANCCA controlled kinesins is associated with poor outcomes in ER-positive breast tumors. A. Kaplan-Meier plots of relapse-free survival (RFS) of tamoxifen-treated patients stratified by high or low (above or below median) expression of indicated kinesins in a microarray data set as described in Materials and Methods. B. Scatter plot showing a positive correlation between ANCCA and indicated kinesin expression in an ER-positive tumor data set. C. Correlation of high expression of indicated kinesins with poor relapse-free survival of patients with ER-positive tumors was determined using an online survival

analysis tool as described in Materials and Methods. The *p* value for A and C was calculated by a log rank test and the *p* value for B was obtained by two-tailed *t*-test.