DATASET BRIEF

Identification of proteins associated with ligand-activated estrogen receptor α in human breast cancer cell nuclei by tandem affinity purification and nano LC-MS/MS

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Estrogen receptor α (ER-α) is a key mediator of estrogen actions in breast cancer (BC) cells. Understanding the effects of ligand-activated ER-α in target cells requires identification of the molecular partners acting in concert with this nuclear receptor to transduce the hormonal signal. We applied tandem affinity purification (TAP), glycerol gradient centrifugation and MS analysis to isolate and identify proteins interacting with ligand-activated ER-α in MCF-7 cell nuclei. This led to the identification of 264 ER-associated proteins, whose functions highlight the hinge role of ER-α in the coordination of multiple hormone-regulated nuclear processes in BC cells.

Keywords:
Breast cancer / Cell biology / Estrogen receptor / Interactome / Nuclear proteins / Tandem affinity purification

Estrogens control multiple cellular functions *via* the ERs (ER-α and ER-β), modular proteins belonging to the steroid/nuclear receptor family of intracellular homeostatic regulators [1]. In mammary epithelial cells, ERs are involved in the pathological processes leading to carcinogenesis and in the maintenance of the hormone-dependent BC phenotype [2]. According to the canonical model of action, 17-β-estradiol binding to cytosolic ER-α induces conformational changes of the receptor that promote its translocation to the nucleus, where it interacts with chromatin and promotes recruitment of co-regulator complexes at specific sites, leading to enhancement or repression of target gene transcription [3, 4]. In addition, an alternative non-genomic pathway of estrogen action has also been described, involving crosstalk of ER with different signaling pathways [5]. Both genomic and non-genomic pathways involve several molecular

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Abbreviations: BC, breast cancer; **CBP,** calmodulin-binding peptide; **ER,** estrogen receptor; **HDAC,** histone deacetylase; **NAM,** nucleophosmin; **NCL,** nucleolin; **TAP,** tandem affinity purification; **TEV,** tobacco etch virus; **TOP,** topoisomerase

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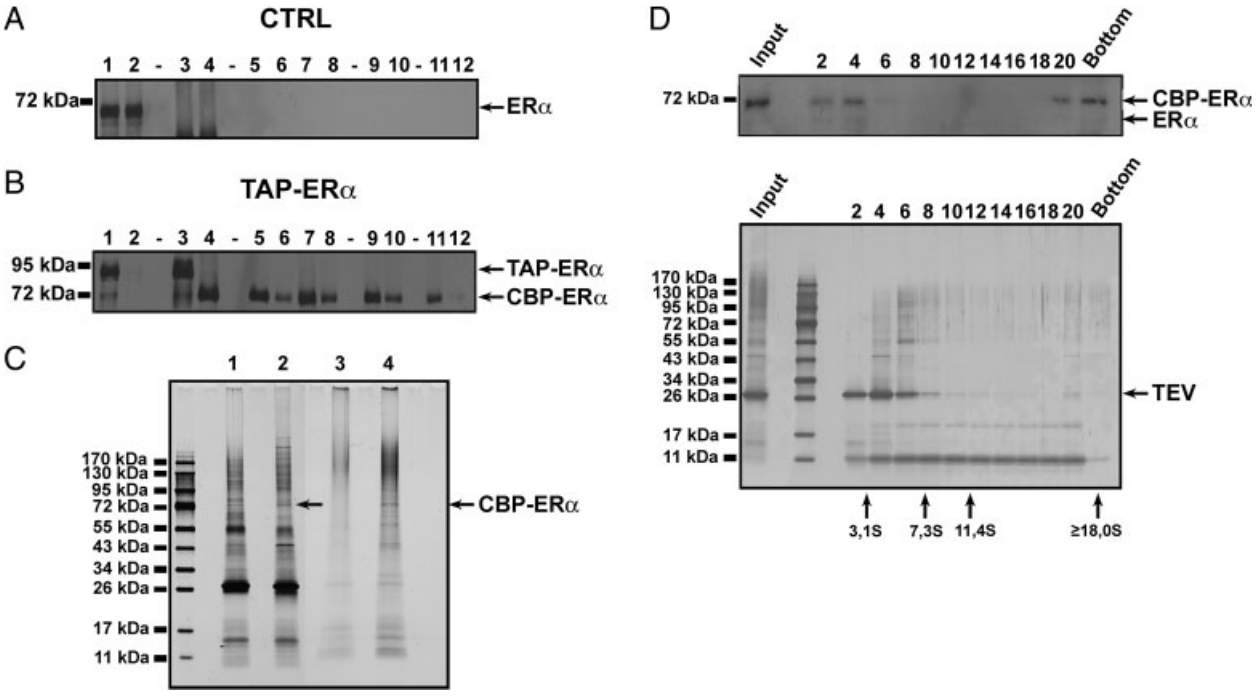

