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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 1, pp. 20–26, January 6, 2006

An hGCN5/TRRAP Histone Acetyltransferase Complex Co-activates BRCA1 Transactivation Function through Histone Modification*

Received for publication, September 15, 2005 Published, JBC Papers in Press, October 31, 2005, DOI 10.1074/jbc.M510157200

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It is well established that genetic mutations that impair BRCA1 function predispose women to early onset of breast and ovarian cancer. However, the co-regulatory factors that support normal BRCA1 functions remain to be identified. Using a biochemical approach to search for such co-regulatory factors, we identified hGCN5, TRRAP, and hMSH2/6 as BRCA1-interacting proteins. Genetic mutations in the C-terminal transactivation domain of BRCA1, as found in breast cancer patients (Chapman, M. S., and Verma, I. M. (1996) *Nature* 382, 678–679), caused the loss of physical interaction between BRCA1 and TRRAP and significantly reduced the co-activation of BRCA1 transactivation function by hGCN5/TRRAP. The reported transcriptional squelching between BRCA1 and estrogen receptor α (Fan, S., Wang, J., Yuan, R., Ma, Y., Meng, Q., Erdos, M. R., Pestell, R. G., Yuan, F., Auborn, K. J., Goldberg, I. D., and Rosen, E. M. (1999) *Science* 284, 1354–1356) was rescued by the overexpression of TRRAP or hGCN5. Histone acetyltransferase hGCN5 activity appeared to be indispensable for co-regulator complex function in both BRCA1-mediated gene regulation and DNA repair. Biochemical purification of the hGCN5/TRRAP-containing complex suggested that hGCN5/TRRAP formed a complex with hMSH2/hMSH6, presumably as a novel subclass of hGCN5/TRRAP-containing known TFTC (TBP-free TAF-containing)-type histone acetyltransferase complex (hTFTC, hPCAF, and hSTAGA) (Yanagisawa, J., Kitagawa, H., Yanagida, M., Wada, O., Ogawa, S., Nakagomi, M., Oishi, H., Yamamoto, Y., Nagasawa, H., McMahon, S. B., Cole, M. D., Tora, L., Takahashi, N., and Kato, S. (2002) *Mol. Cell* 9, 553–562). Unlike other subclasses, the isolated complex harbored a previously unknown combination of components including hMSH2 and hMSH6, major components of the BRCA1 genome surveillance repair complex (BASC). Thus, our results suggested that the multiple BRCA1 functions require a novel hGCN5/TRRAP histone acetyltransferase complex subclass.

Germ line mutations in BRCA1 are known to predispose women to the early onset of breast and ovarian cancer (1, 2). The 1863-amino acid BRCA1 protein is unique in that it harbors an N-terminal RING domain and two tandem copies of BRCT[2] at its C-terminal end (8). The major function of BRCA1 is thought to be as a tumor suppressor via DNA repair and transcriptional control (9) presumably involving chromatin remodeling and histone modification (10). The transcription factor function of BRCA1, as a co-regulator of other classes of sequence-specific regulators, is dependent on the BRCT autonomous transactivation domain (11, 12). The physiological significance of the BRCT domain is further supported by the finding that a number of BRCA1 mutations found in breast cancer patients involve the BRCT domain with resultant loss of transactivation function (3, 13). However, despite the pivotal role of BRCT function in BRCA1-mediated tumor suppression, little is known about the co-regulators and co-regulator complexes that support BRCT function (14).

Recent progress in cell biology has revealed that chromatin remodeling and modification are indispensable for events involving chromosomal DNA. A large number of chromosomal DNA-interacting factors and complexes have been identified, and most of them appear to exhibit specific enzyme activities and chromatin remodeling functions (10, 15). For gene regulation by sequence-specific regulators and co-regulators, chromatin remodeling and modification are thought to be tightly coupled such that histone acetylation appears to often initiate gene regulation and is followed by further histone modification, including methylation and phosphorylation (10, 16, 17). However, the molecular mechanisms by which these processes of histone modification are controlled remain largely unknown with many histone modification complexes still to be identified. Although it is known that several histone acetyltransferases (HATs) and HAT-containing complexes co-regulate sequence-specific regulators (5, 18–21), it is unclear whether each sequence-specific regulator requires a cognate HAT complex (or complexes) or can share common HAT complexes with other sequence-specific regulator classes.

It was reported recently that the transactivation function of BRCA1 was squelched by the transactivation of ERα (4, 22), considered to be a critical regulator of estrogen-dependent breast cancer. ERα is a member of the nuclear receptor gene superfamily, acting as a hormone-dependent transcription factor, and is known to require a number of chromatin remodeling and histone modification complexes. As two HAT co-acti-

* This work was supported in part by a grant-in-aid for priority areas from the Ministry of Education, Science, Sports, and Culture of Japan (to S. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[2] The abbreviations used are: BRCT, BRCA1 C-terminal domain; GCN5, general control non-derepressible 5; TRRAP, transformation/transcription domain-associated protein; HAT, histone acetyltransferase; ERα, estrogen receptor α; LBD, ligand binding domain; GST, glutathione S-transferase; ID, interaction domain; ERE, estrogen-responsive element; TFTC, TATA-binding protein (TBP)-free TBP-associated factor (TAF)-containing; RNAi, RNA interference; MMS, methylmethane sulfonate; BASC, BRCA1 genome surveillance repair complex; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

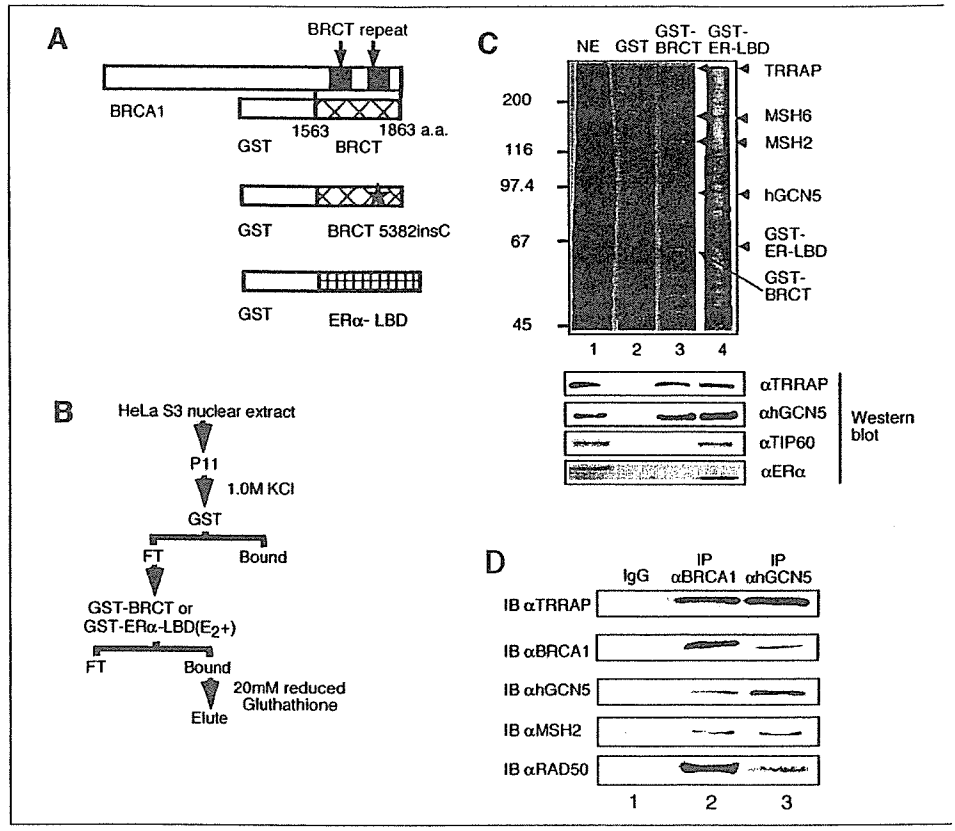

FIGURE 1. **Biochemical purification of a BRCT-interacting hGCN5/TRRAP complex.** *A*, schematic illustration of BRCA1, GST-fused BRCT proteins, and GST-fused ERα-LBD. *B*, purification schema for BRCT or estrogen-bound ERα-interacting proteins. Elution fractions from P11 columns were passed through immobilized GST columns and loaded onto immobilized GST-BRCT or GST-ERα (LBD) in the presence of estrogen (E_2) (10^{-6} M). *C*, identification of BRCT-interacting proteins. BRCT-interacting proteins contained TRRAP, hGCN5, MSH2, and MSH6 in common with liganded ERα fractions (*arrows*). Total HeLa S3 nuclear extracts (*lane 1*), fractions bound by the GST columns (*lane 2*), fractions eluted from the GST-BRCT columns (*lane 3*), and fractions eluted from liganded GST-ERα (LBD) fractions (*lane 4*) were subjected to SDS-PAGE followed by silver staining. Proteins from GST-BRCT columns were examined by MALDI-TOF MS with identified proteins indicated at the *right* side of the panel. The identity of the components in the *lower panel* was further confirmed by Western blotting. *D*, endogenous BRCA1-associated hGCN5/TRRAP complexes were co-immunoprecipitated with anti-BRCA1 and anti-hGCN5 antibodies. Whole cell extracts from subconfluent proliferating MCF7 cells were subjected to immunoprecipitation. RAD50, a major component of the BRCA1-associated repair complex (BASC), co-immunoprecipitated with endogenous BRCA1 and hGCN5. *IB*, immunoblot; *IP*, immunoprecipitation; *FT*, flow-through.

vator complexes have already been reported to support the hormone-induced transactivation function of hERα (19, 21), it is possible that BRCA1 and ERα share a common co-regulator complex. To address this issue, we searched for a co-activator complex common to both hERα and BRCT and biochemically identified a TRRAP/hGCN complex (23, 24). Both TRRAP and hGCN5 potently co-activated hERα transactivation function, and the addition of hGCN5 rescued the transcriptional squelching observed between BRCA1 and hERα. Biochemical purification of the TRRAP/hGCN5-containing complexes associated with BRCA1 identified a novel class of TFTC-type HAT complex. Thus, our study suggested that a novel hGCN5/TRRAP HAT complex subclass is required for the multiple functions of BRCA1.

MATERIALS AND METHODS

Expression Vectors—Full-length BRCA1 and hGCN5 cDNA were inserted into pcDNA3 vectors with FLAG or His/MYC epitope tags (Invitrogen). The BRCT domain was cloned into pGEX4T-1 (Amersham Biosciences). BRCA1 and hGCN5 point mutants were generated using the QuikChange mutagenesis kit (Stratagene). Adenoviral vectors were constructed using the Adeno-X expression system (Clontech) according to the manufacturer's instructions. Sequences for short hairpin RNAi were inserted into the H1 RNA promoter vector (25, 26). Targeted RNAi sequences used were TRRAP-RNAi (5′-CCAGGGGTGCCAGGCGGGGTCCATGCGG-3′), hGCN5 (5′-GCCGGGGTCGGGGCCTGGGAAGGTTCCG-3′), and hMSH2 (5′-CTCTCCAACTGCAGCGTCTCCTTCGGCT-3′). A scrambled sequence (5′-ATCTCACCCATCACACGGAGCCGCTTGC-3′) was used as a negative control. Other vectors were as described previously (16, 21, 27).

Cell Culture—Human MCF7 breast cancer cells were transformed to stably express hGCN5 as described previously (21) and maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum containing 500 μg/ml G418. The HCC1937 cell line was obtained from the ATCC and maintained in RPMI 1640 medium with 10% fetal bovine serum.

