Identification of the MLL2 Complex as a Coactivator for Estrogen Receptor α

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A novel estrogen receptor (ERα) coactivator complex, the MLL2 complex, which consists of MLL2, ASH2, RBQ3, and WDR5, was identified. ERα directly binds to the MLL2 complex through two LXXLL motifs in a region of MLL2 near the C terminus in a ligand-dependent manner. Disrupting the interaction between ERα and the MLL2 complex with small interfering RNAs specific against MLL2 or an MLL2 fragment representing the interacting region with ERα significantly inhibited the ERα transcription activity. The MLL2 complex was recruited on promoters of ERα target genes along with ERα upon estrogen stimulation. Inhibition of MLL2 expression decreased the estrogen-induced expression of ERα target genes cathepsin D and to a lesser extent pS2. In addition, MCF-7 cell growth was also inhibited by the depletion of MLL2. These results demonstrate that the ERα signaling pathway is critically dependent on its direct interaction with the MLL2 complex and suggest a central role for the MLL2 complex in the growth of ERα-positive cancer cells.

The biological effects of estrogen are mediated by estrogen receptors (ER) in estrogen responsive tissues. There are two types of estrogen receptors, ERα and ERβ. The well studied ERα is involved in normal mammary gland development as well as breast cancer initiation and progress (1–4). ERα has two transcriptional activation domains, the N-terminal activation domain AF-1 and the C-terminal activation domain AF-2. Upon estrogen binding, ERα undergoes a conformational change and regulates the expression of its target genes (5, 6). ERα, just as other nuclear receptors, requires coactivators and corepressors for its function. A large number of ERα coactivators, including the three members of the SRC-1 family (SRC-1, SRC-2/GRIP1/TIF2, and SRC-3/AIB1/ACTR/pCID/RAC3/TRAM1) (7–9), CREB-binding protein (CBP/p300), and TRAP220 (DRIP 205, PBP) (10, 11), have been identified to date. Most of the coactivators interact with the AF-2 domain of ERα in a ligand-dependent manner. Some of these cofactors are intrinsic enzymes with the activity of acetyltransferase or methyltransferase or are able to recruit such enzymes (12–14) to modify histone composition of chromatin to make transcription factors accessible to specific regions of the genome. The varying patterns of histone modification are now referred to as a histone code and are proposed to be epigenetic markers for determining gene activation status (15). Some nuclear receptor coactivators (corepressors) are presented as multiprotein complexes, and these steady-state protein complexes probably act as functional units of nuclear receptor coregulators (16). ERα coactivator TRAP220/PBP exists in the multiprotein TRAP complex, which has a molecular mass of ~2 MD and is composed of more than 30 subunits (17). The TRAP complex facilitates ERα actions by synergizing basal transcription machineries. The ERα coactivator PRIP (TRBP, TRAP250, NRC, and AIB3) is also demonstrated to stay in a massive steady-state complex ASCOM (18), which consists of MLL4, PRIP (ASC2), MLL2, ASH2, RBQ3 and α/β-tubulins.

Mixed lineage leukemia (MLL) also termed ALL-1, HRX, and HTRX is often involved in chromosome translocations in human acute leukemia and functionally essential for maintaining the expression of the HOX gene during embryo development (19, 20). MLL is the mammalian homolog of the yeast SET1 protein, which is present as a SET1 complex harboring the methyltransferase to modify chromatin histone H3 (19, 22). MLL is reported to be present within a large multiprotein complex composed of MLL1, ASH2, RBQ3, WDR5, and Menin, which is a product of the MEN1 tumor suppressor gene (23–26). Despite extensive research work being done on MLL, little is known about the function of MLL2 (or ALR), a close homologue of MLL, which is a polypeptide of 5262 amino acids encoded by an mRNA that is over 18-kb long (27).

Identifying ERα interacting complexes is essential to understanding how ERα controls gene transcription and how it is involved in the development of breast cancer. Here we describe the identification of a novel protein complex, the MLL2 complex, consisting of MLL2, ASH2, RBQ3, and WDR5, which is required for ligand-dependent ERα transactivation. Disruption of the complex repressed estrogen-dependent expression of ERα target genes and also inhibited growth of MCF-7 cells.