Figure 1. TAP-ER-α purification. (A and B) TAP-ER-α concentration was monitored by Western blotting using specific antibodies against the ER-α and the CBP moiety of the fusion protein. Nuclear extracts were prepared from TAP (control, A) and TAP-ER-α (B) expressing cells. Fifty milligrams of nuclear extract proteins was used for purification. TAP-ER-α concentration in the nuclear extract before and after IgG-Sepharose binding is shown in lanes 1 and 2, respectively. The amount of IgG-Sepharose bound receptor before and after TEV treatment is shown in lanes 3 and 4, respectively (1:200 of each sample analyzed), whereas the level of receptor in the eluted samples is shown in lanes 5 (first TEV elution) and 6 (second TEV elution; 1:100 of each sample analyzed). In lanes 7 and 8 are shown samples before and after calmodulin binding, respectively (1:200 of each sample analyzed). The amount of calmodulin–agarose bound receptor before and after EGTA elution is shown in lanes 9 and 10, respectively (1:100 of each sample analyzed); EGTA eluted receptor is shown in lanes 11 and 12 (1:65 of each sample analyzed). (C) Partially (lanes 1 and 2) and highly purified samples (lanes 3 and 4) from TAP (lanes 1 and 3) and TAP-ER-α (lanes 2 and 4) expressing cells were separated by SDS-PAGE and silver stained to verify protein content. (D) TEV eluate from TAP-ER-α expressing cells was fractionated by glycerol gradient sedimentation, and the indicated fractions were analyzed by Western blotting (using an antibody against ER-α) and silver staining. Arrows indicate the position of the sedimentation markers within the gradient.

components, each capable of influencing and/or mediating the ER activity by physically interacting with these proteins. Although the general mechanisms for ER regulation of gene transcription are well defined, much is still unknown about the nature and the composition of the multiprotein complexes involving ERs in the various cell compartments [6]. Indeed, the identification of such complexes in hormone-responsive BC cell nuclei is much sought after, to characterize the molecular pathways involved in the hormonal control of cancer cell growth and to identify the key regulators of such cascades, which may also represent new molecular targets for BC therapy.

Native protein purification by affinity chromatography followed by MS analysis of purified samples is the method of choice to identify biologically relevant macromolecular complexes. Recently, Cheng et al. [7] applied QNanoPX, a novel approach of quantitative nanoproteomics for protein complexes, to identify the proteins associated with DNA-bound ER-α from human BC cells. This study led to the identification of more than 250 novel ER partner proteins, indicating that the number of molecular partners of ERs in the nucleus is expected to be very high.