Cell Extraction and Purification—For the purification of BRCT-interacting hGCN5 complexes, nuclear extracts from 10^{10} MCF7 stable transformants were prepared using standard procedures (21). The nuclear extracts were passed through a glutathione *S*-transferase (GST) column and then bound to a GST-BRCT column. After washing with 10 column volumes of washing buffer (20 mM Tris-HCl (pH 7.9), 150 mM KCl, 0.2 mM EDTA, 0.05%, 0.5 mM phenylmethylsulfonyl fluoride, 0.05% Nonidet P-40, and 10% glycerol), complexes were eluted with 20 mM reduced glutathione in 3 ml of elution buffer (50 mM Tris-HCl (pH 7.9), 150 mM KCl, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.08% Nonidet P-40, and 10% glycerol). Bound proteins were then loaded onto 3-ml M2 anti-FLAG-agarose gels (Eastman Kodak Co.) and then eluted by incubation with 10 ml of FLAG peptide at 0.2 mg/ml (Sigma) in binding buffer. For fractionation on glycerol density gradients, 1-ml eluants were layered onto the top of 13-ml linear 100–10% glycerol gradients and centrifuged for 14 h at 40,000 rpm in an SW40 rotor (Beckman). All the procedures were done at 4 °C. Protein standards used were β-globulin (158 kDa) and thyroglobulin (667 kDa).

Immunoprecipitation—For immunoprecipitation of endogenous BRCA1 or hGCN5, subconfluent 5×10^7 MCF7 cells were harvested, washed twice with ice-cold phosphate-buffered saline, and resuspended in 5 ml of ice-cold lysis buffer (50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 1.0% Triton X-100). Whole cell extracts were then immunoprecipitated using 10 μg of each antibody (anti-BRCA1 (BRCA1–17F8, Gene Tex), hGCN5 (H-75, Santa Cruz Biotechnology), or non-immune rabbit IgG (negative control)). The precipitated proteins were collected using protein G-Sepharose beads (Amersham Bio-

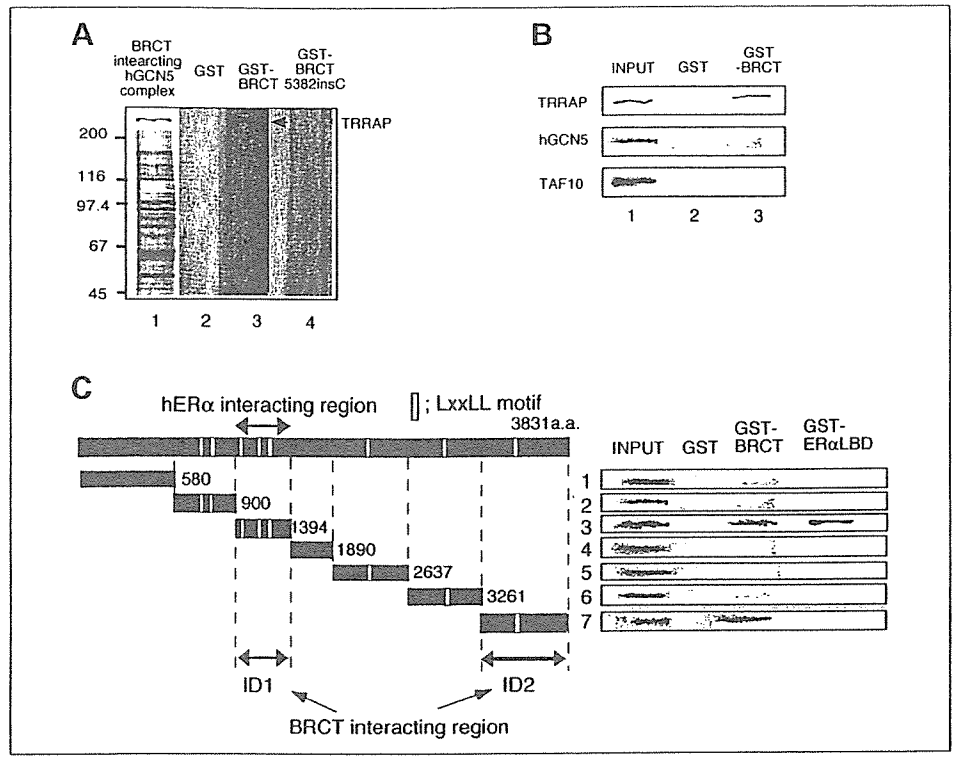

FIGURE 2. **BRCA1 associates with an hGCN5/TRRAP complex in MCF7 cells.** *A*, far Western blotting of BRCT-interacting hGCN5 complex components and BRCT. BRCT-interacting hGCN5 complexes (*lane 1*) were analyzed by far Western blotting and visualized by silver staining (10% of input shown). Each probe (*lanes 2–4*) was detected using an anti-GST-specific antibody specific for GST-fused BRCT (16). At the *right* side of the panel, an *arrow* shows TRRAP as a direct interactant; this was confirmed by Western blotting (data not shown). *B*, physical interaction between the complex components and BRCT was confirmed by GST pull-down assay. TRRAP binding was determined by SDS-PAGE autoradiography. *C*, mapping of the BRCT- or ERα-interacting region of TRRAP by GST pull-down assay using TRRAP fragments and GST-BRCT or GST-ERα-LBD. BRCT-interacting domains were designated as ID1 and ID2 as shown at the *bottom* of the panel. TRRAP ID1 interacts with both BRCT and ERα-LBD. *a.a., amino acids.*

sciences), washed, eluted in sample buffer, and subjected to Western blotting analysis (28).

GST Pull-down Assay—GST fusion proteins were expressed in *Escherichia coli* and bound to glutathione-Sepharose 4B beads (Amersham Biosciences). *In vitro* translated proteins were prepared by *in vitro* translation using the T7 promoter of the pcDNA3 vector. Proteins were labeled using [^{35}S]methionine (Amersham Biosciences), and *in vitro* translation was carried out using the TNT-coupled rabbit reticulocyte lysate system (Promega). The *in vitro* translated proteins were then incubated with beads for 1 h at 4 °C, and free proteins were removed by washing the beads with NET-N$^+$ buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 7.5), 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol). Bound proteins were eluted in boiling sample buffer, separated by SDS-PAGE, and visualized by autoradiography. For all GST pull-down assays, the input lanes show 10% of the protein amount used in the assays.

Antibodies—For Western blotting, antibodies against BRCA1 (BRCA1–17F8, Gene Tex), TRRAP (H-300, Santa Cruz Biotechnology), hGCN5 (C-20, Santa Cruz Biotechnology), hMSH2 (N-20, Santa Cruz Biotechnology), hMSH6 (N-20, Santa Cruz Biotechnology), RAD50 (13B3, Gene Tex), and TIP60 (K-17, Santa Cruz Biotechnology) were used. For the chromatin immunoprecipitation assay, we used antibodies against BRCA1 (MS-BRC14-UP50, Upstate Biotechnology, Inc.), TRRAP (T-17, Santa Cruz Biotechnology), hGCN5 (H-75, Santa Cruz Biotechnology), and acetyl-H3 (Upstate Biotechnology, Inc.).

Chromatin Immunoprecipitation Assay—Preparation of soluble MCF7 chromatin for PCR amplification was performed as described previously (21). Specific primer pairs for the p21 promoter region used were 5′-TCCAGCGCACCAACGC-3′ and 5′-AGCTGCTCACACCTCAG-3′. Subconfluent MCF7 cells were infected with adenoviral vectors (Adeno-LacZ, V; Adeno-BRCA1, BRCA1; and Adeno-BRCA1 5382insC, BRCTmt) for 24 h at a multiplicity of infection of 20 to ensure expression in more than 90% of cells. Cells were then treated with 0.01% methylmethane sulfonate (MMS) for 60 min, washed with phosphate-buffered saline, and incubated for 60 min with fresh medium.

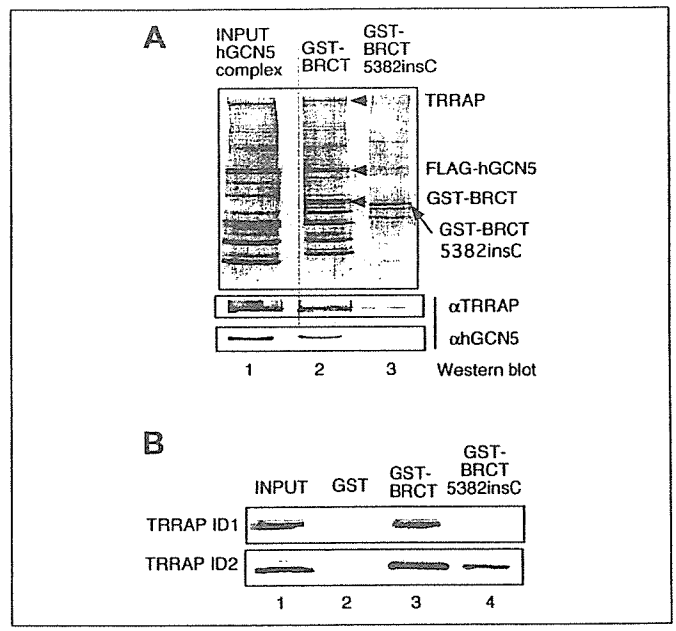

FIGURE 3. **The BRCT mutation BRCT insC abolished interaction with BRCT-interacting TRRAP/hGCN5 complexes.** *A*, hGCN5 complexes were applied to immobilized GST-BRCT beads. *Lane 1* shows hGCN5 complex (5% of input) immunoprecipitated from FLAG-hGCN5 stable transformant MCF7 cells by anti-FLAG antibody. Amounts of GST-fused proteins were equalized. Bound proteins were eluted and subjected to silver staining. *B*, BRCT mutation abolished interaction with TRRAP. The binding of wild-type or clinical mutant BRCT to TRRAP ID1 was analyzed by GST pull-down assay.