EXPERIMENTAL PROCEDURES

Plasmids and Antibodies—pCDNA3.1-ERα, ERE-TK-LUC, and glutathione S-transferase (GST)-ERα (LBD) were described as before (28). GST-MLL2888 was constructed by inserting the C-terminal 172-amino-acid coding region of mouse MLL2 into the EcoRI/XhoI site of pGEX-5X-1 vector (Amersham Biosciences). PCMV-ASH2 and PCMV-RBQ3 clones were purchased from Open Biosystems. PCMV-WDR5 was generated by releasing WDR5 full-length cDNA from pOTT7-WDR5 (Open Biosystems) and inserted into the EcoRI/Sall site of pCMV-Sport6. All constructs were confirmed by sequencing.

QuikChange multisite-directed mutagenesis kit (Stratagene) was used to create amino acid mutations of the fifth and sixth LXXLL motifs in PCMV-MLL2 (from LXXLL to LXXAA) with mutagenic primers 5′-CTGTCGTTCGAGAAGGACGCGCCGACCCGGCCAGAAAGAA-TGTG-3′ and 5′-AAGCGGCTTCGCTGCGGCGGACCATCCAGAAAGGAC-3′. The mutations were confirmed by sequencing.
GST-MLL2<sup>2<sup>nd</sup></sup> proteins were produced in the <i>Escherichia coli</i> BL21 codon plus strain (Stratagene), and purified by glutathione-Sepharose beads 4B. The 172-amino-acid MLL2 fragment was released from the GST fusion protein by treatment with thrombin. Polyclonal rabbit antiserum against the C-terminal 172-amino-acid of MLL2 was developed by the Spring Valley Laboratory. Rabbit polyclonal anti-ASH2 and anti-RBQ3 monoclonal antibody was purchased from Santa Cruz Biotechnology. Polyclonal rabbit anti-PRIP was generated in previous work (29). The polyclonal anti-WDR5 antisemur was a kind gift of Dr. W. Herr (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

Isolation of ERα AF2-associated Complexes—DU4475 cells, an ER-negative breast cancer cell line, were grown in a suspension culture by the National Center Culture Center (Minneapolis, MN). DU4475 cell nuclear extracts were prepared as described (30) and precluded by incubating with an excess amount of GST-Sepharose 4B beads. Immobilized GST-ERα (LBD) fusion proteins were precultured for 1 h at 4°C in GST binding buffer (20 mM Tris-HCl, pH 7.9, 180 mM KCl, 0.2 mM EDTA, 0.05% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) containing bovine serum albumin (1 mg/ml). Bead-immobilized proteins were then incubated at 4°C overnight with precleared DU4475 nuclear extract in the presence or absence of 100 nM 17β-estradiol (E2). After washing with GST wash buffer (GST binding buffer with 0.1% Nonidet P-40) four times, proteins were eluted with binding buffer containing 30 mM reduced glutathione. Elutants were concentrated and layered on top of a 4.5-ml linear 10–40% glycerol gradient in 5-ml tubes. Following a 16-h centrifugation at 4°C using an SW40 rotor (Beckman) with a speed of 40,000 rpm, 200 μl of solution from each fraction was collected from top to bottom. Ovalbumin (44 kDa), β-globulin (158 kDa), and thyroglobulin (667 kDa) were used as protein standards.

Immunoprecipitation—DU4475 nuclear extracts, 2.0 mg, were pre- cleared with polyclonal rabbit anti-PRIP for 2 h at 4°C and high speed centrifugation to remove precipitates. The supernatant was divided equally and immunoprecipitated by specific antibodies or control IgG in GST binding buffer (20 mM Tris-HCl, pH 7.9, 180 mM KCl, 0.2 mM EDTA, 0.05% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). After extensive washing using the same buffer, the bound proteins were eluted by boiling in SDS-PAGE sample buffer, resolved by SDS-PAGE, transferred onto a nitrocellulose membrane, and subjected to Western blot analysis using antibodies as indicated.

**GST Pull-down Assays**—GST and GST fusion proteins were produced in the <i>E. coli</i> BL21 codon plus strain (Stratagene) and purified by glutathione-Sepharose beads 4B according to the manufacturer’s instructions (Amersham Biosciences). Proteins were in vitro translated using TNT quick coupled in vitro transcription and translation kit (Promega) and labeled with [35S]methionine. A 20-μl volume of GST fusion protein bead slurry was incubated with 5 μl of in vitro translated protein for 2 h in 500 μl of NETN (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.7 mM EDTA, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride) in the presence or absence of 100 nM E2. After the beads were washed four times with binding buffer, 30 μl of Laemmli protein loading buffer was added and boiled for 3 min. Samples were separated by SDS-PAGE, amplified, dried, and autoradiographed.