We have coupled TAP, which allows isolation of native multiprotein complexes by affinity chromatography, to glycerol gradient centrifugation and nanoLC-MS/MS to identify partners of ligand-activated ER-α in MCF-7 cell nuclei. To this aim, a TAP-tag comprising the IgG-binding domain of Protein A from *Staphylococcus aureus* and a calmodulin-binding peptide (CBP), separated by the cleavage site for the tobacco etch virus (TEV) protease, was fused to the C-terminus of coding region of human ER-α and stably transfected in MCF-7 cells to generate TAP-ER-α expressing cells [8] that were functionally characterized showing that the presence of the tag did not interfere with the physiological functions of ER-α. These MCF-7 cells stably expressing C-TAP-ER-α were used to purify nuclear ER-α-driven complexes from hormone-stimulated cells. Clones (C-TAP-ER-α) and control (mock transfected) cells were deprived of hormone for 5 days before stimulation with 17-β-estradiol for 2 h. Nuclear extracts (50 mg proteins) from

both cell lines were subjected to a TAP protocol setup in our laboratory [8], described in detail in section on Methods (Supporting Information). Technical and biological replicates were performed to assess the reliability and the reproducibility of the technique, and aliquots of samples derived from each step of the procedure were analyzed in each case by WB to assess the purification yields. In Fig. 1, the results obtained in a representative experiment are reported; to avoid saturation of chemiluminescent signals in some lanes, different amounts of each sample were loaded onto the gel. As shown in Fig. 1A, the amount of ER-α recovered from control cells was negligible. On the other hand, the results displayed in Fig. 1B show that TAP-ER-α could be efficiently purified. The considerable reduction of tagged receptor in the unbound sample (lane 2) with respect to the input proteins (lane 1), together with the high signal in the bound fraction (lane 3), indicates the efficient binding of TAP-ER-α to matrix-immobilized IgG. Although a fraction of cleaved protein (CBP-ER-α) remained in the matrix after elution (lane 4), it was possible to obtain enough partially purified receptor (lanes 5 and 6) to be able to proceed to the next step of purification. TEV eluates were pooled (lane 7) and subjected to calmodulin–agarose chromatography (lane 9). Even though the second affinity chromatography resulted less efficient than the first one, as a part of the fusion protein did not bind the affinity matrix (compare lanes 7 and 8) or was not eluted by EGTA (lane 10), we succeeded in obtaining a sufficient amount of highly purified sample (lanes 11 and 12) for MS analysis. Protein quantification and ER-α ELISA assays performed in all samples (see Table 1 in Supporting Information), confirmed that it was possible to obtain, after the first purification step (binding to IgG-Sepharose followed by TEV protease cleavage) samples with about 15–20% recovery of the tagged 'bait' protein; after the second purification step (binding of the TEV eluates to calmodulin–agarose, followed by extensive washes and elution) we could reach significant ER-α purification, with an overall yield of 8–10% and the receptor representing about 0.1% of the total proteins in the sample. Partially and highly purified samples from control and TAP-ER-α expressing cells were then subjected to SDS-PAGE and silver staining, to verify their complexity (Fig. 1C). Despite the relatively high background, clear difference between the control (lane 1) and the sample (lane 2) is evident already in the TEV eluates from IgG-Sepharose. Such differences are more evident in the EGTA eluates from calmodulin–agarose (compare lanes 3 and 4). In order to evaluate the size of the purified ER-α-containing complexes, TEV eluates were subjected to glycerol gradient fractionation. Analysis of the fractions obtained by WB and silver staining (Fig. 1D) shows that ER-α is mainly localized in two sections of the gradient, corresponding to complexes of low molecular weight (fractions from 1 to 6, where probably ER-α sediments as a dimer – note the co-sedimentation of tagged and endogenous ERs – or associated with small molecules) and high molecular weight (fractions 19 to bottom, where ER-α is associated with large and possibly multiple interactors). The concentration of receptor detectable by ELISA (Supporting Information Table 1) and its behavior in glycerol gradients is consistent with the fact that we achieved isolation of ER-α in medium-to-large complexes, likely to comprise several interacting proteins.

