Supplementary Table 1 *Association between EBAG9 immunoreactivity and clinicopathological parameters in human RCC patients*

Variable	n	EBAG9 immunoreactivity		*P* Value[a]
		Negative	Positive[b]	
Patients	78	10	68	
Lymph node status				> 0.9999
Positive	6	0	6	
Negative	72	10	62	
Metastatic status				0.5857
Positive	7	0	7	
Negative	71	10	61	
Age		57.5 ± 8.1[c]	54.1 ± 11.7	0.3849
Sex				0.3735
Male	64	7	57	
Female	14	3	11	
Infiltration				0.3263
α	43	7	36	
β, γ	35	3	32	
Grade				0.0606
1	24	6	18	
2, 3	54	4	50	
Histological type				0.0126*
Clear cell	51	10	41	
Others	27	0	27	
Vascular infiltration				0.0109*
Positive	29	0	29	
Negative	49	10	39	
Pathological stage				0.0017*
T1	43	10	33	
≥T2	35	0	35	

[a]Evaluated by the student-t test/ Fisher's exact probability test.

[b]Defined positive if >5% of cells were stained.

[c]Mean ± standard deviation.

*$P < 0.05$.

Supplementary Table 2 *Correlation between 5-year cancer-specific survival and clinicopathological parameters in RCC patients*

Variable	n	Survival (%)	*P* Value[a]
Patients	78		
Age			0.5828
<60	49	85.5	
≥60	29	77.4	
Grade			0.0254*
1	24	95.7	
2, 3	54	76.8	
Histological type			0.0205*
Clear cell	51	89.8	
Others	27	70.4	
EBAG9 immunoreactivity			0.0007*
Low (0, 1+ & 2+)	59	91.2	
High (3+)	19	55	
Vascular infiltration			0.0003*
Positive	29	62.4	
Negative	49	93.9	
Lymph node status			0.0002*
Positive	6	33.3	
Negative	72	86.9	
Infiltration			< 0.0001*
α	41	97.5	
β, γ	37	65.5	
Pathological stage			< 0.0001*
T1	43	97.6	
≥T2	35	63.6	
Metastatic status			< 0.0001*
Positive	7	0	
Negative	71	91.2	

[a]Determined by the log-rank test.

*$P < 0.05$.

Supplementary Table 3 *Multivariate anlalysis of prognostic factors in Cox regression hazard model*

Variable	Relative risk	95% confidence interval	*P* value
Grade (2, 3/ 1)	1.081	0.099 - 11.835	0.9490
Vascular infilatration (positive/ negative)	0.938	0.213 - 4.139	0.9326
Pathological stage (≥T2/ T1)	8.702	0.973 - 77.831	0.5290
Lymph node status (positive/ negative)	0.385	0.087 - 1.700	0.2077
Infiltration (β, γ/ α)	4.342	0.485 - 38.883	0.1892
Histological type (others/ clear cell)	3.874	0.817 - 18.365	0.0880
EBAG9 immunoreativity (3+/ 0,1+, and 2+)	5.092	1.010 - 25.662	0.0485*
Metastatic status (positive/ negative)	42.534	7.138 - 253.469	< 0.001*

*$P < 0.05$

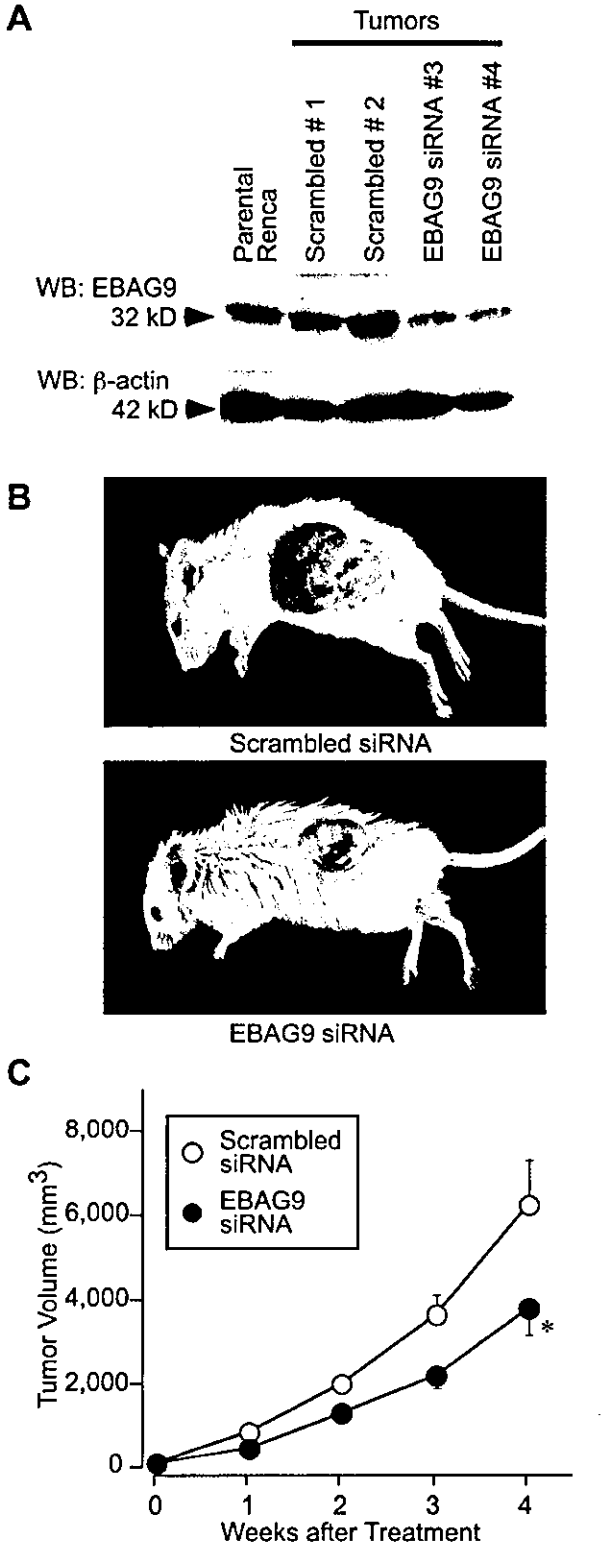
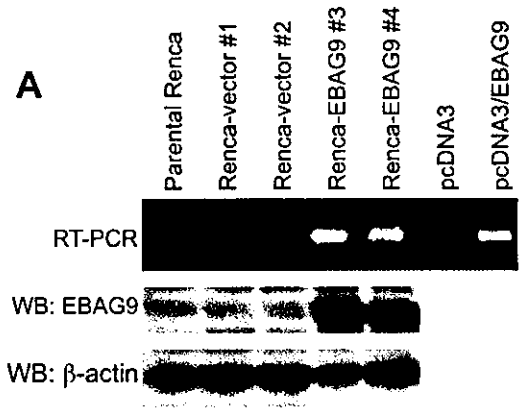

A
Tumors
Parental Renca
Scrambled # 1
Scrambled # 2
EBAG9 siRNA #3
EBAG9 siRNA #4
WB: EBAG9
32 kD
WB: β-actin
42 kD
B
Scrambled siRNA
EBAG9 siRNA
C
Tumor Volume (mm3)
8,000
6,000
4,000
2,000
0
Scrambled siRNA
EBAG9 siRNA
*
0
1
2
3
4
Weeks after Treatment