**Cell Culture and Transfection**—A total of 2×10<sup>5</sup> MCF-7 cells were seeded in 6-well plates 24 h before transfection. Cells were transfected with 1.25 μg of ERE-Luc, 20 ng of pCDNA3.1-ERα, 2 μg of siRNA or plasmid as indicated, and 0.1 μg of pCMVβ (Clontech) using Lipofectamine 2000 reagent (Invitrogen). The cells were split equally into two different wells on the next day, and the cell medium was changed to phenol red-free modified Eagle’s medium containing 10% of charcoal stripped FBS. The cells were treated with or without 100 nM E2 overnight and lysed for luciferase and β-galactosidase activity assays (Tropix, Bedford, MA). Three independent transfections were performed for each assay.

**Chromatin Immunoprecipitation**—MCF-7 cells were maintained in phenol red-free modified Eagle’s medium supplemented with 10% charcoal-dextran-stripped fetal bovine serum. After the hormone treatment for 45 min, cells were cross-linked with 1% formaldehyde at room temperature for 10 min. After the cells were collected, chromatin immunoprecipitation was performed as described (31). Briefly, nuclei were prepared by incubating the cells in cell lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% Nonidet P-40) containing protease inhibitors (phenylmethylsulfonyl fluoride, pepstatin, aprotinin, and leupeptin) for 10 min on ice. Nuclei were precipitated and lysed with nuclear lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, 1% SDS, phosphatase and protease inhibitors) for 10 min on ice. The chromatin was sheared by sonication to an average size of 500–1000 base pairs. Soluble chromatin was diluted 10-fold with dilution buffer (16.7 mM Tris-Cl, pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl) and precleared with salmon sperm DNA-blocked preimmune IgG protein A beads. Immunoprecipitation was performed by incubating the precleared cell lysate with spe-
cific antibodies at 4°C for 12 h. Immune complexes were collected by binding to protein A beads and were washed sequentially for 10 min with washing buffer I (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 150 mM NaCl), buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.1, 500 mM NaCl), and buffer III (0.25 mM LiCl, 1% Nonidet P-40, 1% deoxycholate, 10 mM Tris-Cl, pH 8.1). DNAs were extracted from precipitates and used as templates in PCR amplifications. The primer pair for cathepsin D (CTSD) 5'-GGACGGCCTGAGCCCGG-3' and 5'-GGGACAACCTCGGCGGACG-3' and primer pair for p53 5'-CCACCTGACCT-TAATCCA-3' and 5'-CTGTTTTGCGCTCCAAAA-3' were used in PCRs. In all cases, PCR was performed with a serial dilution of input and various cycles (29–35 cycles) to ensure that amplification was maintained in the linear range.

**siRNA Transfection and Real Time PCR**—siRNA smart pool oligonucleotides targeting human MLL2 and control siRNAs were purchased from Dharmaco. MCF-7 cells were transfected with control siRNA or MLL2 siRNA using Lipofectamine 2000 reagent (Invitrogen) and cultured in phenol red-free modified Eagle's medium containing 10% charcoal stripped fetal bovine serum. After 24 h, the cells were treated with 10 nM E2 for 4 h, and total RNA was then isolated using TRizol reagent. TaqMan probes targeting human MLL2, p53, and cathepsin D mRNA were purchased from Applied Biosystems. The mRNA levels were detected on Applied Biosystems 7900HT fast real time PCR system. The primers for cathepsin D are 5'-GCAGCACAGGGACCCA-3' and primer pair for cathepsin D 5'-TTGCCGAGCAGTGCA-3', and its probe is 5'-CAGACA- GAGACGTGTA-3'. The primer for cathepsin D are 5'-TGATCGAGAGA-GAGCTGTTG-3' and 5'-GAGTCTGTGCCACCCAGCA-3', and its probe is 5'-CCTGAGGACGAGGACC-3'.