Northern Blot Analysis—Total cellular RNA was isolated from the indicated cells using ISOGEN reagent (Wako Co.), and 20 mg RNA were used for Northern blot analysis with digoxigenin-dUTP-labeled cDNA probes. Probes were synthesized using a PCR digoxigenin probe

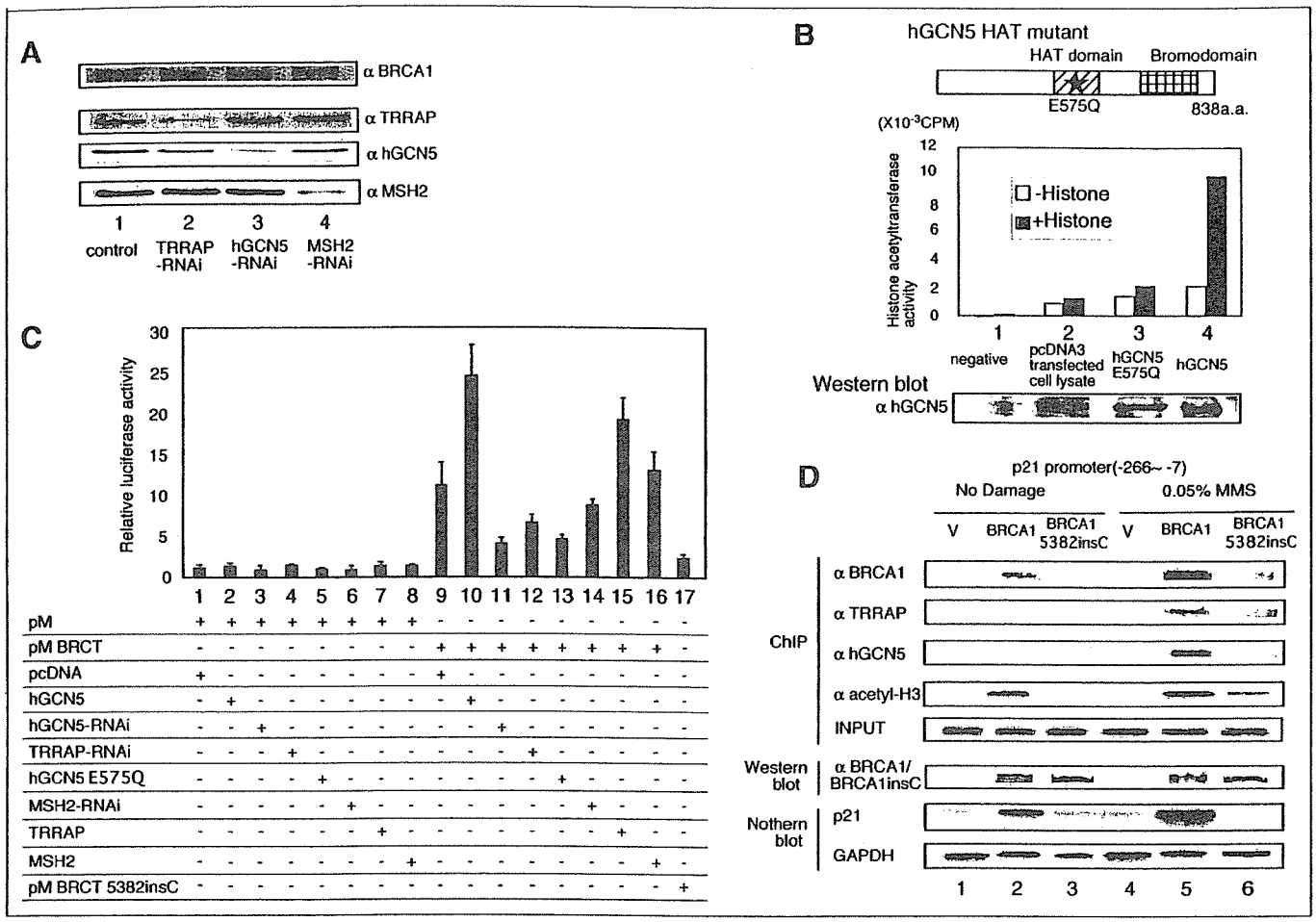

FIGURE 4. **A BRCT-interacting hGCN5 complex co-activates the transactivation function of BRCA1.** *A*, reduced expression of endogenous proteins by RNAi. The indicated RNAi vectors were transfected into MCF7 cells, and resultant whole cell extracts were examined by Western blotting to detect each protein. BRCA1 expression levels were unchanged. For control (*lane 1*), scramble sequence-inserted RNAi vector was used. *B*, the hGCN5 mutation abolished HAT activity. The hGCN5 HAT mutation (E575Q) corresponds to the yeast GCN5 HAT-abolished mutant (34). Subconfluent 293T cells were transfected with the indicated expression vectors (*lanes 2–4*). Whole cell lysates were incubated with either free histones (*closed bars*) or bovine serum albumin (*open bars*) together with ^{3}H-labeled acetyl-CoA and assayed for HAT activity by filter binding assay (21, 28). *Lane 1* shows no cell lysate added. *C*, co-activation of BRCT by hGCN5 and TRRAP. Transient transfection assays of GAL-BRCT with hGCN5, TRRAP, and MSH2 using a luciferase reporter construct containing the GAL4 DNA binding site (17M8) showed specific enhancement of transcription. Subconfluent proliferating MCF7 cells in 12-well dishes were transfected overnight with the indicated expression vectors and reporter plasmid (17M8-luc) (0.2 μg for each lane) using Lipofectamine 2000™ (Invitrogen). Total amount of DNA in each transfection was adjusted to 1.0 μg by supplementing with empty vectors. Luciferase activity is expressed relative to the pM and pcDNA vectors (*lane 1*). Results represent the average of at least three independent experiments; *error bars* indicate standard deviation. *D*, hGCN5/TRRAP is recruited to a p21$^{WAF1/CIP1}$ promoter along with BRCA1. MCF7 cells were infected with Adeno-LacZ (V), Adeno-BRCA1 (BRCA1), or Adeno-BRCA1 5382insC (BRCA1mt) and treated with 0.01% MMS for 60 min. Soluble chromatin fractions were applied to chromatin immunoprecipitation analysis. Induction of the p21 gene by DNA damage was confirmed by Northern blot analysis. *a.a.*, amino acids; *ChIP*, chromatin immunoprecipitation.

synthesis kit. The p21 cDNA probe was amplified using specific primers (5′-ATGTCAGAACCGGCTGGG-3′ and 5′-GGAGGAAGTAGCTGGCAT-3′) (28).

Cell Survival Assay—HCC1937 cells plated on 60-mm dishes at 40% confluency were transfected with pcDNA expression vectors (0.8 μg) and pSh-RNAi vectors (0.2 μg) using Effectene™ (Qiagen). Total DNA amounts were adjusted by supplementing up to 1.0 μg with empty pcDNA vector or scrambled sequence-inserted RNAi vector. After 24 h, transfected cells were treated with medium containing 0.1% MMS for 50 min, washed with phosphate-buffered saline, and maintained for 8 days in fresh medium. Surviving cells were then counted (12).

RESULTS

Biochemical Purification of hGCN5 and TRRAP as BRCA1 Interactants—To identify the co-regulators responsible for BRCT function, we biochemically purified protein complexes that interacted with BRCT. Recombinant BRCT domains (residues 1563–1863) fused to GST were immobilized to glutathione-Sepharose beads and used as a purification probe (Fig. 1, *A* and *B*). A number of BRCT-associating proteins were purified from HeLa S3 nuclear extracts with a 400-kDa protein identified as TRRAP and a 95-kDa protein as hGCN5 by MALDI-TOF MS (Fig. 1*C*). The HAT hGCN5 and the Myc-interacting protein TRRAP (5) are known to be present in several subtypes of TFTC/STAGA complex in combination with other components (6, 7).

Notably hMSH6 and hMSH2 were also identified as BRCT interactants. As hMSH2 and hMSH6 are thought to be major components of the BRCA1 genome surveillance repair complex (BASC) (29, 30) along with other DNA repair response proteins, it was possible that BASC was trapped to the BRCT domain along with the hGCN5/TRRAP complex. Although a TFTC-like complex has been purified previously from HeLa cell nuclear extracts and shown to serve as a ligand-dependent co-activator complex for ERα *in vitro* (21), ERα was undetectable in the BRCT interactants by our purification procedure (Fig. 1*C*). This suggested that we had isolated a novel hGCN5/TRRAP complex subclass that interacted with BRCT but not with ERα. Also as reported previously (31, 32), although TRRAP and hGCN5 were clearly detected by Western blot

analysis, TIP60, a component of another TRRAP-containing HAT complex subtype involved in DNA repair, interacted with ERα but not with BRCT (Fig. 1*C*). Association of BRCA1 with TRRAP, hGCN5, and MSH2 *in vivo* was confirmed by co-immunoprecipitation (Fig. 1*D*), and RAD50, a BASC component, also co-precipitated with BRCA1 as expected.

TRRAP Was a Direct Interactant of BRCT—We then attempted to identify BRCT interactants in the hGCN5/TRRAP-containing complex by far Western blot analysis. Of the purified interactants bound to GST-BRCT probes (Fig. 1*C*), we observed significant binding between TRRAP and BRCT (Fig. 2*A*). Direct and clear interaction of BRCT with TRRAP but not with hGCN5 was confirmed *in vitro* using a GST pull-down assay (Fig. 2*B*). This assay was repeated using TRRAP deletion mutants to map the interacting regions (Fig. 2*C*) and identified two domains in the N- and C-terminal regions, designated as ID1 and ID2, that appeared to serve as the direct interface for BRCT (Fig. 2*C*).

A BRCA1 Mutation Found in Breast Cancer Patients Abrogated the Physical Association with TRRAP—The *LXXLL* motif is well known to serve as a direct interface for liganded nuclear receptors via the C-terminal helix 12, and indeed three *LXXLL* motifs were found to be responsible for stable association with liganded hERα (see Fig. 2*C*) (21). Interestingly ID1, but not ID2, overlapped with the hERα-interacting region (see Fig. 2*C*). Thus, the mode of the BRCT interaction with TRRAP appears to be distinct from that of hERα. The physiological relevance of the observed interaction between BRCT and the hGCN5/TRRAP complex was then verified using a BRCA1 mutant (5382insC) from the breast cancer cell line HCC1937 that exhibits reduced transactivation function (33). When this mutant BRCA1 was used as the purification probe, hGCN5/TRRAP complex components could not be isolated (Fig. 3*A*). Indeed a GST pull-down assay showed that the TRRAP ID2 domain exhibited only poor interaction with the 5382insC mutant (Fig. 3*B*, *lower panel*). Moreover BRCT mutations found in patients resulted in the complete loss of TRRAP ID1 association (Fig. 3*B*, *upper panel*). Far Western blotting also showed no interaction between 5382insC and TRRAP (Fig. 2*A*). Therefore, BRCT mutations often found in breast cancer patients may exhibit decreased transactivation function because of reduced association with TRRAP co-activator complexes.

Co-activation of BRCA1 Transactivation Function by hGCN5 through Histone Acetylation—To determine whether the TRRAP/hGCN5 HAT complex served as a co-activator complex for BRCT, the co-activator functions of the purified interactants were tested using a transient expression assay in MCF7 cells using a chimeric BRCT domain linked to the yeast GAL4 DNA-binding domain. Clear potentiation of BRCT function by hGCN5 overexpression was observed (see Fig. 4*C*, *lane 10*), whereas the RNAi-mediated reduction of endogenous hGCN5 levels (see Fig. 4*A*, *lane 3*) (25, 26) resulted in reduced BRCT transactivation function (Fig. 4*C*, *lane 11*). Likewise both TRRAP and MSH2 alone appeared to co-activate BRCT at least to some extent (Fig. 4*C*). Based on the findings that HAT activity is essential for HAT complex co-activator-mediated transactivation of sequence-specific regulators, we tested an hGCN5 point mutant known to abolish HAT activity through the replacement of a glutamic acid (Glu) with a glutamine (Gln) at position 575 (E575Q) (34). Results of the HAT assay confirmed the loss of HAT activity of this mutant even though the mutant clearly overexpressed (Fig. 4*C*, compare *lanes 3* and *4* with wild-type hGCN5). The E575Q mutation clearly abrogated the hGCN5-mediated co-activator activity on BRCT (Fig. 4*C*, compare *lane 10* with *lane 13*), which suggested that the GCN5 HAT complex was a critical component of the co-activator complex involved in BRCA1 transactivation function.

