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A Novel Genetic System for Analysis of Co-activators for the N-Terminal Transactivation Function Domain of the Human Androgen Receptor

Ken-ichi TAKEYAMA,[1,2] Saya ITO,[1] Shun SAWATSUBASHI,[1] Yuko SHIRODE,[1,2] Ayako YAMAMOTO,[1] Eriko SUZUKI,[1] Akio MAKI,[1] Kaoru YAMAGATA,[1,2] Yue ZHAO,[1] Alexandre KOUZMENKO,[1,2] Tetsuya TABATA,[1] and Shigeaki KATO[1,2,†]

[1]*Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*

[2]*Solution Oriented Research for Science and Technology (SORST), Japan Science and Technology Agency (JST), 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

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Androgen receptor (hAR) regulates transcription of target genes in a ligand-dependent manner and recruits a number of co-activators for the ligand-induced transactivation *via* the N-terminal, activation function-1 (AF-1), and C-terminal, AF-2, transactivation domains. But the co-regulator functions on each of AR domains have not yet been fully understood. We have established a *Drosophila* transgenic system in which hAR and its deletion mutants are ectopically expressed in fly tissues together with an AR response element (ARE)-GFP reporter gene, and have confirmed that hAR was functional in ARE transactivation without affecting the expression of endogenous genes. We found that transcriptional activity of the hAR AF-1 domain was markedly reduced in *Drosophila* deficiency mutants of homologs for known mammalian co-activators of the AR ligand-dependent AF-2 domain. This suggests that hAR AF-1 recruits co-activators previously known only to interact with the AF-2 domain. Therefore, *Drosophila* with the hAR AF-1 transgene provides a relevant genetic system in which to uncover novel functions of vertebrate steroid hormone receptors and to screen for novel AF-1 co-regulators.

Key words: androgen receptor; transactivation; coactivator; histone acetylation; transcriptional mediator

The members of the steroid/thyroid nuclear hormone receptor superfamily act as ligand-inducible transcription factors that regulate transcription of particular sets of target genes involved in diverse physiological processes.[1)] Based on structural and functional similarities, the nuclear receptors are divided into functional domains designated A to E(F). The DNA binding domain is a highly conserved middle region (C domain), while the ligand binding domain (LBD) is located in the less conserved C-terminal E/F domain and is comprised of twelve α-helixes forming a pocket to capture cognate ligands.[2,3)] The N-terminal A/B domain and the C-terminal domain are required for the ligand-induced transactivation function of nuclear receptors. The autonomous activation function-1 (AF-1) in the A/B domain is the least conserved and constitutively active on its own, while AF-2 activation of the LBD E/F domain is dependent on ligand binding. The transactivation function of nuclear receptors requires a variety of common co-activator complexes that in many cases show ligand-dependent interactions (direct or indirect) with the AF-2 domain. A large number of regulatory complexes implicated in AF-2 transcriptional regulation have been identified.[4–6)] Among them, two HAT co-activator complexes have been characterized, one composed of factors belonging to the p160(SRC-1/TIF2/AIB-1) family and another of p300/CBP, both of which function to modify histones through intrinsic histone acetyltransferase (HAT) activities.[7,8)] The other HAT co-activator complex has been recently identified by us.[9)] Besides the HAT complexes, a non-HAT co-activator complex was biochemically identified and designated the thyroid hormone/vitamin D receptor-associated protein (TRAP/DRIP)/ Mediator complex, and represents a different type of non-HAT co-activator complex.[10,11)] This complex was further shown to be essential for TR function in an *in vitro* transcription system.[12,13)] Although the TRAP220 subunit in this complex interacted directly with TR and subsequent analysis with TRAP220$^{-/-}$ fibroblasts confirmed a

† To whom correspondence should be addressed. *Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*; Tel: +81-3-5841-8478; Fax: +81-3-5841-8477; E-mail: uskato@mail.ecc.u-tokyo.ac.jp

Abbreviations: AR, androgen receptor; ARE, AR response element; AF-1, activation function-1; DHT, dehydrotestostesone; HAT, histone acetyltransferase; AIB-1, Amplified in Breast Cancer; CBP, CREB-binding protein; TRAP, thyroid hormone receptor-associated protein

receptor-selective function for TRAP220,[14] the interaction of other subunits, such as TRAP80 and 100, their functional roles are not fully understood. Thus, their functions to the AF-2 have been addressed to some extent, but those for AF-1 are largely unknown.