Fig. 1

A



B

	Doubling Time (h)
Renca-vector #1	25.4 ± 1.1
Renca-vector #2	27.5 ± 0.6
Renca-EBAG9 #3	23.2 ± 1.2
Renca-EBAG9 #4	24.6 ± 1.2

C

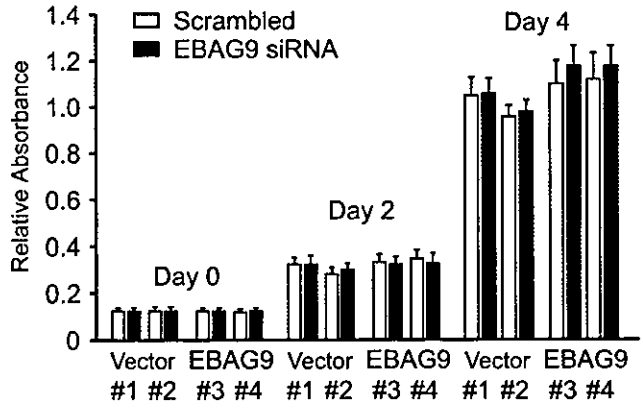


Fig. 2

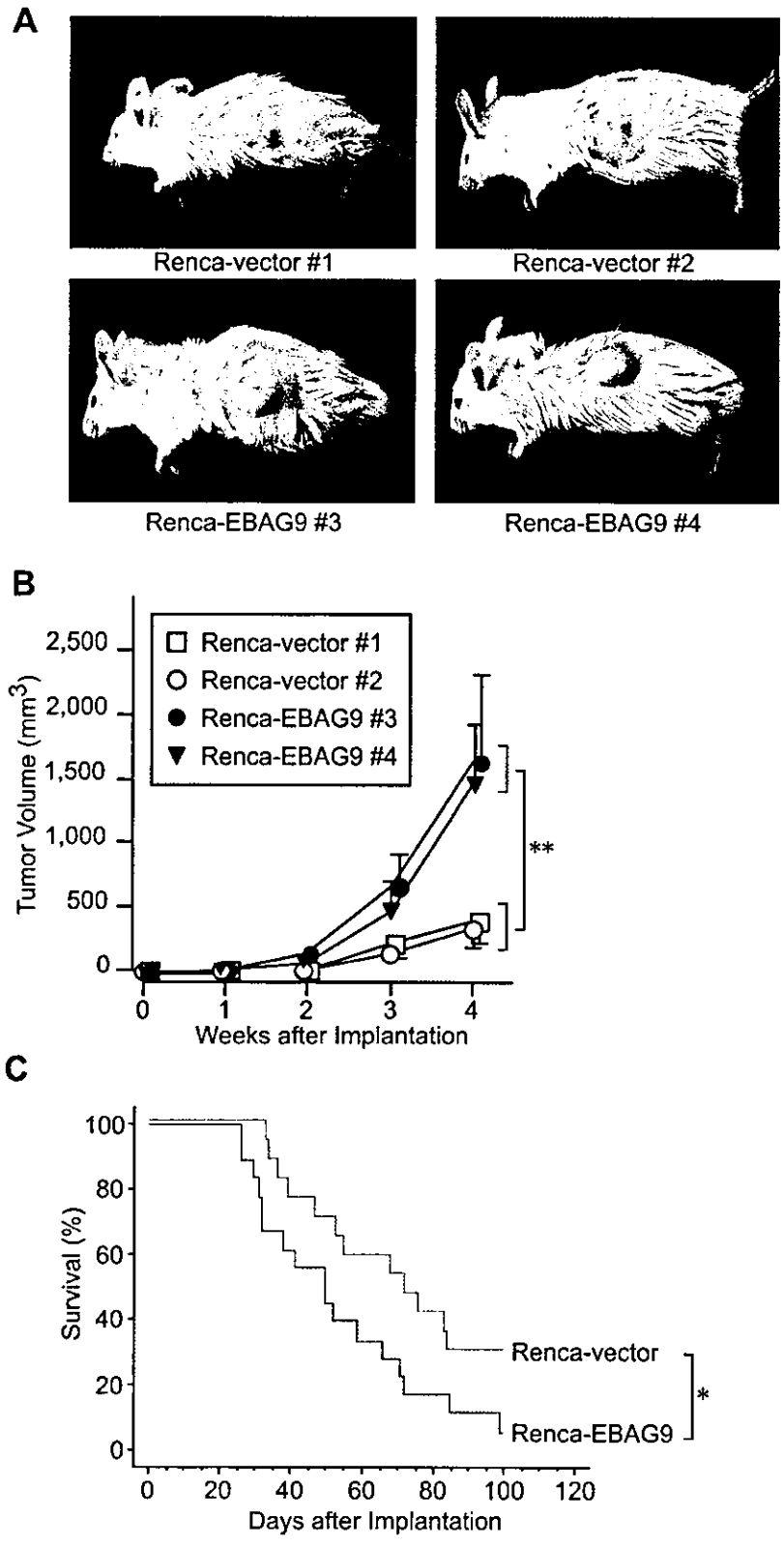
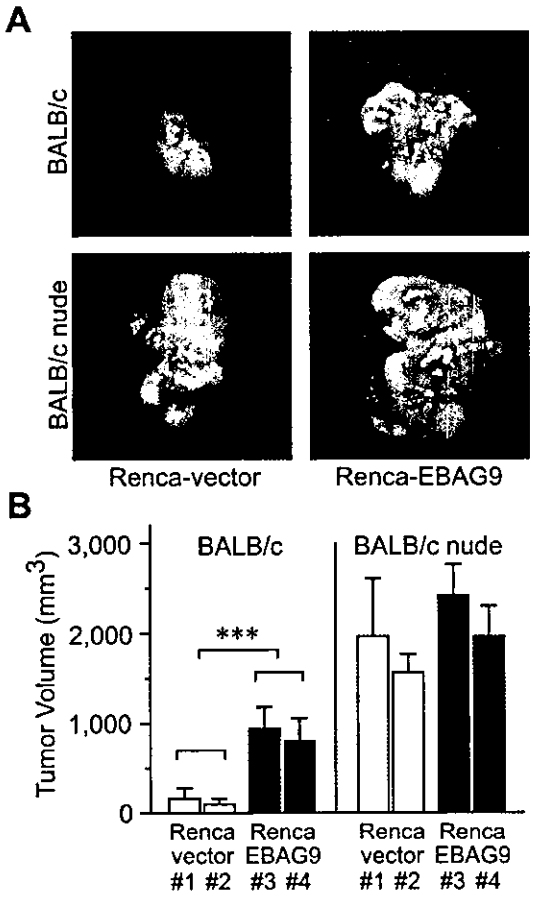
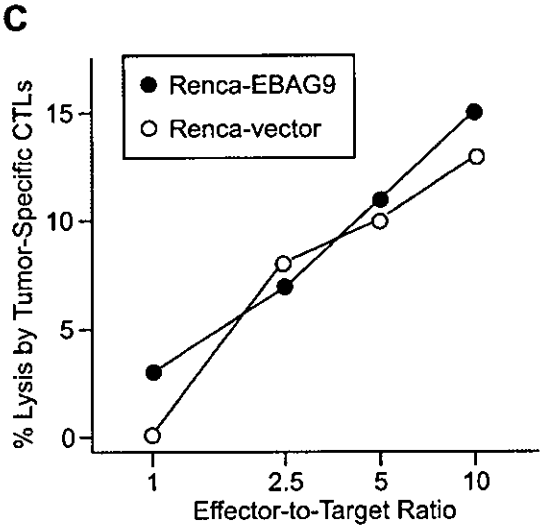