**Cell Proliferation Assay**—MCF-7 cells were plated at a density of 5 × 10^4 cells/well in 6-well plates. The cells were transfected with 2 μg of siRNAs using Lipofectamine 2000 reagent on the following day. After 24 h, the cells were trypsinized, counted, and seeded into 24-well plates with a density of 2 × 10^4 cells/well in fresh phenol red-free modified Eagle's medium with 10% fetal bovine serum. After 24 h, the cells were treated with 10 nM E2 for 4 h, and total RNA was then isolated using TRizol reagent. TaqMan probes targeting human MLL2, p53, and cathepsin D mRNA were purchased from Applied Biosystems. The mRNA levels were detected on Applied Biosystems 7900HT fast real time PCR system. The primers for cathepsin D are 5'-GCAGCACAGGGACCCA-3' and primer pair for cathepsin D 5'-TTGCCGAGCAGTGCA-3', and its probe is 5'-CAGACA-GAGACGTGTA-3'. The primer for cathepsin D are 5'-TGATCGAGAGA-GAGCTGTTG-3' and 5'-GAGTCTGTGCCACCCAGCA-3', and its probe is 5'-CCTGAGGACGAGGACC-3'.

**RESULTS**

**Isolation of the MLL2 Complex Interacting with ERα AF2 Domain**—To isolate potential protein complexes that facilitate the ligand-dependent transcription activity of ERα, the GST-fused ERα ligand binding domain GST-ERα (LBD) was used to pull down its interacting proteins from the DU4475 cell line nuclear extracts in either the presence or absence of E2. Gel retardation gradient was subsequently performed to collect the high molecular mass fractions (>600 kDa), which were subjected to tandem mass spectrometry analyses. As expected, we detected the TRAP/PBP complex, which was abundant and consisted of more than 30 subunits of SRC-1 family, PRIP, and CBP/P300. In addition, mass spectrometry identified multiple peptides from the MLL-related protein MLL2 (ALR-1) (GenBank™ accession number 4505197), (ASH2 (accession number 4009396), retinoblastoma-binding protein RBQ-3 (accession number 755750), and WD repeat domain 5 protein (WDR5) (accession number 12804457) (Fig. 1A), which likely formed a new protein complex. The presence of MLL2, ASH2, RBQ-3, and WDR5 in the proteins pulled down by GST-ERα (LBD) was confirmed by Western blot (Fig. 1B).

To demonstrate that MLL2, ASH2, RBQ3, and WDR5 indeed formed a complex, we generated a rabbit polyclonal antibody against the C-terminal 172 amino acids of mouse MLL2 that specifically recognizes MLL2 of both mouse and human origin. The DU4475 cell nuclear extract was precipitated with anti-MLL2, and the precipitates were then examined by Western blot using antibodies against ASH2, MLL2, and RBQ3, respectively. The immunoprecipitates were separated by SDS-PAGE and then immunoblotted with the antibodies indicated on the right of the panels. MLL2 complex and ASCOM complex are distinct. DU4475 cell nuclear extracts were immunoprecipitated with an excess amount of protein A bound anti-PRIP or preimmune rabbit Iggs. The equal amounts of proteins from the supernatants after depletion were separated by SDS-PAGE and then immunoblotted with indicated antibodies.
FIGURE 3. A, ERα does not bind to ASH2, RBQ3 or WDR5. GST pull-down assays were performed using [35S]methionine-labeled full-length ASH2, RBQ3, or WDR5 with GST-ERα (LBD) fusion protein. B, ERα interacts with the MLL2 complex through two LXXL motifs in a MLL2 region (amino acid 4167–4780). [35S]methionine-labeled truncated MLL2 fragments containing LXXL motifs were pulled down with GST-ERα (LBD). C, the SET domain of MLL2 binds to ASH2 but not to RBQ3 or WDR5. GST pull-down assays were performed using GST-MLL2Set and [35S]methionine-labeled full-length ASH2, RBQ3, or WDR5.

FIGURE 4. MLL2 complex is needed for estrogen-dependent transcription mediated by ERα. A, inhibition of MLL2 expression attenuated the transcriptional activity of ERα. 2.0 μg of siRNA pool against MLL2 (siMLL2) or siRNA pool with no specific target (siControl) were cotransfected with 1.5 μg ofERE-TK-LUC, 20 ng of pCMV-ERα, and 0.1 μg of pCMVβ-gal control vector into MCF-7 cells. Cells were treated with 100 nM estrogen or vehicle for 24 h and collected to measure luciferase and β-galactosidase activity. The luciferase activity in control siRNA group in the absence of a ligand was taken as 1. The results are the mean of three independent experiments with error bars representing the S.D. B, truncated MLL2 carrying LXXL motifs inhibited ERα transcriptional activity. ERE-TK-LUC and pCMV-ERα were cotransfected along with pCMV-MLL2 (amino acids 4167–4780) or control pCMV-FLAG2 plasmid into MCF-7 cells. Luciferase activity is presented as percent where induced ERα activity in the presence of E2 is set as 100%. The experiment was performed independently three times. The results were averaged with error bars showing S.D.