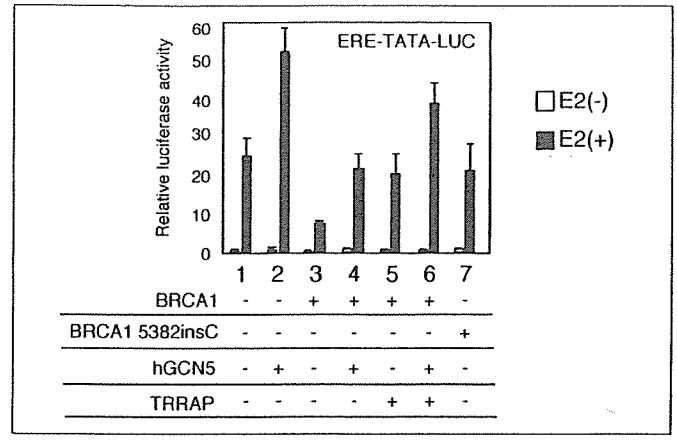

FIGURE 5. **Abrogation of transcriptional interference between BRCA1 and liganded ERα by hGCN5 and TRRAP.** Transcriptional activity in the presence of the indicated factors was examined using a luciferase reporter construct containing the ERE-TATA. Subconfluent proliferating MCF7 cells in 12-well dishes were transfected overnight with the indicated expression vectors and reporter plasmid (ERE-TATA-luc) (50 ng for each lane) using Effectene (Qiagen). Total amount of DNA in each transfection was adjusted to 0.3 μg by supplementing with empty vectors. Luciferase activity is expressed relative to negative control (*lane 1*). Results represent the average of at least three independent experiments; *error bars* indicate standard deviation. E_2, estrogen.

To test whether hGCN5 and TRRAP were indeed recruited to BRCA1, which could then act as a sequence-specific activator at target gene promoters, we performed a chromatin immunoprecipitation assay using the $p21^{WAF1/CIP1}$ gene promoter, a known BRCA1 target (35). As expected from previous reports, clear recruitment of exogenous BRCA1 to the target sequence (−266 to −7 bp) in the p21 promoter was observed in MCF7 cells after BRCA1 was activated by MMS-induced DNA damage (Fig. 4*D*). Reflecting this BRCT recruitment, hGCN5 and TRRAP were also detected in the promoter along with hyperacetylation of histone H3 presumably because of activity of hGCN5 HAT recruited to the complex (Fig. 4*D*).

hGCN5 and TRRAP Overexpression Abrogated Transcriptional Squelching of hERα by BRCA1—We then examined whether hGCN5 and TRRAP were able to rescue the reported transcriptional squelching between ERα and BRCA1. Estrogen-induced transactivation of hERα bound to consensus estrogen-responsive elements (EREs) was potentiated by hGCN5 (Fig. 5, *lane 2*). In contrast, BRCA1 overexpression squelched the ligand-induced transactivation of hERα (Fig. 5, compare *lane 1* with *lane 3*) as previously reported. Interestingly the BRCA1 5382insC mutant failed to squelch hERα transactivation (Fig. 5, *lane 7*), suggesting the possibility of common co-activator(s) between hERα and BRCA1. This hypothesis was supported by the finding that hGCN5 and TRRAP were both able to abrogate the transcriptional squelching of hERα by BRCA1 (Fig, 5, *lanes 4* and *5*) and that an additive effect was observed when both factors were used together (Fig. 5, *lane 7*). These findings suggested that hGCN5 HAT and TRRAP are common co-activators for both hERα and BRCA1.

Biochemical Identification of a Novel hGCN5/TRRAP HAT Complex—To identify the components of the BRCT-interacting hGCN5/TRRAP complex and to test whether the hGCN5/TRRAP complex was trapped to BRCA1, we established an MCF7 stable transformant cell line expressing FLAG-tagged hGCN5. Complexes from this cell line were purified by two-step column chromatography followed by glycerol density gradient sedimentation (Fig. 6*A*) (16). Unlike TFTC-type co-activator complexes, the proteins SAP130, TAF5, and TAF6 appeared to be absent (18, 36), whereas TAF10 was present (37, 38). Notably hMSH2 and hMSH6 were also identified by MALDI-TOF MS and were further confirmed by Western blot analysis (Fig. 6, *B* and *C*). However,

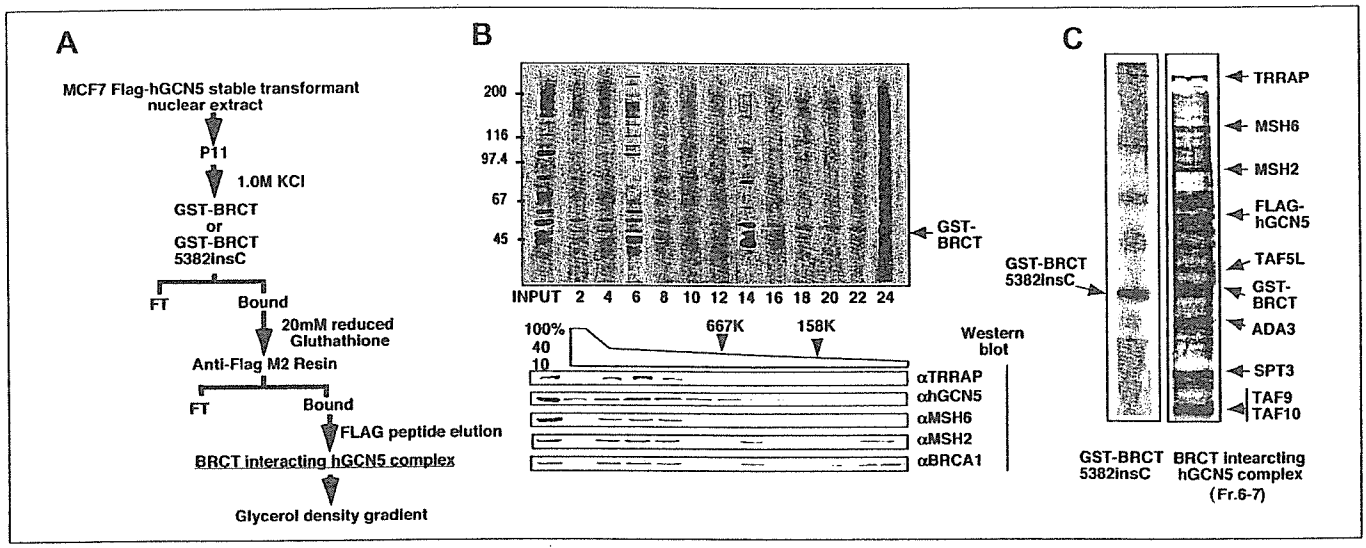

FIGURE 6. **Identification of an hGCN5/TRRAP complex associating with BRCA1.** *A*, purification schema for BRCT-interacting hGCN5 complexes and hGCN5 complexes from MCF7 stable transformants. *B*, fractionation of purified complexes on glycerol density gradients. BRCT-interacting hGCN5 complexes were separated on a 10–40% glycerol gradient by centrifugation. *INPUT* shows GST-BRCT-bound and anti-FLAG-purified material. *INPUT* and fractions from the *top* to *bottom* were resolved by SDS-PAGE and visualized by silver staining. The positions of the marker proteins of known molecular masses are shown as *arrows*. The *lower panel* shows Western blotting analysis of each fraction using specific antibodies against TRRAP, hGCN5, MSH6, MSH2, and BRCA1. Co-fractionation of TRRAP, hGCN5, MSH6, MSH2, and BRCA1 (BRCT) is observed. *C*, purified complexes subjected to SDS-PAGE and silver staining. Purified fractions (*B*, fractions 6 and 7) were separated by SDS-PAGE. GST-BRCT 5382insC shows no bound complex. Components were identified by mass spectrometry and confirmed by Western blotting with specific antibodies (data not shown). *FT*, flow-through.

other BASC components were not detected, suggesting that the purified hGCN5/TRRAP complex(es) did not associate with BASC such that hMSH2 and hMSH6 formed part of the hGCN5/TRRAP complex.

hGCN5 HAT Activity Was Required for hGCN5-mediated Co-activation of BRCA1 DNA Repair Function—Finally to test whether the BRCA1-associated hGCN5/TRRAP complex played a role in BRCA1-mediated DNA repair, we measured cell survival following DNA damage in HCC1937 cells with modulated expression levels of hGCN5/TRRAP components. Although ectopic BRCA1 expression clearly enhanced cell survival, the BRCA1 mutant 5382insC appeared to lower cell survival (Fig. 7, compare *lane 6* with *lane 11*). Interestingly hGCN5 potentiated the DNA repair activity of BRCA1 (Fig. 7, *lane 7*), whereas the hGCN5 HAT mutant (hGCN5 E575Q) appeared to act as a dominant negative hGCN5 (see *lane 10*). Thus, our results suggested that the identified hGCN5/TRRAP complex acted as a co-regulator of BRCA1-mediated DNA repair via its HAT activity.

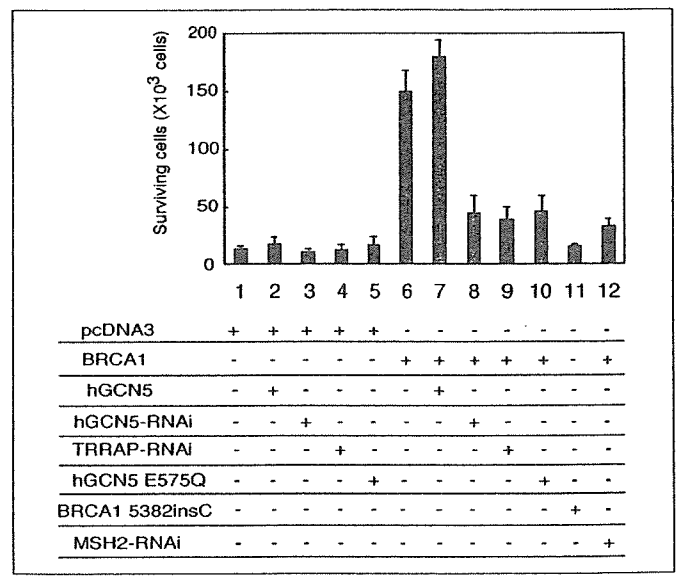

FIGURE 7. **Association between the BRCT domain and hGCN5 complex is required for BRCA1-mediated cell survival after DNA damage.** Parallel cultures of HCC1937 cells transfected with the indicated vectors were treated with 0.1% MMS for 50 min. Cells surviving DNA damage were counted. Although BRCA1-transfected cells restored MMS resistance, loss of BRCT-interacting hGCN5 complex components showed reduced resistance (12).

DISCUSSION

Core Components of the hERα-interacting TFTC-like Complex Were Identified Along with hMSH2 and hMSH6 as BRCT Domain Interactants—BRCA1 has been well established as a tumor suppressor of breast and ovarian cancers through DNA repair control and gene regulation. Although several factors and complexes, including BASC, have been identified as being involved in the BRCA1-mediated DNA repair, it remains unclear how these factors/complexes support BRCA1 functions at specific stages of the DNA repair process. Similarly gene regulation by BRCA1 also appears to require several factors/complexes, but the functional significance of these factors/complexes in BRCA1 function is largely unknown. To address these issues, we biochemically purified and identified protein interactants for BRCT, the C-terminal domain thought to support the transactivation function of BRCA1. Our study identified TRRAP, hGCN5, hMSH2, and hMSH6 as BRCT interactants, and their association with BRCA1 was confirmed using both cellular and *in vitro* approaches. Importantly hGCN5 was found to co-activate BRCA1 function; the HAT activity of hGCN5 apparently was indispensable for BRCT co-activation. In support of these findings, the histone hyperacetylation detected using BRCA1 could be potentiated by hGCN5 overexpression, whereas an hGCN5 mutant lacking HAT activity was unable to co-activate BRCT transactivation function. As hGCN5 is well known to associate with TRRAP to form a transcriptional co-activator complex (6, 7), it was likely that hGCN5 co-activated BRCT function with TRRAP as part of a complex. However, none of the subclasses of hGCN5/TRRAP-containing TFTC-like complexes reported so far also contain hMSH2 and hMSH6.

hGCN5 HAT Co-activated BRCA1 Function through Histone Modification—Biochemical purification of BRCA1-interacting complexes from MCF7 cells stably expressing hGCN5 identified a TFTC/

STAGA complex subclass that appeared to support the transactivation function of BRCA1 via the BRCT domain. The identified hGCN5/TRRAP complex was distinct from other TFTC/STAGA complex subclasses (6, 39) as it contained hMSH2 and hMSH6, major functional components of BASC (30). The presence of these DNA repair-related factors in the hGCN5/TRRAP complex suggested that BRCA1-mediated DNA repair required histone modification via the HAT activity of hGCN5 presumably as part of the TRRAP-containing complex. Given the finding that the physical and functional association between BRCA1 and TRRAP could be disrupted by using BRCT mutants observed in breast cancer patients, it is likely that the hGCN5/TRRAP complex identified in our study plays a pivotal role, via its histone acetylation activity, in the diverse functions of BRCA1, including DNA repair and gene regulation.