A
Renca-vector #1
Renca-vector #2
Renca-EBAG9 #3
Renca-EBAG9 #4
B
Renca-vector #1
Renca-vector #2
Renca-EBAG9 #3
Renca-EBAG9 #4
Tumor Volume (mm3)
2,500
2,000
1,500
1,000
500
0
**
0
1
2
3
4
Weeks after Implantation
C
Survival (%)
100
80
60
40
20
0
Renca-vector
Renca-EBAG9
*
0
20
40
60
80
100
120
Days after Implantation

Fig. 3





D

TILs	Tumors	
	Renca-vector	Renca-EBAG9
$CD3^+$	21.4 ± 1.5	18.6 ± 2.0
$CD4^+$	11.4 ± 1.1	12.8 ± 1.5
$CD8^+$	10.0 ± 1.4	5.8 ± 0.7*

(cells / 10 high power fields)

Fig. 4

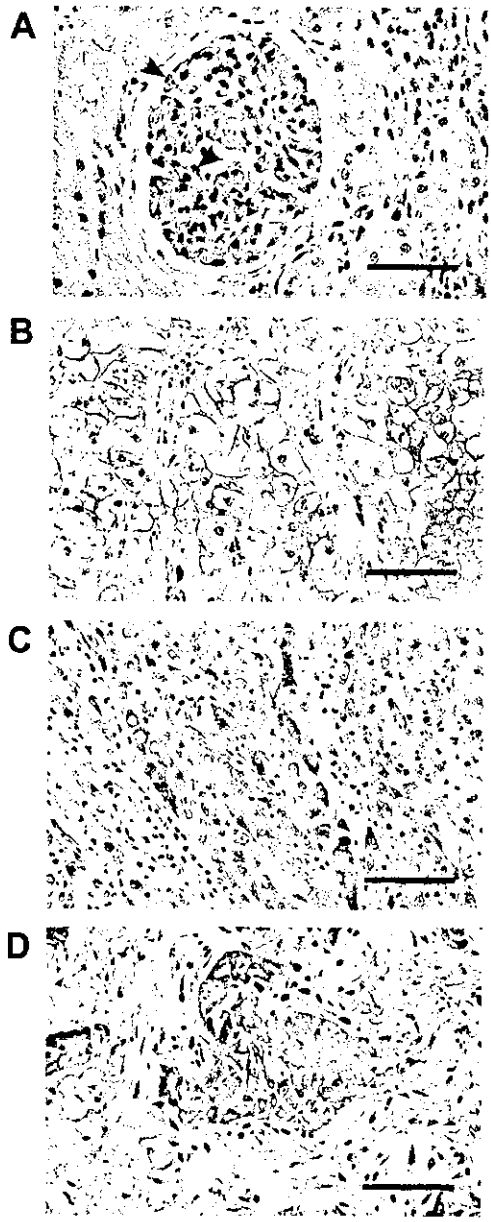


Fig. 5

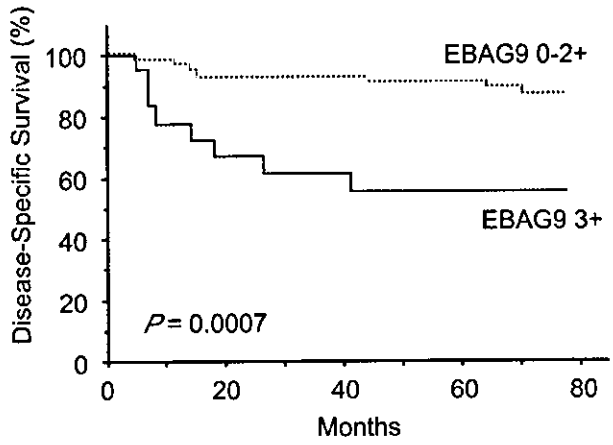

Disease-Specific Survival (%)
100
80
60
40
20
0
EBAG9 0-2+
EBAG9 3+
P= 0.0007
0
20
40
60
80
Months

Fig. 6

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Estrogen receptors and their downstream targets in cancer

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Summary. Estrogen has crucial roles in the proliferation of cancer cells in reproductive organs such as the breast and uterus. Estrogen-stimulated growth requires two estrogen receptors (ERα and ERβ) which are ligand-dependent transcription factors. High expression of ERs is observed in a large population of breast tumors. In addition, the positive expression of ERs correlates with well-differentiated tumors, a favorable prognosis, and responsiveness to an endocrine therapy with anti-estrogen drugs in patients with breast cancer. Transcription activities of ERs can be regulated by interacting proteins such as coactivators and kinases as well as ligand-binding. Moreover, ER isoforms lacking an ability to transactivate are involved in breast cancer. Downstream target genes of ERs have important roles in mediating the estrogen action in breast cancer. We have isolated and characterized several novel estrogen-responsive genes to clarify the molecular mechanism of the estrogen action in target cells. Among these genes, the estrogen-responsive finger protein (Efp) was found to be highly expressed in breast cancer. Efp as a ubiquitin ligase (E3) is involved in the proteasome-dependent degradation of the 14-3-3σ protein, one of cell cycle brakes, this degradation resulting in the promotion of breast cancer growth. A full understanding of the expression and function of ERs and their target genes could shed light on how estrogen stimulates the initiation and promotion of cancer, providing a new approach to diagnose and treat cancer.

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Background

Estrogen, a sex steroid hormone, exhibits important biological functions in the target tissues such as reproductive organs. Among these tissues, the growth of the mammary gland and uterine endometrium during pregnancy and the menstrual cycle is dependent on estrogen. In addition to proliferative effects on normal cells, estrogen is considered as a stimulant for the initiation and promotion of tumors in these organs. Epidemiological studies show that prolonged exposure to estrogen, i.e. early menarche, late menopause, and estrogen replacement therapy, can be a risk factor in breast and uterine cancers (Rose, 1996; Clemons and Goss, 2001). In vitro experiments indicate that cells derived from breast and uterine tumors are capable of growing in response to estrogen administration (Holinka *et al.*, 1986; Foster *et al.*, 2001). It is reasonable to assume that the stimulatory effects of estrogen on cell proliferation also contribute to malignant tumor growth. Following prolonged exposure to estrogen, an increase in cell proliferation would be expected to cause an increase in spontaneous DNA replication errors. When mutated in target cells of estrogen, it would enhance the replication of clones of cells carrying such genetic errors. It is, therefore, important to understand mechanisms by which estrogen increases cell proliferation in estrogen-associated cancer.

The estrogen-stimulated growth in tumor cells as well as in normal cells requires the estrogen receptor (ER). It has been shown that about two-thirds of human breast tumors express higher concentrations of ERs than normal breast tissues (Early Breast Cancer Trialists' Collaborative Group, 1998). The ER expression status is related to a variety of histologic characteristics of breast cancer. Most tumors with low grades are ER-positive but, in contrast, tumors demonstrating histologic evidence of poor tumor differentiation are frequently ER-negative (Millis, 1980; Fisher *et al.*, 1981). Breast tumors which lack any ER expression often reveal more aggressive phenotypes (Clarke *et al.*,

1994).