Drosophila not only possesses homolog of most mammalian signaling proteins, transcriptional co-regulators, and basal transcription factors, but also expresses nuclear receptors like the ecdysone receptor (EcR), which is structurally and functionally homologous with the members of the vertebrate nuclear hormone receptor superfamily.[15,16] The fact that *Drosophila* homologs for mammalian HAT co-activators (CBP and AIB1) and TRAP/Mediator have been identified recently, and the findings of their co-activator functions on glucocorticoid receptor (GR) in cultured *Drosophila* embryonic Schneider's cell line 2, indicate that *Drosophila* and mammalian transcription factors are functionally interchangeable.[17–19]

In the present study, to identify and characterize co-activators for AF-1 of the hAR, we established a transgenic fly model system by ectopically expressing hAR wild type (wt) and its deletion mutants in *Drosophila* tissues. When the hAR expression was induced by tissue-specific GAL4-divers, ligand-dependent hAR transactivation was observed in a given tissue. No abnormality was detected, however, in the expression of either EcR or its downstream genes. The transactivation function of hAR(AF-1) was significantly reduced in all of the fly deletion mutants of homologs for known mammalian AF-2 interacting co-activator complex components, TRAP80, 100, p160, and CBP, showing that the fly co-activators for the AF-2 function also act to enhance the AF-1 function. Taken together, our data provide evidence that transgenic *Drosophila* expressing human ARs represents a potent and functionally relevant system in which to evaluate AR synthetic ligands and androgen-like compounds and to identify and characterize novel nuclear receptor co-regulators.

Materials and Methods

Fly stocks and genetics. All general fly stocks were obtained from the Bloomington *Drosophila* Stock Center. The AR mutant cDNAs in pCaSpeR3 and an ARE-GFP reporter construct (GFP-TT in pCaSpeR3 with a consensus ARE in its promoter) were used to generate several transgenic lines, as previously described.[20] Target chromosomes were separated from those carrying the *Gal4*-driver by crossing with flies harboring second and third balancer chromosomes CyO and TM3. The *Gal4*-driver lines used were as follows: the *gmr-Gal4* line, expressing GAL4 in the retina driven by the *Glass Multimer Reporter*;[21] the *dpp-Gal4* line, expressing GAL4 in the anterior-posterior boundary area in developing wing discs driven by the *dpp* (blk) promoter;[22] and the *ptc-Gal4* line, expressing GAL4 in the anterior portion of embryonic segments driven by a patched (ptc) gene promoter.[23] The TRAP80 delution line, *l(3)s2956*, was obtained from Szeged while the TRAP100 deletion line, *BL-01670*, the AIB-1 deletion line, *l(2)01351*, and the AIB-1 dominant overexpression line, *BL-6378 (UAS-Tai)*, were obtained from BDGP. The CBP deletion line, *nej*, was obtained from S. Ishii.[17]

Immunofluorescence and Histology. Tissues were dissected and fixed for 20 min in 4% formaldehyde and incubated with a primary antibody, either hAR (N-20) or (C-19) (Santa Cruz Biotechnology, Inc.), then Cy5-conjugated AffiniPure donkey anti-rabbit IgG (Jackson Immunoresearch) was used as a secondary antibody for immunofluorescence staining.[24] Conforcal microscopy was performed on the Zeiss conforcal laser scanning system 510, and images were assessed using the Adobe Photoshop 5.0 (Adobe) software program.

RNA isolation and Northern blotting. Total RNA was extracted from third instar larva using ISOGEN (Nipongene, Co.). Polyadenylated RNA (poly(A)$^+$-RNA) was purified by oligo(dT) affinity chromatography[25] and then subjected to Northern blot analysis using cDNAs for ecdysone receptor (EcR), Eip75B, β-actin, and human AR as probes.

Cell Culture and Transactivation assay. Drosophila embryonic Schneider's cell line 2 were maintained in Schneider's *Drosophila* medium, supplemented with 5% fetal calf serum. S2 cells were cotransfected with 1 μg ARE-tk-luc and 0.1 μg AR expression vector (wt or AF-1). Cells were incubated for 18 h in the absence or presence of 10^{-8} M DHT, hydroxyflutamide (HF), and bicalutamide (BIC) and then assayed for luciferase activity as previously described.[26,27]

RNAi experiments. The dsRNAs were transfected after they were annealed. The dsRNAi constructs of TRAP80-RNAi, TRAP100-RNAi, and CBP(*nejire*)-RNAi were from Open Biosystems Co. Original dsRNAs of the AIB-1(*taiman*)-RNAi are constructed from the 5′-region (1 to 700 bases) in AIB-1 ORF cDNA.