Although hMSH2 and hMSH6 were present in the biochemically purified hGCN5/TRRAP complex, other BASC components were not. Given that RAD50, a major BASC component (29, 30), was co-immunoprecipitated with BRCA1 in agreement with previous reports (12), the hGCN5/TRRAP complex may form part of a larger complex with BASC to fully support BRCA1 functions. As BRCT mutations found in breast cancer patients prevented interaction with the hGCN5/TRRAP complex, it is possible that the hGCN5/TRRAP complex is recruited first to allow histone modification as part of the transcriptional process involving hGCN5-mediated histone acetylation and perhaps allowing the recognition of damaged DNA. BASC may then associate with BRCA1 bound to the modified chromosome. To better understand the molecular basis of BRCA1 function, it would be interesting to investigate the cooperative functions between the two complexes in terms of both transcriptional control and DNA repair involving histone modification.

Multiple Nuclear Complexes Support BRCA1 Functions?—It has become clear that DNA-binding transcriptional activators require a number of nuclear complexes to control transcription and that these complexes associate with activators in a sequential and "hit-and-run" manner. As BRCA1 harbors a transactivation function in its C-terminal domain, it is likely that BRCA1 recruits several transcriptional co-regulator complexes in a sequential but highly regulated manner. However, unlike other DNA-binding factors, BRCA1 is presumably able to recruit a number of factors/complexes involved in the DNA repair process.

Acknowledgments—We thank Dr. I. Davidson and Dr. S. B. McMahon for providing antibodies and Dr. H. Nagasawa for technical support. We also thank Y. Mezaki, Dr. F. Ohtake, Dr. M. Kim, Y. Yogiashi, Dr. M. Igarashi, R. Fujiki, A. Baba, Y. Sasaki, and Dr. K. Ishitani for helpful technical support and H. Higuchi for preparation of the manuscript.

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Premature ovarian failure in androgen receptor-deficient mice

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Edited by Bert W. O'Malley, Baylor College of Medicine, Houston, TX, and approved November 10, 2005 (received for review August 5, 2005)

Premature ovarian failure (POF) syndrome, an early decline of ovarian function in women, is frequently associated with X chromosome abnormalities ranging from various Xq deletions to complete loss of one of the X chromosomes. However, the genetic locus responsible for the POF remains unknown, and no candidate gene has been identified. Using the Cre/LoxP system, we have disrupted the mouse X chromosome androgen receptor (*Ar*) gene. Female $AR^{-/-}$ mice appeared normal but developed the POF phenotype with aberrant ovarian gene expression. Eight-week-old female $AR^{-/-}$ mice are fertile, but they have lower follicle numbers and impaired mammary development, and they produce only half of the normal number of pups per litter. Forty-week-old $AR^{-/-}$ mice are infertile because of complete loss of follicles. Genome-wide microarray analysis of mRNA from $AR^{-/-}$ ovaries revealed that a number of major regulators of folliculogenesis were under transcriptional control by AR. Our findings suggest that AR function is required for normal female reproduction, particularly folliculogenesis, and that AR is a potential therapeutic target in POF syndrome.

male hormone | nuclear receptor | female physiology | folliculogenesis | kit ligand

Premature ovarian failure (POF) is defined as an early decline of ovarian function after seemingly normal folliculogenesis (1). Genetic causes of POF have been frequently associated with X chromosome abnormalities (1, 2). Complete loss of one of the X chromosomes, as in Turner syndrome, and various Xq deletions are commonly identified as a cause of POF. However, responsible X-linked genes and their downstream targets have not been identified so far.

The androgen receptor (*Ar*) gene, which is the only sex hormone receptor gene on the X chromosome, is well known to be essential not only for the male reproductive system, but also for male physiology. In contrast, androgens are considered as male hormones; therefore, little is known about androgens' actions in female physiology, although AR expression in growing follicles has been described (3). However, because excessive androgen production in polycystic ovary syndrome causes infertility with abnormal menstrual cycles (4, 5), it is possible that AR-mediated androgen signaling also plays an important physiological role in the female reproductive system. Recently, using Cre/LoxP system, we generated an AR-null mutant mouse line (6) and demonstrated that inactivation of AR resulted in arrest of testicular development and spermatogenesis, impaired brain masculinization, high-turnover osteopenia, and late onset of obesity in males (7–9). At the same time, no overt physical or growth abnormalities were observed in female $AR^{-/-}$ mice. Therefore, to further examine potential role of AR in female physiology, we characterized female reproductive system in $AR^{-/-}$ females. Herein we show that female $AR^{-/-}$ mice develop the POF phenotype. At 3 weeks of age, $AR^{-/-}$ females had apparently normal ovaries with numbers of follicles similar to those in the wild-type females. However, thereafter the number of healthy follicles in the $AR^{-/-}$ ovary gradually declined, with a marked increase of atretic follicles, and by 40 weeks $AR^{-/-}$ mice became infertile, with no follicle detectable in the ovary. Reflecting this age-dependent progression in ovarian abnormality, several genes known to be involved in the oocyte–granulosa cell regulatory loop were identified by microarray analysis as AR downstream target genes. These findings clearly demonstrate that AR-mediated androgen signaling is indispensable for the maintenance of folliculogenesis and implicate impaired androgen signaling as a potential cause of the POF syndrome.

Materials and Methods

Generation of AR Knockout Mice. *AR* genomic clones were isolated from a TT2 embryonic stem cell genomic library by using human *AR* A/B domain cDNA as a probe (6). The targeting vector consisted of a 7.6-kb 5′ region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and a neo cassette with two loxP sites (10). Targeted clones (FB-18 and FC-61) were aggregated with single eight-cell embryos from CD-1 mice (11, 12). Floxed *AR* mice (C57BL/6) were then crossed with CMV-Cre transgenic mice (6). The two lines exhibited the same phenotypic abnormalities. The chromosomal sex of each pup was determined by genomic PCR amplification of the Y chromosome *Sry* gene (13).

Western Blot Analysis. To detect AR protein expression, ovarian cell lysates were separated by SDS/PAGE and transferred onto nitrocellulose membranes (14). Membranes were probed with polyclonal AR antibodies (N-20; Santa Cruz Biotechnology), and blots were visualized by using peroxidase-conjugated second antibody and an ECL detection kit (Amersham Pharmacia Biosciences).

Morphologic Classification of Growing Follicles. Sections were taken at intervals of 30 μm, and 6-μm paraffin-embedded sections were mounted on slides. Routine hematoxylin and eosin staining was performed for histologic examination by light microscopy. Follicle numbers in 12 sections per ovary were evaluated as primary follicles (oocyte surrounded by a single layer of cuboidal granulosa cells), preantral follicles (oocyte surrounded by two or

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AR, androgen receptor; DHT, 5α-dihydrotestosterone; POF, premature ovarian failure.

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www.pnas.org/cgi/doi/10.1073/pnas.0506736102