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ASCOM consisting of MLL4, PRIP (ASC2), MLL2, ASH2, RBQ3, and α/β-tubulins (18) is a complex that shares components with the MLL2 complex. However, the results from mass spectrometry indicated that the MLL2 complex and ASCOM complex belong to two distinct complexes. This was evident as the abundance of MLL2 in the pull-down proteins as revealed by mass spectrometry was 20 times more than that of PRIP, and no MLL4 was detected by mass spectrometry was 20 times more than that of PRIP and full-length ASH2, RBQ3, or WDR5. WDR5 is present in MLL2 complex but not in ASCOM complex. To confirm that the MLL2 protein complex is not an ASCOM complex, we performed an immunodepletion in which we incubated DU4475 nuclear extract with an excess amount of protein A-bound anti-PRIP. As a control, a same amount of DU4475 nuclear extract was immunodepleted with preimmune rabbit IgG. The remaining proteins from both groups were subjected to Western blot to compare relative abundances of individual proteins. As shown in Fig. 2B, although PRIP was almost completely cleared by precleaning with anti-PRIP, AHS2, RBQ3, and MLL2 proteins remained at similar levels to those in the preimmune rabbit IgG immunodepletion group, indicating that the vast majority of these proteins did not associate with PRIP in DU4475 nuclear protein. These results suggest that the MLL2 complex was a novel protein complex distinct from the ASCOM complex.

ERα Interacts with the MLL2 Complex through MLL2 in a Ligand-dependent Fashion—To address which protein of the MLL2 complex interacts directly with ERα, we carried out an in vitro pull-down assay with each component of the complex and GST-ERα (LBD). As shown in Fig. 3A, no detectable interactions were seen between GST-ERα (LBD) and full-length ASH2, RBQ3, or WDR5 in the presence or absence of estrogen. As MLL2 is a huge protein with 5262 amino acids, we tested those regions containing LXXL motifs, which are required for the interaction between nuclear receptor and coactivators in a ligand-dependent fashion. GST-ERα (LBD) efficiently bound to a [35S]methionine-labeled fragment of MLL2 (amino acids 4167–4780) containing two LXXL motifs only in the presence of estrogen. When this region was divided into two fragments (amino acids 4167–4471 and amino acids 4471–4780) with each containing one LXXL motif, the in vitro binding assay demonstrated that both fragments interacted with estrogen-bound GST-ERα (LBD). When the two LXXL motifs were mutated, the fragment no longer interacted with GST-ERα (LBD) (Fig. 3B). The other two MLL2 regions (amino acids 2377–2835 and amino acids 3783–3905), each carrying two LXXL motifs, showed no interactions with GST-ERα (LBD) (Fig. 3B). These results suggest that ERα binds to the MLL2 complex through two LXXL motifs in MLL2.
Estrogen Receptor, Coactivator

A.

| Ligand: | cathepsin D | pS2(ERE) |
|--------|-------------|----------|
|        |            | Input    |
| E2     | ER         | E2       |
|        | ER         | E2       |
|        | MLL2       | E2       |
|        | RBQ3       | E2       |
|        | ASH2       | E2       |
|        | Control IgG| E2       |

FIGURE 5. Requirement of the MLL2 complex for the expression of endogenous estrogen inducible genes. A, E2 induces the recruitment of the MLL2 complex to the promoters of estrogen-responsive genes. Chromatin extracts were made from MCF-7 cells following treatment with or without 100 nM E2 for 45 min and chromatin immunoprecipitation assays were performed using antibodies against ERα, MLL2, ASH2, RBQ3, or control IgG. PCR was performed with theestrogen D promoter region containing ERE, the pS2 gene promoter region spanning ERE, and pS2 gene regions upstream (+2523 to -2315 bp) or downstream (+2306 to +2512 bp) of ERE, which served as an additional negative control. Results shown here are representative of three independent experiments. B, depletion of MLL2 reduces the expression of estrogen target genes. MCF cells were transfected with control siRNA or MLL2 siRNA. 24 h later, cells were treated with 100 nM E2 for 4 h and collected for the preparation of total RNAs. Quantitative real time PCR was performed to determine cathepsin D, pS2, and MLL2 mRNA levels, which were normalized to internal control β-actin mRNA levels. The results represent the average of three independent experiments.