The first protein identifications were performed on calmodulin–agarose eluates from replicates of control and TAP-ER-α samples. The purified protein mixtures were analyzed directly using in-solution digestion of the proteins followed by sensitive nanoLC-MS/MS analysis of the resulting peptides (see section on Methods in Supporting Information). This led to the identification of 13 proteins in highly purified samples from TAP-ER-α but not from control cells (listed in Table 2 of Supporting Information), including human ER-α. This result was not in accordance with the expected results, suggesting that a large fraction of the ER-α interactors was lost during the final purification step, due to the low sample yield. For this reason, TEV eluates before and after glycerol gradient sedimentation (high molecular weight fractions) were analyzed by nanoLC-MS/MS. Samples prepared in the same way from control cells were analyzed in parallel and any protein identified in purified samples from TAP-ER-α cells that was also present in any purified sample from control cells was discarded. This analysis led to the identification of 216 TAP-ER-α-specific proteins in whole IgG eluates and 147 in the same eluates fractionated by glycerol gradient centrifugation (listed in Table 3 of Supporting Information), including 4 of the proteins identified also in calmodulin eluates. A summary of all the results obtained is reported in the Venn diagram shown in Fig. 2A, where the overlaps between the different data sets are summarized. It is worth mentioning that the differences observed for IgG eluates before and after glycerol gradient fractionation could be due either to the dissociating conditions inherent to the ultracentrifugation, which caused dissociation of molecules from the ER-α-containing complexes, or to the selection of the high molecular weight fractions. Moreover, the fractionation procedure might have caused removal of abundant and/or peripheral molecules, thus facilitating the identification of less abundant protein species tightly associated with the receptor. This would explain the identification in the post-gradient fractions of ER-α partners other than those identified in the whole IgG eluates. When combined, these results show that we were able to identify 264 ER-α-interacting proteins. A comparison of this list with those relative to known ER-α interactors from UniHi and PINA databases and that recently reported by Cheng et al. [7] shows that 164 proteins reported here represent newly identified molecular partners of ER-α in BC cell nuclei (see Tables 2 and 3 of Supporting Information).

The set of 108 TAP-ER-α-specific interactors common between the two analyses carried out on the IgG eluate and comprising also four proteins identified in calmodulin eluates was selected for functional characterization. A Gene