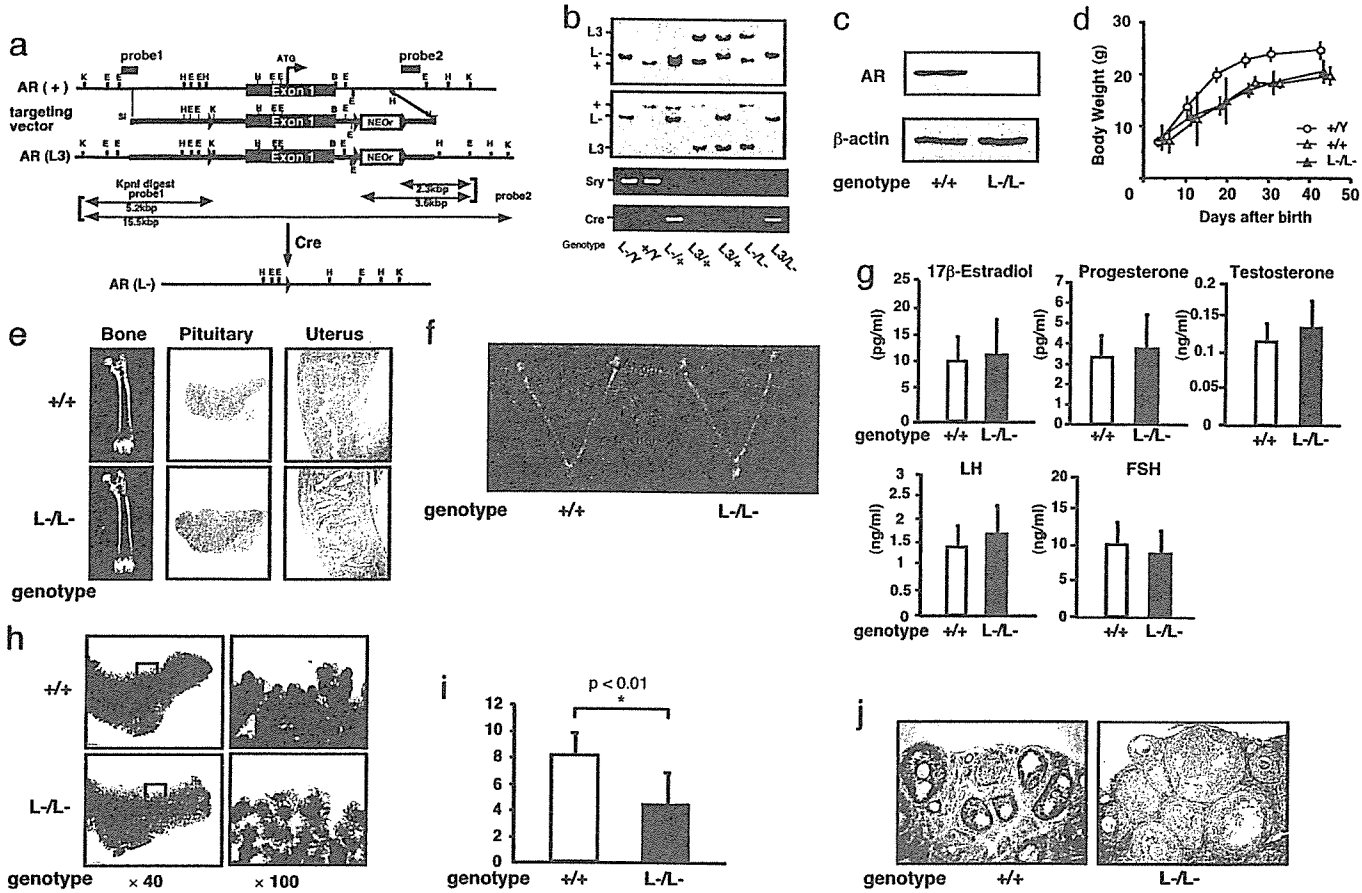

Fig. 1. Phenotypic characterization of AR knockout female mice. (*a*) Diagram of the wild-type *Ar* genomic locus (+), floxed *AR* L3 allele (L3), and *AR* allele (L-) obtained after Cre-mediated excision of exon 1. K, KpnI; E, EcoRI; H, HindIII; B, BamHI. LoxP sites are indicated by arrowheads. The targeting vector consisted of a 7.6-kb 5′ homologous region containing exon 1, a 1.3-kb 3′ homologous region, a single loxP site, and the neo cassette with two loxP sites. (*b*) Detection of the Y chromosome-specific *Sry* gene in $AR^{-/Y}$ mice by PCR. (*c*) Absence of AR protein in $AR^{-/-}$ mice ovaries by Western blot analysis using a specific C-terminal antibody. (*d*) Normal weight gain in $AR^{-/-}$ females. (*e*) Histology of pituitary, uterus, and bone tissues in $AR^{+/+}$ and $AR^{-/-}$ females at 8 weeks of age. (*f*) Female reproductive organs were macroscopically normal in $AR^{-/-}$ mice. (*g*) Serum hormone levels at the proestrus stage in $AR^{-/-}$ mice were not significantly altered. Serum 17β-estradiol, progesterone, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels in $AR^{+/+}$ ($n = 13$) and $AR^{-/-}$ ($n = 10$) females at 8–10 weeks of age are shown. (*h*) Lobuloalveolar development is impaired in $AR^{-/-}$ mammary glands. Whole mount of inguinal mammary glands (*Left*) and its higher magnification (*Right*) were prepared on day 3 of lactation. (*i*) Average number of pups per litter is markedly reduced in $AR^{-/-}$ mice at 8 weeks of age. Data are shown as mean ± SEM and analyzed by using Student's *t* test. (*j*) AR immunocytochemistry in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Sections were counterstained with eosin.

more layers of granulosa cells with no antrum), or antral follicles (antrum within the granulosa cell layers enclosing the oocyte). Follicles were determined to be atretic if they displayed two or more of the following criteria within a single cross section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane, or uneven granulosa cell layers (15).

Immunohistochemistry. Sections were subjected to a microwave antigen retrieval technique by boiling in 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min (16). The cooled sections were incubated in 1% H_2O_2 for 30 min to quench endogenous peroxidase and then incubated with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in normal goat serum for 1 h at 4°C. Sections were then incubated with anti-AR (1:100) or anti-cleaved caspase-3 (1:100) in 3% BSA overnight at 4°C. Negative controls were incubated in 3% BSA without primary antibody. The ABC method was used to visualize signals according to the manufacturer's instructions. Sections were incubated in biotinylated goat anti-rabbit IgG (1:200 dilution) for 2 h at room temperature, washed with PBS, and incubated in avidin–biotin–horseradish peroxidase for 1 h. After thorough washing in PBS, sections were developed with 3,3′-diaminobendizine tetrahydrochloride substrate, slightly counterstained with eosin, dehydrated through an ethanol series and xylene, and mounted.

Estrus Cycles and Fertility Test. To determine the stage of the estrus cycle (proestrus, estrus, and diestrus), vaginal smears were taken every morning and stained with Giemsa solution. For evaluation of female fertility for 15 weeks, an 8- or 24-week-old wild-type or $AR^{-/-}$ female was mated with a wild-type fertile male, replaced every 2 weeks with the other fertile male. Cages were monitored daily and for an additional 23 days, and the presence of seminal plugs and number of litters were recorded.

RNA Extraction and Quantitative Competitive RT-PCR. Total ovarian RNA was extracted by using TRIzol (Invitrogen) (16). Oligo-dT-primed cDNA was synthesized from 1 μg of ovarian RNA by using SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20-μl reaction volume, 1 μl of which was then diluted serially (2- to 128-fold) and used to PCR-amplify an internal control gene, *cycA*, to allow concentration estimation.

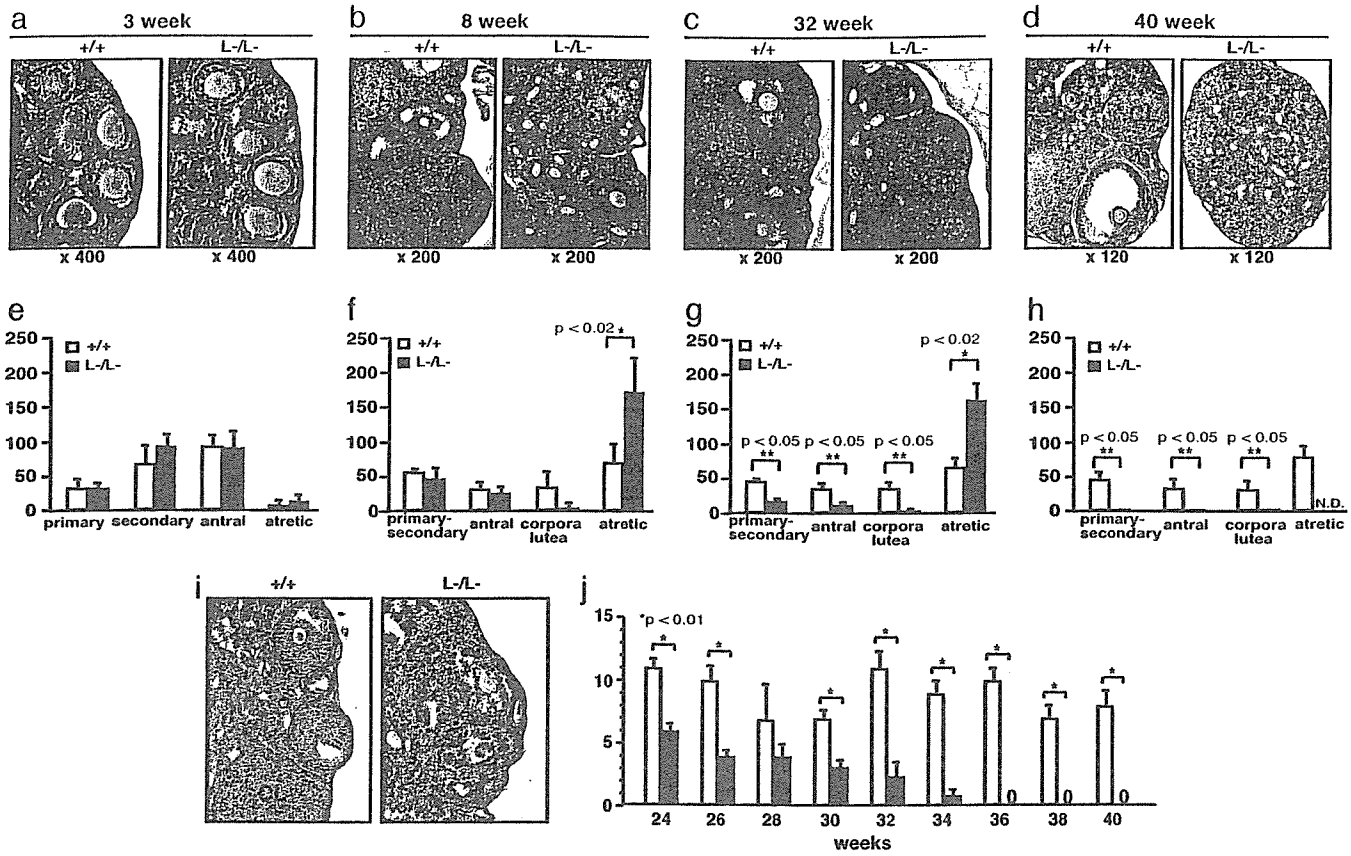

Fig. 2. POF in $AR^{-/-}$ female mice. (*a–d*) Histology of $AR^{+/+}$ and $AR^{-/-}$ ovaries at 3 weeks, 8 weeks, 32 weeks, and 40 weeks of age. All sections were stained with hematoxylin and eosin. An asterisk marks the atretic follicle. CL, corpus luteum. (*e–h*) Relative follicle counts at 3 weeks (*e*), 8 weeks (*f*), 32 weeks (*g*), and 40 weeks (*h*) of age. Numbers represent total counts of every fifth section from serially sectioned ovaries ($n = 4$ animals per genotype). (*i*) Immunohistochemical study for activated, cleaved caspase-3 revealed increased positive cells (apoptotic cells) in $AR^{-/-}$ ovaries. Sections were counterstained with hematoxylin. An asterisk marks the caspase-3-positive cell. CL, corpus luteum. (*j*) Age-dependent reduction in the number of pups per litter in $AR^{-/-}$ female mice. A continuous breeding assay was started at 24 weeks of age ($n = 6–10$ animals per genotype). For all panels, data are shown as mean ± SEM and were analyzed by using Student's *t* test.

Primers were designed from cDNA sequences of *Kitl* (M57647; nucleotides 1099–1751), Gdf9 (NM008110; nucleotides 720-1532), *Bmp15* (NM009757; nucleotides 146–973), *Ers2* (NM010157; nucleotides 1139–1921), *Pgr* (NM008829; nucleotides 1587–2425), *Cyp11a1* (NM019779; nucleotides 761-1697), *Cyp17al* (M64863; nucleotides 522–932), *Cyp19* (D00659; nucleotides 699-1049), *Fshr* (AF095642; nucleotides 625-1427), *Lhr* (M81310; nucleotides 592-1331), *Ptgs2* (AF338730; nucleotides 3–605), and *Ccnd2* (NM009829; nucleotides 150-1065) and chosen from different exons to avoid amplification from genomic DNA.

GeneChip Analysis. Ovaries were isolated and stabilized in RNA-*later* RNA Stabilization Reagent (Ambion, Austin, TX) before RNA purification (17). Total RNA was purified by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of RNA by using 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-$(dT)_{24}$ primer [5′-GGCCAGTGAATTGTAATACGACTCAC-TATAGGGAGGCGG-$(dT)_{24}$-3′], 1× first-strand buffer, and 0.5 mM dNTPs at 42°C for 1 h. Second-strand synthesis was performed by incubating first-strand cDNA with 10 units of *Escherichia coli* ligase (Invitrogen), 40 units of DNA polymerase I (Invitrogen), 2 units of RNase H (Invitrogen), 1× reaction buffer, and 0.2 mM dNTPs at 16°C for 2 h, followed by 10 units of T4 DNA polymerase (Invitrogen) and incubation for another 5 min at 16°C. Double-stranded cDNA was purified by using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions and labeled by *in vitro* transcription by using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, dsDNA was mixed with 1× HY reaction buffer, 1× biotin-labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1× DTT, 1× RNase inhibitor mix, and 1× T7 RNA polymerase and incubated at 37°C for 4 h. Labeled cRNA was then purified by using GeneChip Sample Cleanup Module and fragmented in 1× fragmentation buffer at 94°C for 35 min. For hybridization to the GeneChip Mouse Expression Array 430A or 430B or Mouse Genome 430 2.0 Array (Affymetrix), 15 μg of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1× eukaryotic hybridization control, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, and 1× hybridization buffer in a 45°C rotisserie oven for 16 h. Washing and staining were performed by using a GeneChip Fluidic Station (Affymetrix) according to the manufacturer's protocol. Phycoerythrin-stained arrays were scanned as digital image files and analyzed with GENECHIP OPERATING SOFTWARE (Affymetrix) (17).