MLL2 Is Required for the Expression of Endogenous Estrogen-inducible Genes—To investigate if the MLL2 complex is recruited to liganded ERα in the nuclei of living cells, we performed chromatin immunoprecipitation assay with the promoters of endogenous estrogen-responsive target genes cathepsin D and pS2. The antibodies against ASH2, RBQ3, and MLL2, along with the antibody against ERα could efficiently precipitate the estrogen-responsive regions of cathepsin D and pS2 (Fig. 5A) only when MCF-7 cells were treated with estrogen. These results suggest that estrogen treatment induced the recruitment of ERα and the MLL2 complex onto the promoter regions of estrogen-responsive target genes, which contain the estrogen-responsive element (ERE) in MCF-7 cells.

The effect of MLL2 depletion on the expression of endogenous ERα target genes was assessed by quantitative real time reverse transcriptase-PCR following siRNA transfection in MCF-7 cells. TaqMan probes for human MLL2, cathepsin D, pS2, and β-actin were used in the experiments, and all data were normalized over endogenous β-actin levels. As seen in Fig. 5B, siMLL2 transfection led to nearly an 80% reduction in the MLL2 mRNA level compared with MLL2 mRNA levels in cells transfected with siControl, indicating the efficiency of the transfection method and the specificity of siMLL2. Consistent with the chromatin immunoprecipitation experiment, estrogen-dependent activation of cathepsin D mRNA expression was significantly reduced to ~50% of the control level by siMLL2 transfection (Fig. 5B). We also observed a consistent, but to a lesser extent, reduction of pS2 mRNA,
suggested that MLL2 is differentially required for the mRNA expression of different estrogen-responsive ERα target genes. 

MLL2 Is Required for Estrogen-dependent Growth of MCF-7 Cells—ERα regulates mammary gland development and is involved in the growth of ER-positive breast tumor cells. Because silencing of MLL2 by siRNA inhibited ERα-mediated transcription in MCF-7 cells, we sought to determine whether estrogen-dependent growth of MCF-7 cells was affected by depletion of MLL2. In comparison with the transfection of control siRNA, MLL2 siRNA treatment significantly inhibited the estrogen-dependent growth of MCF-7 cells (Fig. 6), suggesting that MLL2 complex is involved in estrogen-dependent growth of breast cancer cells.

DISCUSSION

In an effort to identify novel molecules involved in ERα-mediated signal transduction in mammary gland epithelium, we employed GST-Eras (LBD) to capture its interacting proteins from human breast carcinoma cell line DU4475 nuclear extract, followed by glycerol gradient centrifugation to fractionate associated proteins according to their molecular masses. We identified the MLL2 complex, which contains MLL2, ASH2, RBQ3, and WDR5. The MLL2 complex is a novel Set1-like complex in mammalian cells (23–25, 34). The yeast Set1-like complex COMPASS, containing proteins homologous to ASH2 and Trithorax, is involved in chromatin remodeling through the methylation of histone H3 at lysine residue 4. Although yeast only contains one Set1 complex, humans appear to have multiple Set1-like complexes, including the MLL1 complex and ASCOM. A major feature of the MLL1 complex is that it contains tumor suppressor menin, which should not be a component of MLL2 complex as it was not detected by mass spectrometry in our proteins pull downs with GST-Eras (LBD). The MLL2 complex is closely related to ASCOM as they share three components, but we demonstrated that they are distinct complexes. As MLL2 and ASCOM were isolated from different cells, it is possible that different cells possess different Set1-like complex for the ERα signaling pathway. 