Clinically, endocrine therapy with anti-estrogen drugs or aromatase inhibitors is utilized to treat hormone-related cancer (Howell, 2000; Ali and Combos, 2002). It is expected that tamoxifen, an anti-estrogen drug, binds to ER, making it nonfunctional, while aromatase inhibitors reduce estrogen levels. As discussed below, most breast tumors expressing ER are primarily able to respond to tamoxifen. Aromatase inhibitors such as anastrozole and letrozole are especially useful in patients who are or become resistant to tamoxifen. However, a substantial portion of patients with breast cancer eventually acquire resistance against these treatments. In addition, most of the ER-negative breast tumors can not respond to the anti-estrogen drug. Furthermore, several side effects by treatment with tamoxifen and aromatase inhibitors to ER positive cancer such as breast and cancer, have been reported (Wiseman and Adkins, 1998; Buzdar and Hortobagyi, 2000; Howell, 2000; The ATAC Trialists' Group, 2002).

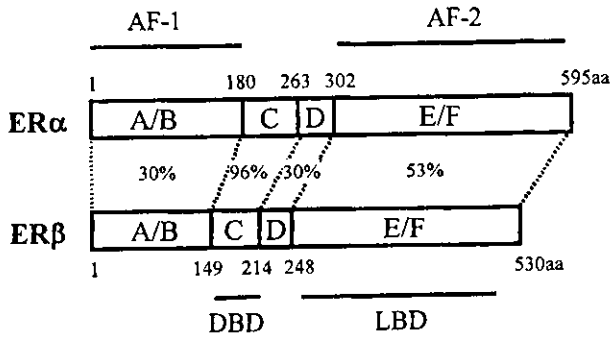

Fig. 1. Schematic representation of human ERα and ERβ. The ERα and ERβ are transcription factors whose activities are regulated by their ligand binding. ERs are members of a nuclear receptor superfamily comprised of six regions (A–F). The ligand-binding domain (LBD) in region E also contains an estrogen-inducible transcription-activating function called AF-2. A constitutively active transcription-activating function (AF-1) is located in the A/B region. Percentages of amino acid identities between the corresponding regions are represented.

It is thus important to uncover the precise mechanism of the estrogen action in breast cancer. In particular, the elucidation of regulatory mechanisms for the expression and function of ERs could provide useful information to predict the responsiveness to endocrine therapy and the prognosis. Moreover, it is important to reveal roles of downstream target genes for ERs, which mediate the effects of estrogen on the proliferation of cancer cells while these genes can be targeted to treat and diagnose estrogen-associated tumors.

Estrogen receptors

As stated above, ER has two subtypes, ERα and ERβ. They belong to the superfamily of nuclear receptors that share similar structures and modes of action (Nuclear Receptors Committee, 1999) (Fig. 1). Namely, estrogen-bound ERs bind as a homodimer or as a heterodimer to an estrogen-responsive element (ERE) with their DNA-binding domain and regulate the transcription of the target genes. ERs contain two independent transcriptional activation functions (AF): the N-terminal A/B domain possesses an autonomous

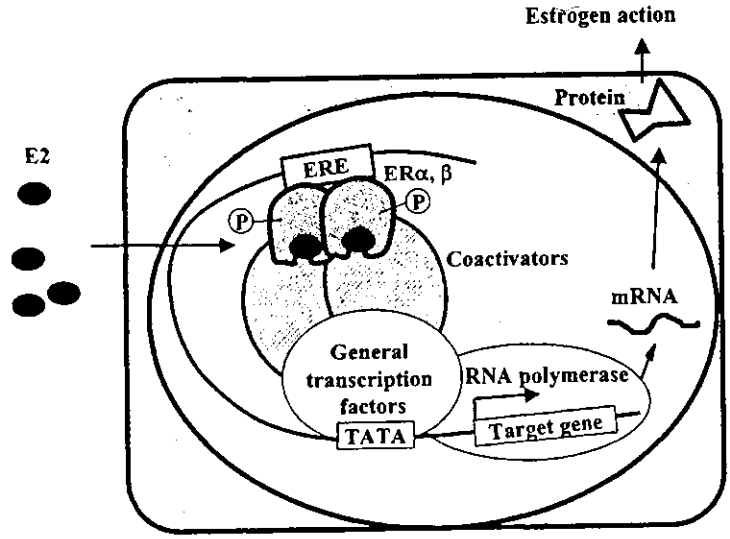

Fig. 2. A model for the regulation of estrogen receptor (ER)-mediated transcription of estrogen-responsive genes. Liganded ERα and ERβ bind as a homodimer or as a heterodimer with an estrogen-responsive element and regulate the target gene transcription. Coactivators are required to mediate ligand-activated transcription by enhancing nuclear receptor transactivation through contacts with the basal transcriptional machinery. Phosphorylation of the ER also modulates the transcription activity.

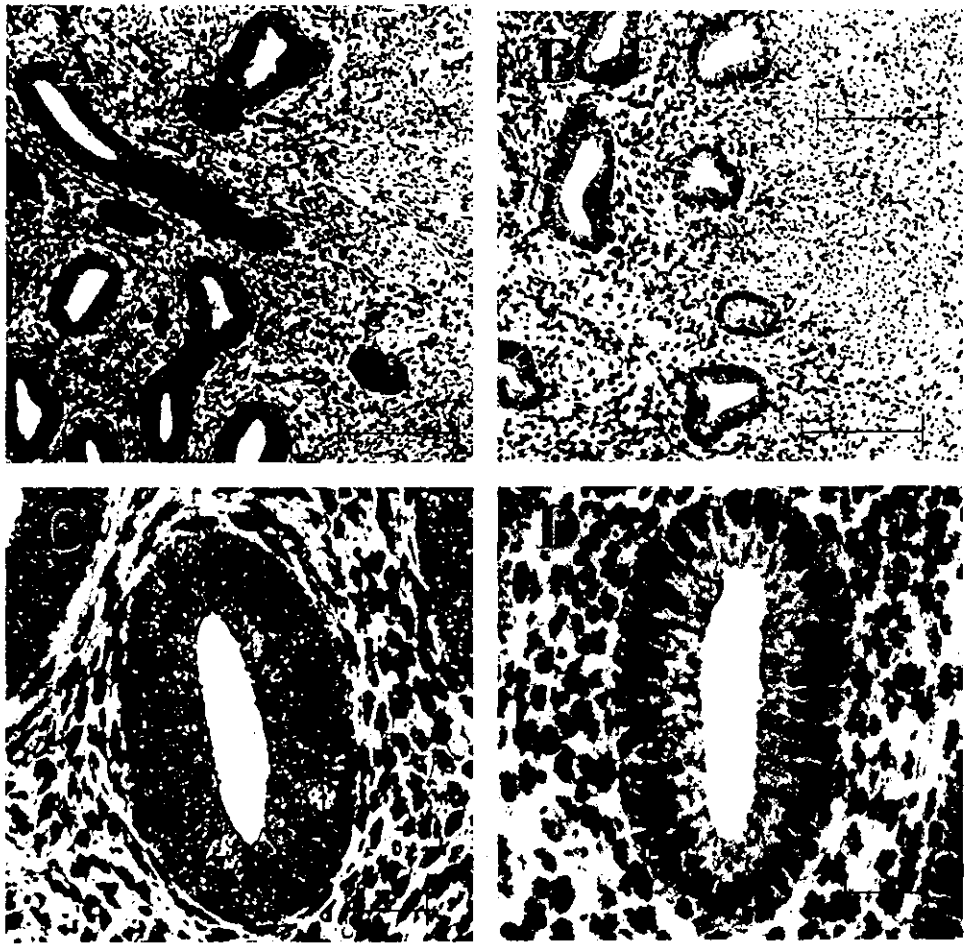

Fig. 3. Immunohistochemical staining of COX7RP (**A** and **C**) and ERα (**B** and **D**) in human endometrium in the proliferative phase of the menstrual cycle. ERα and COX7RP immunoreactivities were detected in the nucleus and cytoplasm, respectively. Strong immunoreactivities of ERα and COX7RP were detected in the glandular epithelia. Scale bar=100 μm (A, B); 10 μm (C, D).