Luciferase Assay. The *Kitl* promoter region (−2866 to −1 bp) was inserted into the pGL3-basic vector (Promega) for assay using the Luciferase Assay System (Promega) (14, 16). Cells at 40–50% confluence were transfected with a reference pRL-CMV

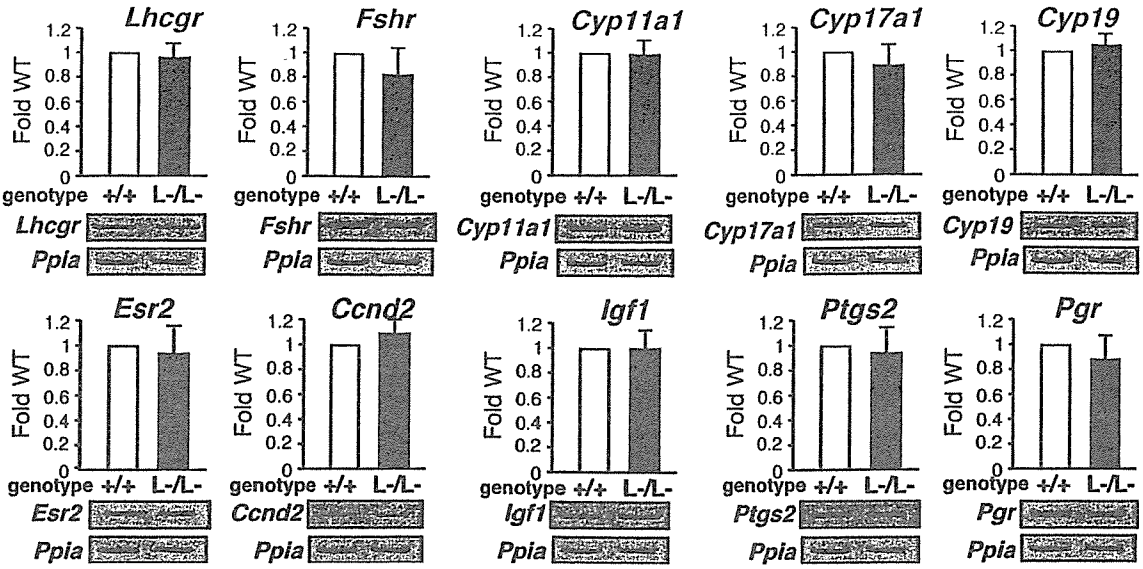

Fig. 3. No significant alterations in mRNA levels of several major regulators in folliculogenesis. Shown is semiquantitative RT-PCR of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), Aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), insulin-like growth factor 1 (*Igf1*), cyclooxygenase 2 (*Ptgs2*), or progesterone receptor (*Pgr*) gene expression in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Results shown were representative (using one ovary per genotype in each experiment) of five independent experiments.

plasmid (Promega) using Lipofectamine reagent (GIBCO/BRL, Grand Island, NY) to normalize transfection. Results shown are representative of five independent experiments.

Results and Discussion

Subfertility of $AR^{-/-}$ Female Mice at 8 Weeks of Age. The *Ar* gene located on the X chromosome was disrupted in mice by using the Cre/Lox P system (6) (Fig. 1 *a–c*). Female $AR^{-/-}$ mice showed normal growth compared with the wild-type littermates (Fig. 1*d*), with no detectable bone loss (Fig. 1*e*) or obesity common for male $AR^{-/Y}$ mice (8, 9). Young (8-week-old) $AR^{-/-}$ females appeared indistinguishable from the wild-type littermates, displayed normal sexual behavior (7), and produced the first offspring of normal body size at the expected age. Macroscopic appearance of their reproductive organs, including uteri, oviducts, and ovaries, also appeared normal (Fig. 1*f*). Histological analysis showed no significant abnormality in the uterus or pituitary (Fig. 1*e*), whereas mammary ductal branching and elongation were substantially reduced, as revealed by whole-mount analysis (Fig. 1*h*). Serum levels of 17β-estradiol, progesterone, testosterone, luteinizing hormone, and follicle-stimulating hormone were also within normal range in 8-week-old mutant females at the proestrus stage (Fig. 1*g*), suggesting that the two-cell two-gonadotrophin system in female reproductive and endocrine organs (18) was intact in $AR^{-/-}$ mice at 8 weeks of age. The most obvious early sign of abnormal reproductive function in the $AR^{-/-}$ females was that their average numbers of pups per litter were only about half of those of the wild-type littermates, ($AR^{+/+}$, 8.3 ± 0.4 pups per litter; $AR^{-/-}$, 4.5 ± 0.5 pups per litter) (Fig. 1*i*).

***$AR^{-/-}$* Female Mice Developed POF Phenotypes.** Histological analysis of 8-week-old $AR^{-/-}$ ovaries clearly showed that numbers of atretic follicles were significantly increased, with decreased numbers of corpora lutea (Fig. 2 *b* and *f*). This finding suggests that the reduced pup numbers were due to impaired folliculogenesis in AR-deficient ovaries. Indeed, AR protein expression was readily detectable in the wild-type 8-week-old ovaries (Fig. 1*j*), with AR expressed at the highest levels in growing follicle granulosa cells at all developmental stages and at relatively low levels in corpora lutea. Thus, AR appears to play a regulatory role in granulosa cells during their maturation to the luteal phase.

To investigate this possibility, we examined the ovarian phenotype of female $AR^{-/-}$ mice at different ages. At 3 weeks, ovaries contain various stages of follicles, including primary, secondary, and antral follicles in wild-type animals (Fig. 2*a*) (19). In $AR^{-/-}$ ovaries at 3 weeks of age, the folliculogenesis appeared to be unaltered, with normal numbers and localization of primary and secondary follicles (Fig. 2 *a* and *e*). However, degenerated folliculogenesis became evident with further aging. Although follicles and corpora lutea at all developmental stages were still present, corpora lutea numbers were clearly reduced in 8-week-old $AR^{-/-}$ mutants (Fig. 2 *b* and *f*), similar to that observed in another mouse line (20). Expected apoptosis was seen in atretic follicles by activated caspase-3 immunohistochemistry assays (Fig. 2*i*). But, by 32 weeks of age, defects in folliculogenesis in $AR^{-/-}$ ovaries became profound, with fewer follicles observed and increased atretic follicles (Fig. 2 *c* and *g*), and >40% (5 of 12 mice) of the $AR^{-/-}$ females were already infertile. By 40 weeks, all $AR^{-/-}$ females became infertile, with no follicles remaining (Fig. 2 *d* and *h*); at the same age, $AR^{+/+}$ females were fertile and had normal follicle numbers. Consistent with progressive deficiency in folliculogenesis, the pup number per litter steadily decreased in aging $AR^{-/-}$ females (Fig. 2*i*). These data indicate that AR plays an important physiological role at the preluteal phase of folliculogenesis.

Alteration in Gene Expressions of Several Major Regulators Involved in the Oocyte–Granulosa Cell Regulatory Loop. To explore the molecular basis underlying the impaired folliculogenesis in $AR^{-/-}$ ovaries, we analyzed expression of several major known regulators and markers of folliculogenesis (21–23). Surprisingly, no significant alterations in mRNA levels of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17-α-hydroxylase (*Cyp17a1*), aromatase (*Cyp19*), estrogen receptor-β (*Esr2*), cyclin D2 (*Ccnd2*), or insulin-like growth factor 1 (*Igf1*) of 8-week-old $AR^{-/-}$ ovaries at the proestrus stage, and further cyclooxygenase 2 (*Ptgs2*) or progesterone receptor (*Pgr*) at the estrus stage, were detected by

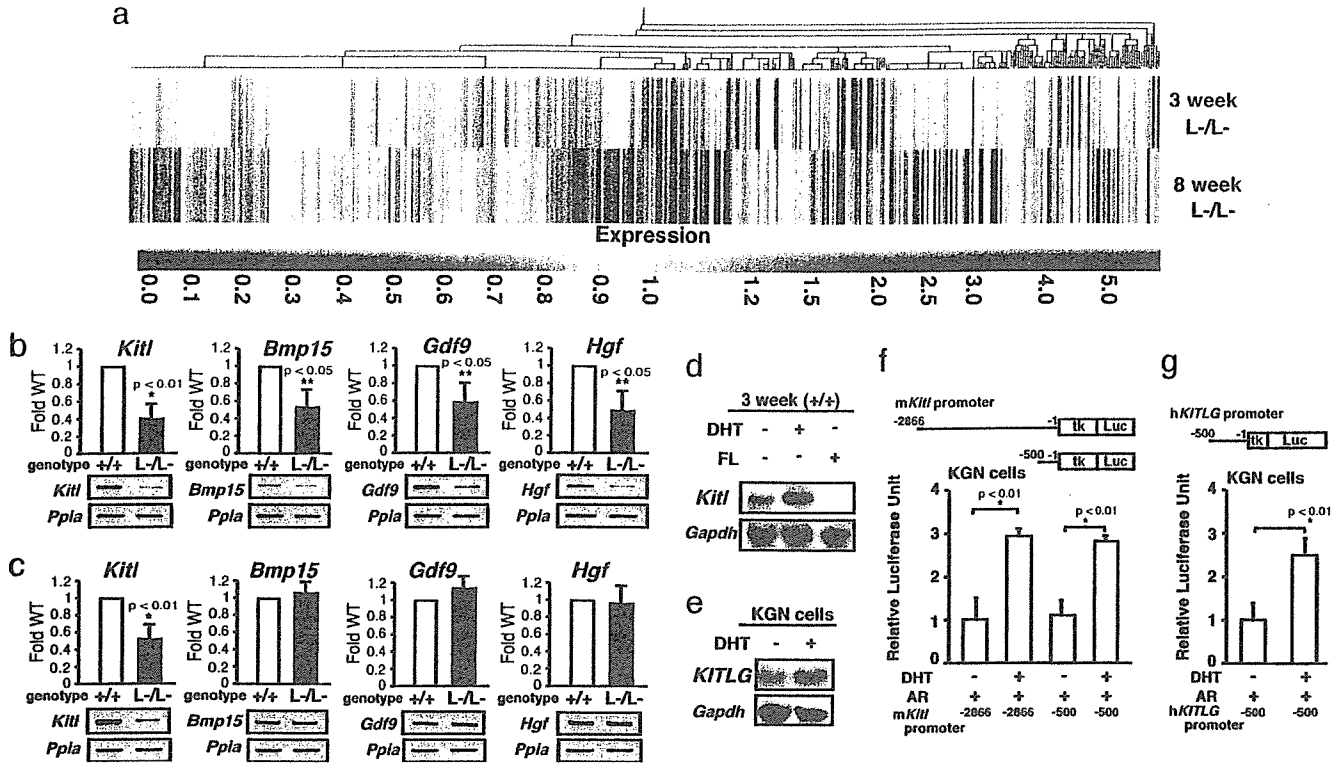

Fig. 4. Genome-wide microarray analysis and semiquantitative RT-PCR revealed that expression of the oocyte–granulosa cell regulator loop was down-regulated in $AR^{-/-}$ ovaries. (*a*) Microarray analysis of $AR^{-/-}$ compared with $AR^{+/+}$ ovaries at 3 and 8 weeks of age. Data obtained from microarray analysis as described in *Materials and Methods* were used to generate a cluster analysis. Each vertical line represents a single gene. The ratios of gene expression levels in $AR^{-/-}$ ovaries compared with wild type are presented. (*b* and *c*) Semiquantitative RT-PCR analysis of AR-regulated genes identified from the microarray study. Results shown are representative (using one ovary per genotype in each experiment) of five independent experiments. Data are shown as mean ± SEM and were analyzed by using Student's *t* test. (*d*) Comparison of *Kitl* gene expression by Northern blot analysis among placebo-, DHT-, and flutamide (FL)-treated $AR^{+/+}$ mouse ovaries. (*e*) Induction of *KITLG* gene expression by DHT treatment in KGN cells. (*f* and *g*) Androgen responsiveness in the mouse and human *kit ligand* promoters by a luciferase assay performed by using KGN cells. Data are shown as mean ± SEM and were analyzed by using Student's *t* test.