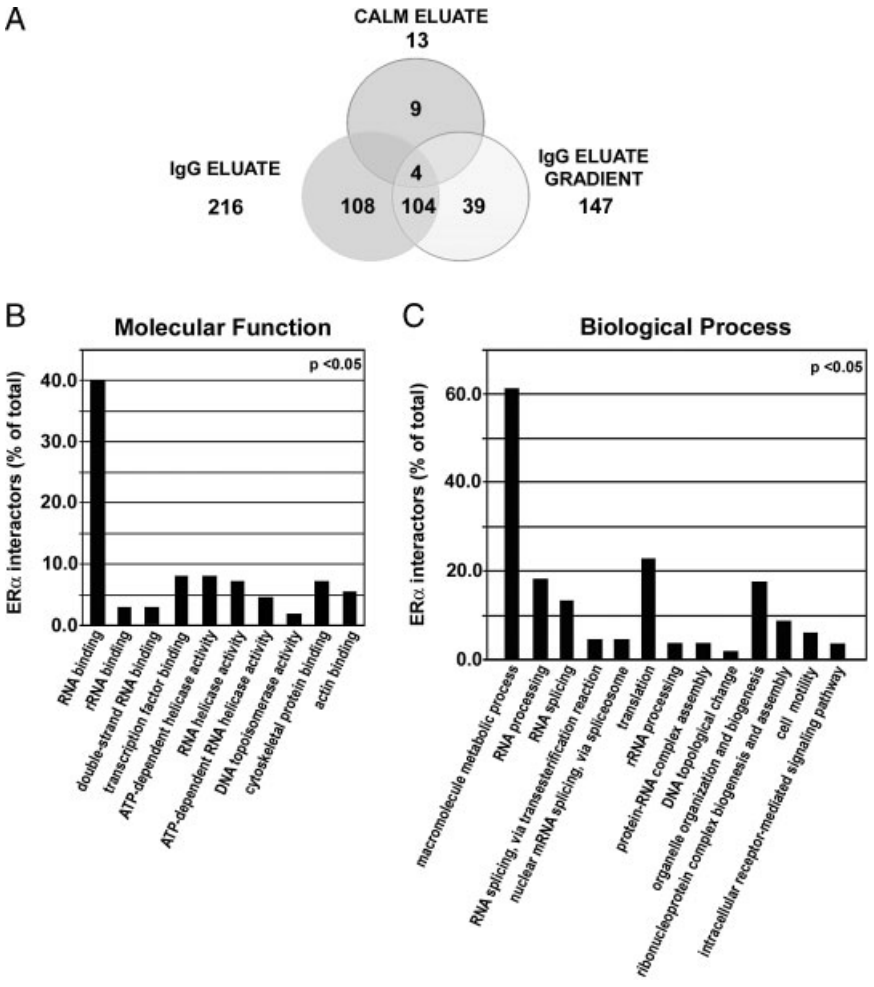

Figure 2. Protein identification results. (A) Venn diagram of all the TAP-ER-α-specific proteins identified in the different samples analyzed. (B and C) Functional analysis according to Gene Ontology of the 108 ER-α interactors identified in both IgG-Sepharose eluates (IgG ± gradient), including 4 interactors identified also in the calmodulin–agarose (CALM) eluate.

Ontology analysis (see section on Methods in Supporting Information) was performed on this protein set to identify molecular functions (Fig. 2B) and biological processes (Fig. 2C) significantly represented. The most representative molecular functions resulted to be: RNA binding, transcription factor binding, ATP-dependent and RNA helicase activity, DNA topology, cytoskeletal and actin-binding activity. On the other hand, the most significant biological processes involving the ER-α interacting proteins identified are represented by metabolic processes, RNA processing and splicing, translation, organelle organization and biogenesis, ribonucleoprotein complex biogenesis and assembly, cell motility and intracellular receptor-mediated signaling. Taken together, this indicates the involvement of ER-α in the control of most fundamental processes and functions taking place in BC cell nuclei including, among others, RNA biosynthesis and maturation and β-actin nuclear network [1–3, 8].

Finally, a computational network analysis based on both online databases and bibliography (see section on Methods in Supporting Information) was performed, to search for known evidence of physical/functional interactions between the various components of the ER-α nuclear interactome identified. This was performed by combining the results obtained with Cytoscape software with those of a UniHi network analysis described earlier [8]. The resulting network (Fig. 3) comprises several 'branches', all either directly or indirectly linked to ER-α, suggesting recruitment to ER-α of complexes comprising transcriptional co-regulators, such as PELP-1, enzymes affecting DNA topology and chromatin remodeling (helicases, histone deacetylase (HDACs), topoisomerases (TOPs), etc.), proteins regulating pre-mRNA splicing (SR proteins), components of the β-actin nuclear network (ACTB, GSN and MYO1C, see also [8]) and ribosomal proteins. Taken together, the Gene Ontology and network analyses indicate that the key processes controlling central events in the cell are tightly interconnected and co-regulated. Indeed, the results of this study suggest that ligand-activated ER-α may act as a bridging factor, linking transcription with RNA splicing and processing, cell cycle and ribosome biogenesis in hormone-stimulated cells. The most compelling evidence supporting this view is represented by the presence within the identified protein set of several DEAD/H box RNA helicases, known to be involved