AF-1, while the E-domain possesses a ligand-dependent AF-2. Biological activities of ERs could be controlled by a number of interacting proteins. The ligand-dependent transactivation of ERs requires the recruitment of coactivators such as TIF2 and SRC-1 (Glass and Rosenfeld, 2000). Transcription activities of ERs are also regulated by phosphorylation. In particular, the serine residue at 118 within the A/B domain of human ERα is a major target site of phosphorylation by MAPK in the presence of growth factors (Kato *et al.*, 1995) and by Cdk7 in a ligand-dependent manner (Chen *et al.*, 2000). Recently, we demonstrated that this serine residue is opposingly dephosphorylated by protein phophatase 5 (Ikeda *et al.*, 2004) (Fig. 2). The protein level of ERα is regulated by the ubiquitin-mediated proteasomal degradation (Nawaz *et al.*, 1999; Tateishi *et al.*, 2004). In addition, some elements in the promoter region have been shown to be responsible for a high expression of ER α in breast cancer cells (Hayashi *et al.*, 1997; Tanimoto *et al.*, 1999). Collectively, it is reasonable to assume that these regulatory mechanisms of ERs are closely associated with oncogenesis and tumor growth. Moreover, it is also indispensable for the diagnosis and treatment of estrogen-associated cancer to reveal the regulatory mechanisms for expression levels of the ER mRNA and protein.

The expression of ERβ has been detected in various tumors including breast cancer (Omoto *et al.*, 2002), uterus

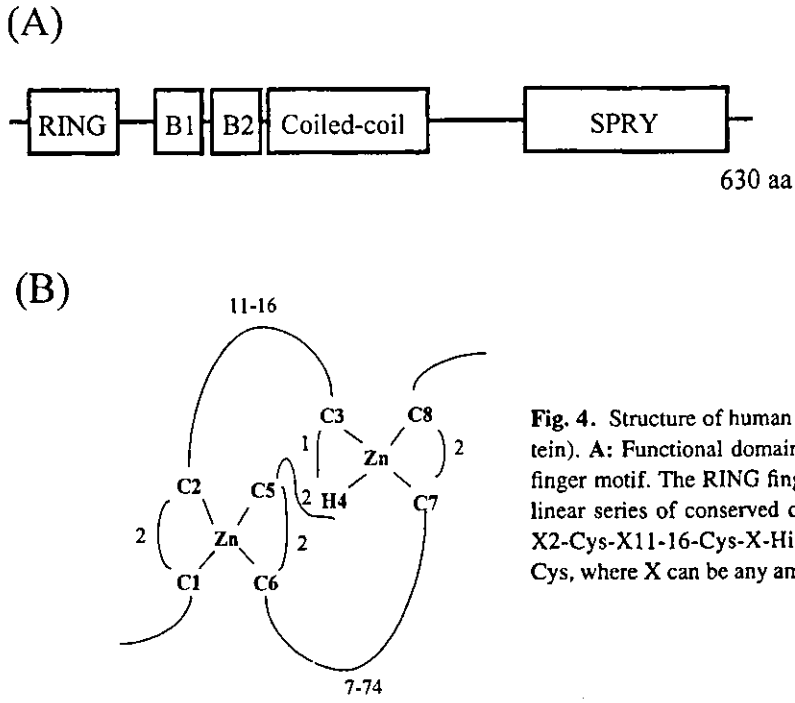

Fig. 4. Structure of human Efp (estrogen-responsive finger protein). **A:** Functional domains of the Efp. **B:** Structure of RING finger motif. The RING finger motif can be defined as a unique linear series of conserved cysteine and histidine residues: Cys-X2-Cys-X11-16-Cys-X-His-X2-Cys -X2-Cys-X7-74-Cys-X2-Cys, where X can be any amino acid.

cancer (Sasano *et al.*, 1999), and prostate cancer (Fujimura *et al.*, 2001). In breast cancer, ERβ shows a tendency to be expressed in ERα-positive carcinomas, while ERα and ERβ double positive cells are also detected. ERβ, as well as ERα, serves as an indicator of a good prognosis in breast cancer (Omoto *et al.*, 2002). It has been found that several variants of ERβ are expressed in breast cancer cells (Leygue *et al.*, 1999). We originally isolated an ERβ isoform, ERβcx (Ogawa *et al.*, 1998), which lacks the last 61 C-terminal amino acids and has an alternative 26 unique amino acids. The ERβcx isoform shows no ligand binding ability and has no capacity to activate transcription in response to estrogen (Ogawa *et al.*, 1998). Moreover, ERβcx shows preferential heterodimerization with ERα rather than with ERβ, inhibiting ERα DNA binding and transactivation. In ERα positive breast cancer, the presence of ERβcx is significantly correlated with the absence of a progesterone receptor (PR) which is a downstream target of activated ER, indicating that ERβcx is a dominant repressor of the ER function in breast cancer. (Saji *et al.*, 2002). These lines of evidence suggest that ERβisoforms are important functional modulators of estrogen-signaling pathways in breast cancer cells and may affect the clinical outcome of patients with breast cancer.

Estrogen-responsive genes in cancer

Estrogen modulates transcription of downstream target genes through ERs. It is thus fundamentally important to identify genes whose expression is regulated by estrogen and to reveal the functions of their protein products. Although a list of ER-target genes has been accumulating, the entire mechanism by which ER enhances the proliferation and progression of tumors remains unknown. In particular, only a few genes are known to be directly regulated by ER through EREs. In order to isolate estrogen-responsive genes having EREs in their transcription regulatory region, we have developed the genomic binding-site cloning (GBSC) method (Inoue *et al.*, 1991, 1999). Using this method, several genomic sequences containing EREs were successfully isolated. Subsequently, novel estrogen-responsive genes were identified nearby the EREs (Inoue *et al.*, 1993; Watanabe *et al.*, 1998). Protein products of these genes include the estrogen-responsive finger protein (Efp), the cytochrome c oxidase subunit VIIa-related polypeptide (COX7RP), and the estrogen receptor-binding fragment-associated antigen 9 (EBAG9).