ERα strongly binds to the MLL2 protein through two LXXLL motifs in the region (amino acids 4167–4780) but does not bind to the other three components of the complex, indicating the MLL2 as a key component linking the MLL2 complex with ERα. Full-length MLL2 protein, structurally similar to MLL, contains a SET domain and five PHD fingers. Although SET domains have been shown to exhibit histone methylytransferase activity (23, 35), we were unable to demonstrate that the SET domain from MLL2 possessed this activity (data not shown). The function of the PHD domains in MLL family proteins remains to be understood. MLL2 also contains an N-terminal Ring finger domain, often harboring the ubiquitin ligases (36, 37), which is not found in the MLL molecule. It is well established that the activation of ERα is coupled with its degradation (38, 39, 40). Hence, it is intriguing to question whether the MLL2 complex participates in the ligand-induced degradation of ERα through adding ubiquitin to ERα.

Because of the gigantic molecular size of MLL2, which spans over 18 kb of mRNA, it is difficult to obtain its full-length cDNA for performing cotransfection to evaluate the effect of overexpression of MLL2 on ERα transcriptional activity. Indeed, silencing MLL2 by specific siRNA in MCF-7 cell did impair ERα transcription activity. In addition, the MLL2 fragment, which only contains the region necessary for binding to ERα, acted as a potent dominant-negative inhibitor of the ERα transactivation. Just as the MLL1 complex, which is recruited to the HOX gene promoter (41), we observed that components of the MLL2 complex were recruited onto the promoters of ERα target genes directly after ligand induction in MCF-7 cells. Consistent with this observation, suppression of MLL2 by its specific siRNA decreased estrogen-induced expression of ERα target genes. Suppression of MLL2 also inhibited estrogen-dependent growth of MCF-7 cells. Taken together, these results strongly suggest a key role of the MLL2 coactivator complex in ERα-mediated signal transduction pathways. It should be noted that certain coactivators such as SRC-3 and PBP are amplified and overexpressed in some breast cancers (42, 43). Besides, the MLL2 gene was mapped to chromosome band 12q12–13 (27), a region involved in duplications and translocations associated with cancers (21, 44). Therefore it is tempting to propose that the MLL2 complex may be involved in the pathogenesis of breast neoplastic diseases.

REFERENCES

1. Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5925–5930
2. Ali, S., and Coombe, R. C. (2000) J. Mammary Gland Biol. Neoplasia 5, 271–281
3. McDonnell, D. P., and Norris, J. D. (2002) Science 296, 1642–1644
4. Anderson, E. (2002) Breast Cancer Res. 4, 197–201
5. Tora, L., White, J., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) Cell 59, 477–487
6. Webster, N. J., Green, S. J., Mir, J. R., and Chambon, P. (1988) Cell 54, 199–207
7. Xu, J., and Li, Q. (2003) Mol. Endocrinol. 17, 1681–1692
8. Leo, C., and Chen, J. D. (2000) Gene (Amst.) 245, 1–11
9. McKenna, N. J., Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O’Malley, B. R. (1999) J. Steroid Biochem. Mol. Biol. 69, 3–12
10. Kang, Y. K., Guermah, M., Yuan, C. X., and Roeder, R. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2642–2647
11. Hanstein, B., Eckner, R., DiRenzo, J., Halachmi, S., Liu, H., Searcy, B., Kurokawa, R., and Brown, M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11540–11545
12. Kitagawa, H., Yanagisawa, J., Fuse, H., Ogawa, S., Yoshizaki, Y., Okuno, A., Nagasawa, H., Nakajima, T., Matsumoto, T., and Kato, S. (2002) Mol. Cell. Biol. 22, 3698–3706
13. Lee, Y. H., Koh, S. S., Zhang, X., Cheng, X., and Stalkup, M. R. (2002) Mol. Cell. Biol. 22, 3621–3632
14. Briggs, S. D., Bryk, M., Strahl, B. D., Cheung, W. L., Davie, J. K., Dent, S. Y., Winston, F., and Allis, C. D. (2001) Genes Dev. 15, 3286–3295
15. Jennewein, T., and Allis, C. D. (2001) Science 293, 1074–1080
16. Xu, L., Glass, C. K., and Rosenfeld, M. G. (1999)Curr. Opin. Genet. Dev. 9, 140–147
17. Yuan, C. X., Ito, M., Fendell, J. D., Fu, Z. Y., and Roeder, R. G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7939–7944
18. Goo, Y. H., Sohn, Y. C., Kim, D. H., Kim, S. W., Kang, M. J., Jung, D. J., Kwak, E., Barlev, N. A., Berger, S. L., Chow, V. T., Roeder, R. G., Azorsa, D. O., Meltzer, P. S., Shul, P. G., Song, E. J., Lee, K., Lee, Y. C., and Lee, J. W. (2003) Mol. Cell. Biol. 23, 140–149
19. Ayton, P. M., and Cleary, M. L. (2001) Oncogene 20, 5695–5707
20. Hughes, C. M., Rosenblatt-Rosen, O., Milne, T. A., Copeland, T. D., Levine, S. S., Lee, J. H., Manner, D., and Heyward, K. N., Hess, J. L., and Meyerson, M. (2004) Mol. Cell 13, 587–597
21. Mittelman, F. (1983) Cytogenet. Cell Genet. 36, 1–15
22. Roguev, A., Schaft, D., Shvchenko, A., Pijnappel, W. W., Wilm, M., Aasland, R., and
Estrogen Receptor, Coactivator