semiquantitative RT-PCR analysis (Fig. 3). Genome-wide microarray analysis (17) of RNA from 8-week-old $AR^{-/-}$ ovaries at the proestrus stage has been undertaken to identify AR-regulated genes. In comparison with $AR^{+/+}$ ovaries, expressions of 772 genes were down-regulated, whereas 351 genes were up-regulated in $AR^{-/-}$ ovaries (Fig. 4*a*; see also Tables 1 and 2, which are published as supporting information on the PNAS web site). Several genes known to be involved in the oocyte–granulosa cell regulatory loop (24) were identified as candidate AR target genes, including KIT ligand (*Kitl*) (25), morphogenetic protein 15 (*Bmp15*) (26), growth differentiation factor-9 (*Gdf9*) (27), and hepatocyte growth factor (*Hgf*) (28). Impaired folliculogenesis had been reported in mice deficient in each of these three regulators (26, 27, 29). To validate the microarray data, we performed semiquantitative RT-PCR analysis of 8-week-old $AR^{-/-}$ ovary RNA and confirmed that expression of these factors was down-regulated (Fig. 4*b*). To identify a regulator downstream of the AR signaling at an earlier stage of folliculogenesis, 3-week-old $AR^{-/-}$ ovaries that, as pointed out earlier, display no apparent phenotypic abnormality were examined. Fewer genes had altered expression levels (519 genes up-regulated; 326 genes down-regulated) (Fig. 4*a*; see also Tables 3 and 4, which are published as supporting information on the PNAS web site), and, of the four regulators tested by RT-PCR, only *Kitl* was found to be down-regulated at this age (Fig. 4*c*). Because *Kitl* is a granulosa cell-derived factor and stimulates oocyte growth and maturation (29–31), down-regulation of the *Kitl* expression in 3-week-old or even younger $AR^{-/-}$ ovaries may trigger impairment in folliculogenesis at a later age. To test for possible *Kitl* gene regulation by AR, 3-week-old wild-type females were treated with 5α-dihydrotestosterone (DHT). At 4 h after hormone injection, a clear induction of *Kitl* expression was observed in the ovaries, whereas a known antiandrogen flutamide attenuated the induction by DHT (Fig. 4*d*). The induction of endogenous human *kit ligand* (*KITLG*) gene by DHT was also observed in human granulosa-like tumor cells (KGN) in culture (Fig. 4*e*). Furthermore, androgen-induced transactivation of mouse and human *kit ligand* promoters (32) was observed by a luciferase reporter assay (33) in KGN (Fig. 4 *f* and *g*), 293T, and HeLa (data not shown) cells. However, no response to DHT was detected in the similar assay using promoters of the *Bmp15*, *Gdf9*, and *Hgf* genes (data not shown). Thus, we have shown that, in a regulatory cascade controlling folliculogenesis, *Kitl* represents a direct downstream target of androgen signaling.

As an upstream regulator, AR may also be indirectly involved in control of expression of other genes critical for folliculogenesis, because an age-dependent down-regulation of *Bmp15*, *Gdf9*, and *Hgf* gene expression was also observed in $AR^{-/-}$ ovaries. *Bmp15* and *Gdf9* are oocyte-derived factors that promote the development of surrounding granulosa cells in growing follicles (34, 35), whereas *Hgf* is secreted by theca cells and acts as a granulosa cell growth factor (36). Down-regulation of these factors, presumably due to decreased *Kitl* expression, may lead to impaired bidirectional communication between oocyte and granulosa cells (24) and, eventually, to early termination of folliculogenesis, as in POF syndrome.

Thus, we have identified AR as a novel regulator of follicu-

logenesis that apparently acts in the regulatory cascade upstream of the major factors controlling ovarian function, confirming the previous findings of the AR expression in granulose cells of growing follicles (3). Although not immediately relevant to the ovarian physiology, abnormal development of the mammary glands observed in our AR-deficient mice adds further strong evidence of an essential role of the AR not only in male, but also in female, reproductive function.

With increasing age of the first childbirth by women in the modern society, POF syndrome has become an important social and medical problem. Our findings suggest that POF syndrome may be caused by an impairment in androgen signaling and that X chromosomal mutations affecting the *AR* gene function may play a key role in hereditary POF. From clinical perspective, the present study provides evidence that AR can be a beneficial therapeutic target in treatment of POF syndrome patients.

We thank T. Iwamori and H. Tojo for expert advice on mammary gland anatomy, Y. Kanai for ovarian phenotypic analysis, members of the KO project team at the laboratory of Nuclear Signaling (Institute of Molecular and Cellular Biosciences) for their support, A. P. Kouzmenko for helpful suggestions, and H. Higuchi for manuscript preparation. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences and priority areas from the Ministry of Education, Culture, Sports, Science, and Technology (to S.K.).

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免疫系の老化と機能回復，特に免疫力評価の重要性について

Senescence of immune system and its restoration, laying particular stress on the assessment of immunological vigor

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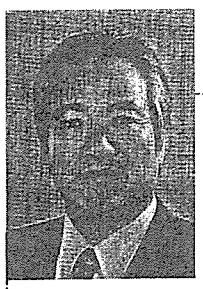
Key Words
- 免疫力
- 感染症
- 免疫力評価
- 免疫機能回復
- 健康維持

Summary

病理解剖による検索では，感染症が日本人の死因の3位以内に入り，特に高齢者では第1位となる。その理由は，高齢者や疾病にかかっている患者の免疫機能が低下していることが多いからである。免疫機能低下の原因の第一は老化・加齢であり，第二は生活習慣，ストレス，疾病とそれに対する治療などである。免疫系はホメオスターシスの維持にも関与するので，その機能低下はQOLの低下も引き起こす。したがって，健康状態を維持し，あるいは疾病の進行を阻止するためには，免疫機能のレベルを適切に評価し，低下があれば，その回復が必要になる。免疫機能は多岐にわたっているので，その評価には，さまざまな免疫細胞のマーカーや機能を包括的に測定し，個体全体の免疫力として評価する方法が必要となる。低下した免疫機能の回復には，その回復効果が体質や生活習慣に左右されるので，免疫力の適切な評価をしながら，個々の人に合った免疫機能回復方法を選ぶ必要がある。



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免疫と老化の研究に関する第一人者。東京医科歯科大学医学部を卒業，病理学を専攻。その後NIHに留学，東京都老人総合研究所免疫病理部長，東京医科歯科大学病理学教授を経て，現在は健康ライフサイエンス代表取締役。

いまだに怖い感染症

日本では年間約100万人の人が亡くなるが，厚生労働省発表によると死因の上位3つは悪性腫瘍，心疾患，脳血管障害のいわゆる成人病である。しかし，よくみると，第4位に感染症というのがみられる。それは平均寿命が延びて，老人が増え，その老人が感染症で亡くなることが多いからである。

厚生労働省の死因統計は臨床医の提出する死亡診断書をもとにしている。しかし，年間2万数千例行われる病理解剖学的報告で死因をみると，感染症がトップ3に入ってくる。

病理学的にみた場合，感染症が死因のトップ3に入ることは，どこの病院でもほぼ同じである。その原因の第一は免疫機能の低下した高齢者の増加であり，第二は年々増加する癌患者が感染症で亡くなることが少なくないからである。癌患者は高齢者であることが

多い上に，化学療法などの治療による免疫機能の低下のため，感染症にかかりやすくなっている。

加齢あるいは老化とともに免疫機能が低下することはよく知られている[1)2)]。HIVウイルスに感染しなくても，年をとるに従い免疫機能は低下する。2003年の冬に東南アジア，中国で重症急性呼吸器症候群（severe acute respiratory syndrome：SARS）によって亡くなったのも圧倒的に老人が多かった。そして今年は鳥インフルエンザの脅威が伝えられているが，この場合も犠牲のトップになるのは高齢者である。

厚生労働省の統計でみても，毎年8万人は肺炎で亡くなり，また，癌で亡くなる人は約30万人であるが，そのうちの約4分の1の7万人強は病理学的にみれば感染症で亡くなっていると推測される。これらを考慮すると，約15万人の人が感染症で亡くなっていることになる。この感染症はSARSでも鳥インフルエンザでもなく，日和見感染を含めた普通の感染症なのである。

感染症に対抗する免疫システム

感染に対抗する防御システムは次に述べる3段階のバリアからなり，感染に対抗する能力は免疫系を中心としながら，これら異なるシステムの総合的な能力からなる。

①皮膚粘膜の物理的バリア：この中で最も大事なのは感染予防の関門となる口腔・鼻腔領域である。

②好中球（顆粒球）やマクロファージなどからなる自然免疫系：これらの細胞は，「病原性微生物」と白兵戦を行い，病原体の種類を問わずに貪食・消化することにより身体を守る。この自然免疫系の働きはヒトの誕生後，すぐに機能し始める。

③リンパ球は好中球とは異なり，病原体をはじめとする無数の抗原に対する無数のクローンからなる。機能的には異なるさまざまなサブグループから構成され，サイトカインという物質を介して，オーケストラのように協調的な共同作業により，感染防御能を発揮する。この系は誕生直後には十分に機能せず，環境にある無数の抗原に曝され，クローンが拡大することにより，免疫機能を「獲得」する。それゆえに，「獲得」免疫系といわれる。後述するように，リンパ球からなる免疫系が老化やストレスによる影響を最も受けやすいシステムなのである。

神経系・内分泌系と協調してストレスに対抗する免疫系

免疫系の主役であるリンパ球はサイトカインや抗体を作るだけでなく，さまざまなホルモンや神経系伝達物質を産生し，それらの受容体ももっている。一方，神経・内分泌系でも，さまざまなサイトカインが産生され，それらの受容体が機能している。感染などでリンパ球系が働き，サイトカインが産生されれば，それは神経系，内分泌系にも作用し，一方，ストレスによる神経・内分泌系の働きは免疫系の機能に影響する。すなわち，免疫系は神経系，内分泌系と協調して感染やストレスに対抗し，身体の内部環境の恒常性を維持（ホメオスターシス）する上で，重要な役割を果たしている[1)-3)]。

免疫系を脅かすもの

物理的バリアである皮膚にとっては外傷が脅威である。口腔・鼻腔領域では，粘膜の乾燥状態が大きな脅威となる。つまり，口腔領域の唾液の分泌は乾燥を防ぎ，バリアを守る最大の武器なのである。冬の乾燥の進んだ時期には，唾液の分泌が追いつかないため，バリアが働かず，咽頭から始まる感染症にかかることが多い。うがいや部屋の湿度を上げることが感染予防になるのはこうした理由による。

好中球，リンパ球にとって怖いのは老化，ストレス，病気，不適切な生活習慣，極端に不潔な環境である。病気に対する薬剤や治療も免疫系を脅かすことが少なくない。これらは我々の周囲に満ちあふれていて，免疫力を常に脅かしている（図1）。

加齢・老化により最も大きな影響を受けるのは獲得免疫系である。リンパ球からなる獲得免疫系は生後急激に発達し，思春期くらいにはピークに達する。こうしてでき上がった獲得免疫系の機能は顆粒球・マクロファージからなる自然免疫系の機能よりずっと強固なものになり，生体防御の中核を形成する。しかし，このリンパ球からなる免疫機能はピークに達した後，その高いレベルを維持することなく徐々に低