COX7RP has a well conserved region with cytochrome c oxydase subunit VIIa (Watanabe *et al.*, 1998). Expression of the COX7RP mRNA was up-regulated by estrogen in

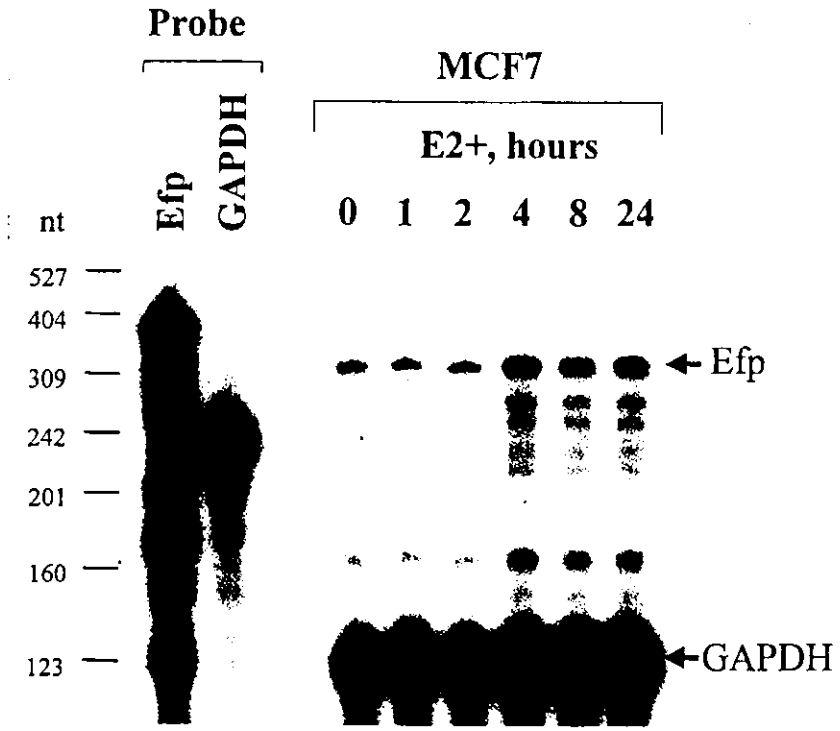

Fig. 5. Estrogen-induced expression of Efp mRNA in MCF7 cells. Total RNAs were isolated from MCF7 cells treated with 10^{-8} M 17β-estradiol at the indicated times. Twenty μg of total RNA was examined by a RNase protection assay using Efp and GAPDH probes. The full-length protected fragment for each probe is indicated.

MCF7 cells. The perfect palindromic ERE found in the first intron possesses an estrogen-dependent enhancer activity in these cells. In addition, an immunohistochemical study demonstrated that the COX7RP protein is co-expressed with the ERα protein in the endometrial glandular epithelium of the human uterus (Fig. 3). We speculate that COX7RP is involved in the regulation of energy production in target cells by estrogen.

Molecular mechanism of Efp function in breast cancer

Among ER-downstream molecules isolated by the GBSC method, we have clarified the molecular mechanism of Efp, which possesses a RING finger motif, two B-boxes, α-helical coiled-coil domains, and a C-terminal SPRY domain (Inoue *et al.*, 1993) (Fig. 4). The RING finger motif is comprised of a unique linear series of conserved cysteine and histidine residues that features a 'cross-brace' arrangement with two zinc ions (Pickart, 2001). Members of the RING finger family grow enormously; some of them have been shown to be responsible for malignant tumors. For instance, PML is responsible for acute premyelocytic leukemia when it forms a fusion protein with the retinoic acid receptor (RAR)α by chromosomal translocation (Jensen *et al.*, 2001). Loss of the tumor suppressor BRCA1 results in chromosomal instability leading to the development of familial breast and ovarian tumors (Ruffner *et al.*, 2001). Efp is predominantly expressed in estrogen target tissues and cells including the mammary gland and uterine epithelial cells (Orimo *et al.*, 1995). Efp is also highly expressed in breast tumors (Ikeda *et al.*, 2000). Expression of the Efp mRNA was shown to be elevated after estrogen treatment in MCF7 cells (Fig. 5). Thus, Efp could function as an estrogen-responsive gene that mediates the estrogen action in cancer. The estrogen-responsive proliferation of the uterine endometrium which expresses abundant ERα was

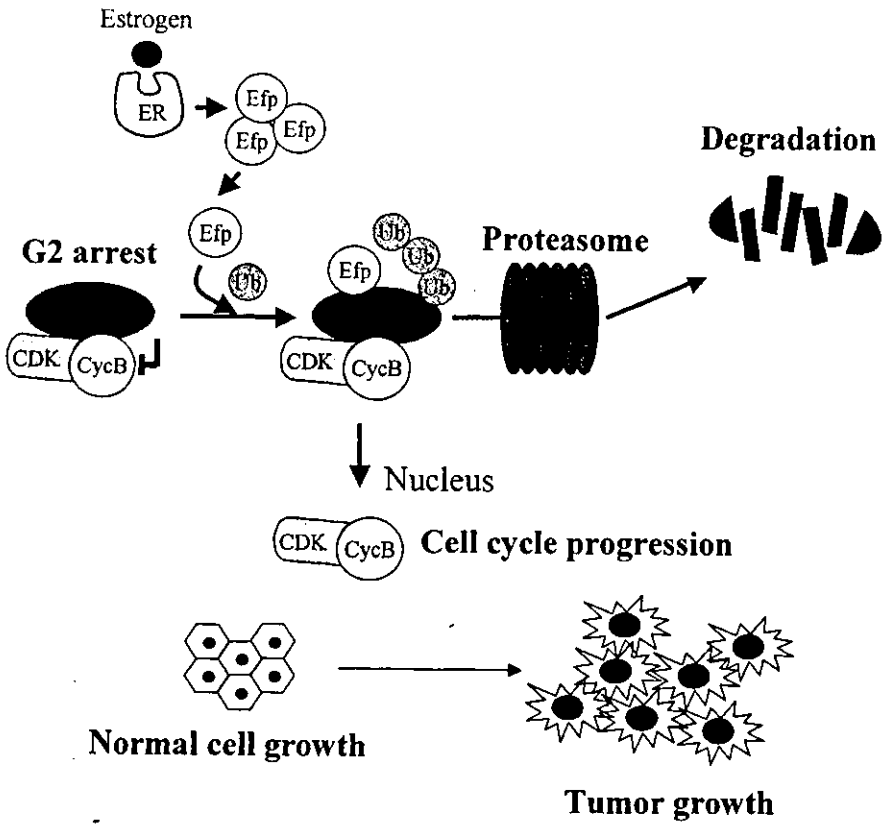

Fig. 6. A simulation model for tumor growth controlled by Efp. The Efp targets 14-3-3σ for proteolysis as a ubiquitin ligase and stimulates tumor growth.

shown to be impaired in Efp knockout mice, suggesting that Efp is a mediator of estrogen-dependent cell growth (Orimo *et al.*, 1999).

To investigate the role of Efp in breast tumor growth, we examined the effects of Efp antisense oligonucleotides on tumor formation in female nude mice inoculated with MCF7 cells (Urano *et al.*, 2002). These mice were ovariectomized or administrated with antisense/sense Efp oligonucleotides. We revealed that the Efp antisense oligonucleotide effectively inhibits the tumor growth generated by MCF7 cells in the recipient mice. MCF7 cells stably expressing Efp (Efp-MCF7) could proliferate even in estrogen-deprived ovariectomized mice. The Efp-MCF7 cells have lower concentrations of the 14-3-3σ protein, which is a negative regulator of the cell cycle progression. The 14-3-3σ protein is important for maintaining G2 arrest by sequestering phosphorylated Cdc2-cyclin B1 from the nucleus into the cytosol (Chan *et al.*, 1999). Interestingly, the expression level of this protein is significantly low in breast tumors (Vercoutter-Edouart *et al.*, 2001; Umbrich *et al.*, 2001). We found that Efp associates with the 14-3-3σ protein. We then demonstrated that Efp functions as a ubiquitin ligase, E3, that ubiquitinates the 14-3-3σ protein, this ubiquitination resulting in the cell cycle progression via the proteasome-dependent degradation of the 14-3-3σ protein (Urano *et al.*, 2002) (Fig. 6).