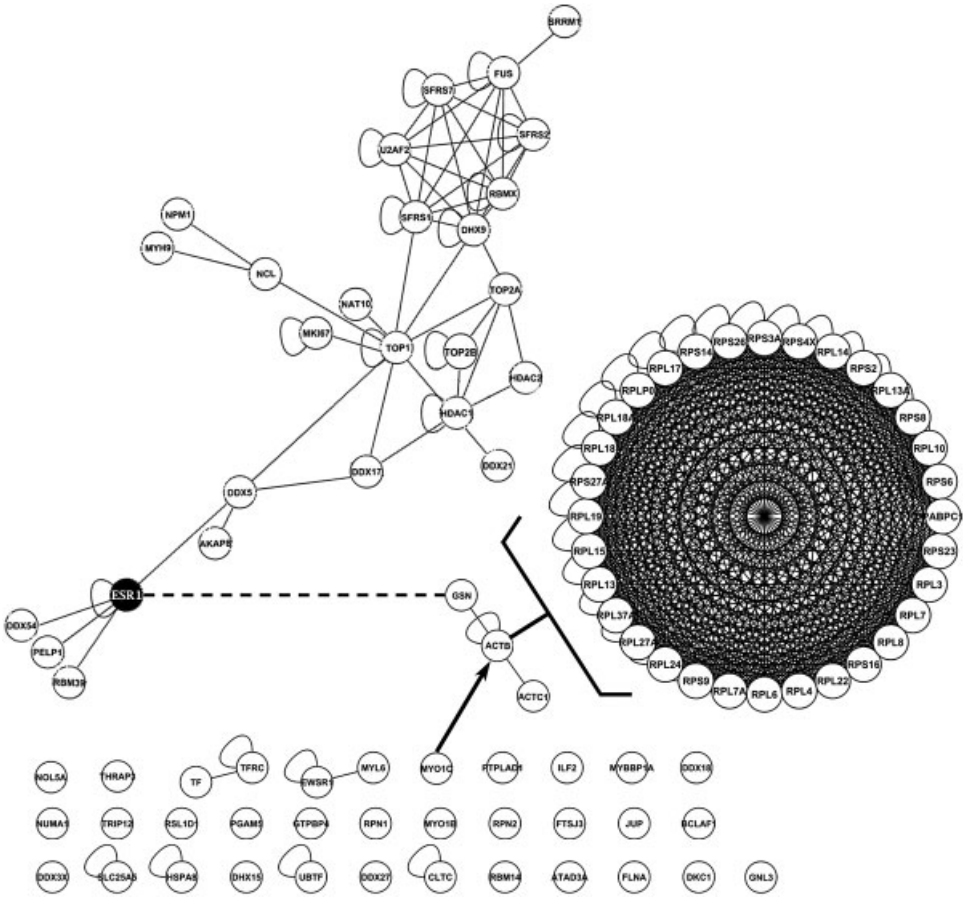

Figure 3. Map of the ER-α-dependent protein interaction network identified in hormone-stimulated MCF-7 cell nuclei. To draw the map of ER-α interactions shown, the results of *in silico* networking analysis were combined with those demonstrated experimentally under the same conditions (dotted line [8]). Solid lines shows protein–protein physical/functional interactions derived from Cytoscape; solid bold lines and arrow indicate protein–protein interactions reported in UniHI database.