Results and Discussion

Just as we previously showed that the selective and ectopic expression of hAR mutants with expanded polyglutamine in fly eyes caused neurodegeneration, the hARs were expressed in selected tissues using the GAL4-UAS system.[20] The cDNA for wild-type hAR [hAR(wt)] or AR(AF-1) was inserted into an expression vector under the control of the hsp70 promoter containing UAS. After establishment of UAS transgenic lines, the flies were crossed with *Drosophila* from GAL4-driver lines that expressed GAL4 under a tissue-specific promoter. To monitor the ligand-induced transactivation

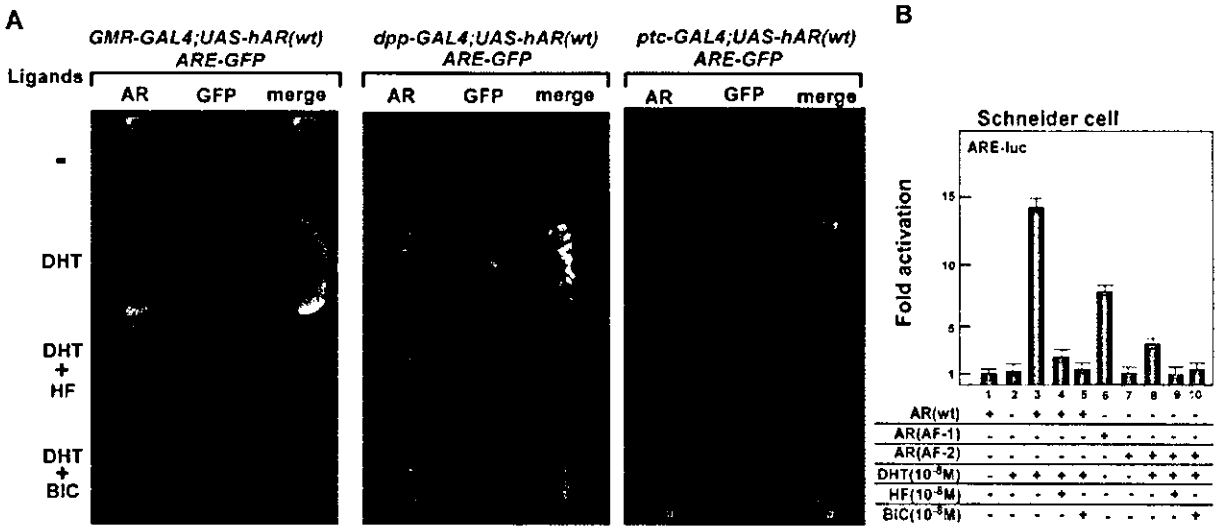

Fig. 1. Targeted Expression and Ligand-induced Transactivation Function of Human AR.

A. Targeted expression and function of hAR(wt) in *Drosophila* tissues. Expression in third instar eye imaginal discs driven by *GMR-GAL4* (right), third instar wing discs driven by *dpp-GAL4* (middle), and embryonic segments driven by *ptc-GAL4* (left) was stained (red) with anti-hAR N-20 antibody, shown in the left panel. GFP expression and green fluorecence (in the middle panel) reflects ligand-induced transactivation by hAR, and merged images are shown in the right panel. A consensus androgen response element (ARE) was introduced into the promoter of GFP to produce an ARE-GFP reporter plasmid. Transgenic flies were cultured on medium containing vehicle or ligands (DHT and/or antagonists 10^{-5} M). B. ARs transactivate in the *Drosophila* Schneider's cell line 2. Transactivation of the ARE-luciferase reporter gene was analyzed in Schneider's cell line 2 expressing wild type or mutant hAR. Ligand DHT (1×10^{-8} M) and its antagonists [hydroxyflutamide (HF) and bicalutamide(BIC)] (1×10^{-6} M) were added as indicated. AR (wt) means wild type AR. AR(AF-1) and AR(AF-2) are the AR mutants deleting the C-terminal E/F domain and A/B domain respectively. The data presented are from a representative experiment out of the five independent experiments performed. Luciferase reporter activity of AR transactivation in the absence of DHT is presented as fold induction. Values are mean ± SD.

function of hAR, these flies were further crossed with flies bearing a GFP reporter gene with consensus sequences for androgen response element (ARE) in their promoter.