Perspective

A better understanding of the molecular mechanisms by which estrogen stimulates cell growth can provide new insights into diagnosis, treatment and prevention in estrogen-associated tumors. For this reason, it is indispensable to reveal the expressional and functional regulation of ERs and their target genes. Especially, the identification of estrogen-responsive genes which are closely related to the cancer biology could provide us new approaches for these fields.

Efp, an estrogen-responsive gene, would contribute to the disregulated proliferation of breast cancer cells by the accelerated destruction of a cell cycle regulator, 14-3-3σ. We speculate that Efp could promote tumor growth even in the absence of estrogen and, therefore, the high expression of Efp might be one of reason for acquiring the ability to proliferate independently of estrogen. The future investigation of the relationship between Efp expression and clinical or pathological features could indicate its usefulness as a potential prognostic factor. These trials may lead to the utilization of Efp as a prognostic marker and a therapeutic target in breast cancer. Thus, the accumulation of experimental evidence concerning the estrogen-responsive genes such as Efp can allow us to develop novel cancer treatments separately targeted for each downstream molecule that directly mediates the estrogen action.

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Vitamin D receptor (VDR) promoter targeting through a novel chromatin remodeling complex☆

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Abstract

We have purified nuclear complexes for Vitamin D receptor (VDR), and identified one of them as a novel ATP-dependent chromatine remodeling containing Williams syndrome transcription factor (WSTF), that is supposed to be responsible for Williams syndrome. This complex (WSTF including nucleosome assembly complex (WINAC)) exhibited an ATP-dependent chromatin remodeling activity in vitro. Transient expression assays revealed that WINAC potentiates ligand-induced function of VDR in gene activation and repression. Thus, this study describes a molecular basis of the VDR function on chromosomal DNA through chromatine remodeling.

Keywords: Vitamin D receptor; WINAC; Histone acetyltransferase; Chromatin remodeling

1. Transcriptional controls by Vitamin D receptor (VDR) requires chromatin remodeling

VDR is a member of the nuclear receptor (NR) gene superfamily, and acts as a ligand-inducible transcriptional factor [1]. At transcriptional initiation sites in the VDR target promoters, distinct classes of multiprotein complexes are thought to be indispensable for controlling transcriptions of VDR through chromatin remodeling [2,3]. These complexes modify the chromatin configuration in a highly regulated manner, like nucleosome rearrangement, and bridge the functions between regulators and basal transcription factors, along with RNA polymerase II. Two major classes of chromatin-modifying complexes have been well characterized and their anchoring to the promoters presumably requires enzyme-catalyzed modifications of histone tails in chromatin [4]. One class contains several discrete subfamilies of transcription co-regulatory complexes with either histone acetylase (HAT) or histone deacetylase (HDAC) activities to covalently modify histones through acetylation. In ligand-induced transactivation processes of nuclear receptors like VDR, the complexes containing HDAC first act to co-repress transactivation of unliganded NRs, while upon ligand binding, two HAT complexes, p160/CBP and TRRAP/GCN5, co-activate the NR function, like the other non-HAT DRIP/TRAP/SMCC co-activator complexes [5–7].

Another class of complexes are chromatin remodeling complexes to use ATP hydrolysis to rearrange nucleosomal arrays in a noncovalent manner, and make chromosomal DNA accessible for sequence-specific regulators like VDR [4]. These ATP-dependent chromatin-remodeling complexes act on transcription, DNA repair, and DNA replication as well. These complexes are further classified into subfamilies based on the major catalytic components, ATPases (SWI2/SNF2, ISWI, and Mi-2) [8,9]. Indeed, recently ligand-induced transactivation function of VDR in vitro has been shown to require a SWI2/SNF2-type chromatin remodeling complex containing pBAF180.

2. Identification of a novel Williams syndrome transcription factor (WSTF)-containing nuclear complex associate with VDR

To identify a novel co-regulator complex for VDR, HeLa cell nuclear extracts were incubated with a chimeric VDR-DEF region protein (VDR-DEF) fused to glutathione-*S*-transferase (GST), in the presence or absence of 1α,25(OH)2D3. Mass spectrometry and the apparent molecular weights of the different proteins associated with the VDR-DEF in a ligand-dependent way led to the identification of several known components of the DRIP/TRAP/SMCC complex,

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in agreement with previous observations [5,7]. One of the ligand-independent VDR-specific interactants turned out the WSTF/WBSCR9/BAZ1B [10,11]. WSTF has been supposed to be a candidate responsible for the diverse WS disorders [10,12]. This possibility is raised by the fact that WSTF is highly homologous to hACF1 as one of the WAC family proteins [11]. Also, hACF1 is a partner of hSNF2h (a *Drosophila* ISWI homologue) to form well-characterized ISWI-based chromatin remodeling complexes [13].

To purify a WSTF-containing complex, we established a MCF7 stable transformant overexpressing FLAG-tagged WSTF. With the nuclear extracts of the stable transformants, WSTF containing complexes were isolated by multi-step purification using the GST-VDR column and an anti-FLAG affinity resin column. On the glycerol density gradient, the purified complexes with a molecular weight of greater than 670 kDa bound to the GST-VDR column and these large molecular weight fractions contained WSTF. With the mass fingerprinting, we identified all the components of the purified complex containing WSTF, and designated this complex as WSTF including nucleosome assembly complex (WINAC) [14]. WINAC consists of at least 13 components, but unexpectedly contains neither hSNF2h nor the components of known ISWI-based complexes (Table 1). Rather, the SWI/SNF type ATPases (Brg1 and hBrm) and several BAF components share with the SWI2/SNF2-based complexes [2]. Interestingly, WINAC appears to harbor three components (TopoIIb, FACTp140, and CAF-1p150) [15–17], which have not yet been found in any known ATP-dependent chromatin remodeling complexes.

3. INAC rearranges the nucleosome array around VDRE through ATP-dependent chromatine remodeling in vitro

By a standard micrococcal nuclease assay, an ATP-dependent chromatin assembly reaction was clearly induced by WINAC, indicating that Brg1/hBrm in WINAC serves as an ATPase for this ATP-dependent chromatin remodeling process. We examined the ability of WINAC to disrupt nucleosome arrays through VDR bound DNA since the known ATP-dependent chromatin remodeling complexes are potent to recognize the nucleosomal array around the binding sites of a sequence-specific regulator [18,19]. By Southern blot analysis with a pair of oligonucleotides complementary to a region in the vicinity (Promoter Probe) or to a site about 900 bp upstream (Distal Probe) of the GAL4 DBD binding sites for a chimeric VDR-DEF protein (GAL-VDR), disruption of the nucleosome arrays in the GAL4 binding site vicinity was induced only when both VDR and WINAC were present (Fig. 1A), while the other regions appeared unaffected in the nucleosome arrays. Reflecting the VDR-specific nucleosome disruption by WINAC among tested receptors (Fig. 1B), ligand-induced transactivation in vitro was potentiated by WINAC only for VDR, but for neither ERa nor PPARg (data not shown).