in processes requiring a remodeling of RNA structure, such as pre-mRNA processing [9] but playing important roles also in different cellular processes [10]. Interestingly, experimental evidence indicates that these proteins act in concert with ER-α in transcriptional regulation of estrogen target genes, by first inducing stabilization of the initiation complex of transcription, acting as bridges between CREB-binding protein and RNA polymerase II, and then by promoting recruitment of co-regulators to ER-responsive promoters [10–12]. Furthermore, RNA helicases like RNA helicase A (DHX9) can also regulate transcription in an ATP-dependent manner, inducing chromatin remodeling through their interaction with TOP2A [10, 13]. In BC cells DHX9 regulates the transcription of genes involved in DNA repair also by mediating the interaction between RNA polymerase II holoenzyme and BRCA1 [14, 15]. Moreover, DP97 (DDX54), p68 (DDX5) and p72 (DDX17) can act as ER-α co-repressors either mediating directly receptor-mediated transrepression [16] or through their interaction with HDAC1 and -2 [17]. Furthermore, RNA helicases are also involved in pre-mRNA processing, splicing, ribosome biogenesis, RNA turnover, RNA export and translation [18, 19]. A possible link between RNA processing and transcriptional activity of steroid nuclear receptors has already been suggested [16, 20]. The data presented here are in agreement with such hypothesis, that is further strengthened by the presence in the complexes identified of splicing factors such as FUS and RNA-binding protein 39 (RBM39), known to be involved in the coordination of nuclear receptors mediated transcription and splicing processes [20–22]. Moreover, U2AF2, a protein present in this data set but not previously associated with ER-α or other steroid hormone receptors, is an interactor of RNA-binding protein 39, belonging to the same family of pre-mRNA splicing factors [23], which includes the heterogeneous nuclear ribonucleoprotein G (RBMX) [24] and other ER-α-associated proteins belonging to the SR family, including splicing factor arginine/serine-rich 1, -2, -7 (SFRSs) and SRm160 (SRRM1). SR proteins are known to modulate alternative splicing of specific pre-mRNA [25] and to exert a role in tumorigenesis by regulating the processing of mRNAs, such as splicing of CD44 pre-mRNA during the early and intermediate stages of mammary carcinogenesis [26]. Furthermore, the RNA-binding protein FUS is believed to play, together with SRm160 and other SR proteins, an important role in spliceosome assembly and splice site selection, once associated with the nuclear matrix [27]. Although there is no previous evidence of a direct connection between ER-α and SR

proteins, several reports demonstrated how this family of proteins links RNA processing with transcription through their interaction with RNA polymerase II [28, 29] and is thought to be implicated in cell-cycle control [30] and to be subject to phosphorylation by DNA TOP1 [31], all processes directly involving ER-α in hormone-responsive BC. Indeed, the identification of nucleolar (nucleolin, NCL and nucleophosmin, NPM1) and ribosomal proteins associated with the receptor provides further evidence of an existing cross-talk between the distinct cellular processes described above in estrogen-stimulated BC cells. Previous studies have shown a role of estrogen in the regulation of nucleolar RNA synthesis [32] and the co-localization of ER-α and NCL in cell nucleolus [33, 34] where NPM1 is also localized. Both NCL and NPM1 are mainly not only responsible for controlling ribosome biogenesis [35, 36] but also involved in other key cellular events, like cell-cycle progression [37–39] and tumorigenesis [40]. Ribosomal proteins (RPLs and RPSs) are mainly involved in ribosome biogenesis and structure [41], but recently different functions have been associated with these molecules, such as regulation of transcription and translation [42, 43] and cell proliferation [44]. NPM1 and ribosomal proteins are also associated with β-actin and its interacting proteins that all co-purify with ER-α [8] and play a role in ribosome biogenesis and maturation [45] and in dynamic remodeling of multiprotein complexes in the nucleus during estrogen-regulated transcriptional regulation [8]. In the scheme reported in Fig. 3, it appears that, once activated by its cognate hormone and bound to DNA, ER-α recruits co-regulators (PELP-1, RBM39, DDX54, etc.) [16, 23, 46] to control target genes transcription and associates with helicases mediating transcriptional initiation, chromatin remodeling and cell cycle (TOPs, HDACs, MKI67, etc.), all processes probably occurring in the nuclear matrix, where ER-α has been found anchored together with AKAP8 and DHX9 [47, 48]. Concomitantly, ER-α also recruits SR proteins to regulate pre-mRNA processing, and through NCL, NPM1 and β-actin, together with its interacting proteins and ribosomal proteins in nucleoli [8, 37, 38, 49], controls ribosome biogenesis and assembly, a fundamental process for effective cell growth and duplication.

In conclusion, the results described here provide a molecular chart useful to dissect and analyze multiple components and pathways of the estrogen-dependent regulatory cascade stemming from ligand-activated ERs in hormone-responsive BC cells.

The protein interactions from this publication have been submitted to the IMEx (http://imex.sf.net) consortium through IntAct [50] and assigned the identifier IM-13780.

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The authors declare no conflict of interest.

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