Stewart, A. F. (2001) EMBO J. 20, 7137–7148
23. Nakamura, T., Mori, T., Tada, S., Krajewski, W., Rozovskaia, T., Wassell, R., Dubois, G., Mazo, A., Croce, C. M., and Canaani, E. (2002) Mol. Cell 10, 1119–1128
24. Dou, Y., Milne, T. A., Tackett, A. J., Smith, E. R., Fukuda, A., Wysocka, J., Allis, C. D., Chait, B. T., Hess, J. L., and Roeder, R. G. (2005) Cell 121, 873–885
25. Yokoyama, A., Wang, Z., Wysocka, J., Sanwal, M., Auferro, D. J., Kitabayashi, I., Herr, W., and Cleary, M. L. (2004) Mol. Cell Biol. 24, 5639–5649
26. Miller, T., Krokan, N. J., Dover, J., Erdjument-Bromage, H., Tempst, P., Johnston, M., Greenblatt, J. F., and Shilatifard, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 12902–12907
27. Prasad, R., Zhadanov, A. B., Sedikov, Y., Bullrich, F., Druck, T., Rallapalli, R., Yano, T., Alder, H., Croce, C. M., Huebner, K., Mazo, A., and Canaani, E. (1997) Oncogene 15, 549–560
28. Qi, C., Chang, J., Zhu, Y., Yeldandi, A. V., Rao, S. M., and Zhu, Y. J. (2002) J. Biol. Chem. 277, 28624–28630
29. Zhu, Y., Qi, C., Cao, W. Q., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 10380–10385
30. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
31. Shang, Y., Hu, X., DiRienzo, J., Lazar, M. A., and Brown, M. (2000) Cell 103, 843–852
32. Deleted in proof
33. Deleted in proof
34. Milne, T. A., Hughes, C. M., Lloyd, R., Yang, Z., Rozenblatt-Rosen, O., Dou, Y., Schneppe, R. W., Krankel, C., Livolsi, V. A., Gibbs, D., Hua, X., Roeder, R. G., Meyerson, M., and Hess, J. L (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 749–754
35. Rea, S., Eisenhaber, F., O’Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000) Nature 406, 593–599
36. Jackson, P. K., Eldridge, A. G., Freed, E., Furstenhull, L., Hu, Y. J., Kaiser, B. K., and Reimann, J. D. (2000) Trends Cell Biol. 10, 429–439
37. Vaux, D. L., and Silke, J. (2005) Nat. Rev. Mol. Cell. Biol. 6, 287–297
38. Alam, E. T., Bakopoulos, N., and Solodin, N. (1999) Mol. Endocrinol. 13, 1522–1534
39. El Khissini, A., and Leclercg, G. (1999) FEBS Lett. 448, 160–166
40. Navaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L., and O’Malley, B. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1585–1586
41. Milne, T. A., Briggs, S. D., Brock, H. W., Martin, M. E., Gibbs, D., Allis, C. D., and Hess, J. L. (2002) Mol. Cell 10, 1107–1117
42. Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X. Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) Science 277, 965–968
43. Zhu, Y., Qi, C., Jain, S., Le Beau, M. M., Espinosa, R., III, Atkins, G. B., Lazar, M. A., Yeldandi, A. V., Rao, M. S., and Reddy, J. K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10848–10853
44. Zucman, J., Delattre, O., Desmaze, C., Epstein, A. L., Stenman, G., Speleman, F., Flecters, C. D., Aurias, A., and Thomas, G. (1993) Nat. Genet. 4, 341–345