4. WSTF potentiated ligand-induced functions of VDR in gene induction and repression

In a transient expression analysis, 1α,25(OH)2D3 (10^{-9} M) was effective to induce VDR AF-2 transactivation function and WSTF co-activated this ligand-induced AF-2 function of VDR, but not ERα (Fig. 2A). Both Brg1 and hBrm were also effective to enhance the transactivation functions of VDR and ERα (Fig. 2A) as previously reported [20,21]. Interestingly, such co-activator-like activity of WSTF was selective for VDR, and not detected for ERa, even in the presence of Brg1/hBrm (Fig. 2A).

ChIP analysis revealed that VDR and the WINAC components were constitutively associated with the promoter irrespective of ligand binding. In the contrast, ligand-induced occupancy in the promoter was seen in TRAP220 and TIF2 with ligand-induced histone H4 acetylation though the

Table 1
Major ATP-dependent chromatin remodeling complexes

Complex name	BAF	PBAF	WINAC	hCHRAC	hACF	NoRC	xWICH
Complex size (kDa)	~2,000	~2,000	~2,000	~600	~600	~600	~600
ATPase subunit	Brgl, Brm	Brgl	Brgl, Brm	hSNF2h	hSNF2h	hSNF2h	xISWI
Chromatin remodeling			WSTF	hACF1	hACF1	TIP5	WSTF
	BAF250		BAF250				
		BAF180					
	BAF170	BAF170	BAF170				
	BAF155	BAF155	BAF155				
	BAF60a	BAF60a	BAF60a				
	BAF57	BAF57	BAF57				
	BAF53	BAF53	BAF53				
	Ini1	Ini1	Ini1				
				p17		p80	
				p15		p50	
DNA replication			TopoIIβ CAF-1 p150				
Transcription elongation			FACT p140				

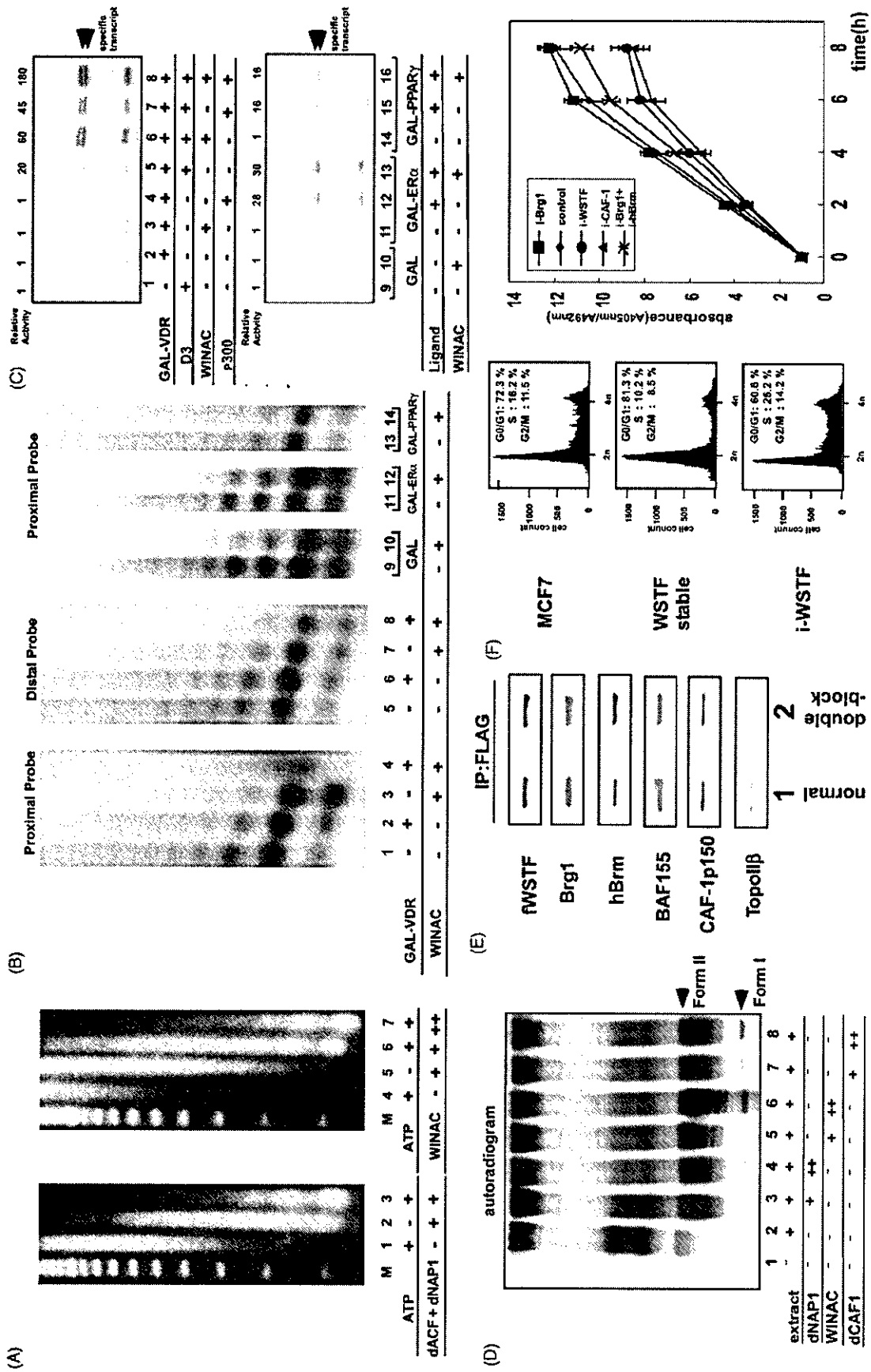

Fig. 1. WINAC as an ATP-dependent chromatin remodeling complex. (A) Chromatin reconstitution activity of WINAC. The reacted samples were subjected to partial micrococcal nuclease digestion. The molecular mass marker (M) is the 200 bp ladder. (B) Chromatin disruption by WINAC is specifically VDR dependent. Oligonucleotide probe corresponds to either a sequence between the GAL4 sites and the RNA start site (proximal probe) or 900 bp upstream of the start site (Distal Probe). (C) Potentiation of VDR transactivation by WINAC in vitro. Arrows indicate specific transcripts by transcription reactions by GAL4 derivatives. A representative result is displayed, and relative activities were calculated from three independent assays with PGI-0 vector as an internal control. (D) WINAC functions as a chromatin reconstitution factor during DNA replication in vitro. During DNA replication induced by SV40 T antigen in vitro, WINAC could form chromatin with negatively supercoiled DNA. Form I: a perfect supercoiled DNA. Form II: a relaxed form. (E) WINAC formation is unchanged in S phase. MCF7 stable transformants were cultured under either normal conditions or double thymidine block treatments. (F) Modulation of the cell cycle by altered WSTF expression. Left panel: DNA histogram of the MCF7 cells [MCF7], WSTF stably expressing MCF7 cells [WSTF stable] and MCF7 cells transfected with WSTF-RNAi [i-WSTF]. Right panel: BrdU incorporation during S phase of the MCF7 cells transfected with RNAi from the indicated proteins during double thymidine treatment. After the final release (time 0), cells were collected every 2 h, for up to 8 h. The average values of triplicate analyses are shown.