Using this system, tissue-specific expression of hAR(wt) was induced in the developing eye disc under a *Glass Multimer Reporter* (*gmr*) gene promoter[21] (left panel in Fig. 1A), in the middle area in the developing wing disc under a *decapentaplegic* (*dpp*) gene promoter[22] (middle panel), and in the anterior portion of embryonic segments under a patched (*ptc*) gene promoter[23] (right panel). No phenotypic abnormalities were observed in flies with any of the transgene constructs. Expressed hARs were detected *in situ* using an immunofluorescent antibody, and ingestion of DHT induced GFP expression, observed as green fluorescence, only in tissues that ectopically expressed hAR (Fig. 1A). Significantly, since no green fluorescence was detected in flies cultured on the medium without androgens (Fig. 1A), it appears that *Drosophila* does not produce endogenous hAR ligands. Well-known androgen antagonists, such as hydroxyflutamide (HF) and bicalutamide (BIC), did not induce GFP expression in the presence of DHT in the fly and S2 cells (Fig. 1A and 1B, lanes 3–5). Antagonized effects of AR (AF-2) transactivation were also detected (Fig. 1B, lanes 8–10). Both unliganded and liganded hAR (wt, AF-1 and AF-2) were non-toxic in the transgenic flies under all conditions studied as no phenotypic abnormalities were detected in any of the tissues. The biological activity in *Drosophila* of ingested AR ligands appeared to be identical to ligand activity in cultured mammalian cells or intact mammals.[27,28]

Targeted expression of hAR(wt) in several tissues at different developmental stages caused no overt abnormalities in the transgenic fly, even after 5 d of dietary ingestion of DHT and/or antagonists. Nonetheless, it is difficult to exclude the possibility that the function of endogenous coactivators, essential for endogenous nuclear receptor action,[17,18] was affected by the overexpressed hAR, resulting in altered endogenous nuclear receptor function. The *Drosophila* nuclear receptor Eip75B is known to be under the transcriptional control of EcR/USP heterodimers as a response element for the ecdysone receptor has been identified in its gene promoter.[29,30] In our experimental system, DHT ingestion did not appear to affect the expression of endogenous Eip75B or EcR genes even in tissues that expressed high levels of hAR (Fig. 2). Thus it seems unlikely that hAR expression in the fly significantly interferes with endogenous regulatory processes mediated by *Drosophila* nuclear receptors.

The properties of hAR (AF-1) are distinct from those of AF-2 in mammalian cells[28,30] and it is not clear whether cofactors interact specifically with one domain or the other or both. In this study, we tested the transactivation function of the AF-1 domain in *Drosophila* deficiency mutants of homologs of mammalian coactivators for nuclear receptors known to interact with

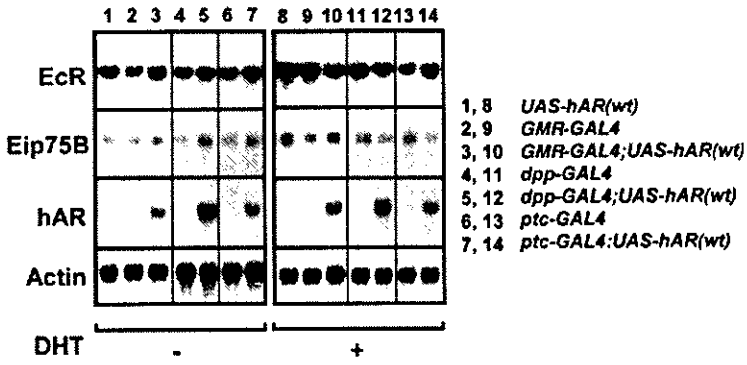

Fig. 2. Transgenic Flies Do Not Display Aberrant Expression of Endogenous Ecdysone Receptor (EcR) and Eip75B Genes.

Expression of EcR and Eip75B was measured in third instar larvae of *UAS-hAR* (lanes 1 and 8), *GAL4-driver* (lanes 2, 4, 6, 9, 11, and 13), and *GAL4-driver: UAS-AR* (3, 5, 7, 10, 12, and 14) lines in the absence or presence of DHT (1×10^{-5} M). Polyadenylated RNA (poly(A)$^+$-RNA) was purified by oligo(dT) affinity chromatography and 10 μg poly(A)$^+$-RNA was separated by electrophoresis on 1% agarose-1.1 M formaldehyde gels, and transferred to nitrocellulose membranes. Northern blots were probed with cDNA for the Ecdysone receptor (EcR), Eip75B, and β-actin.

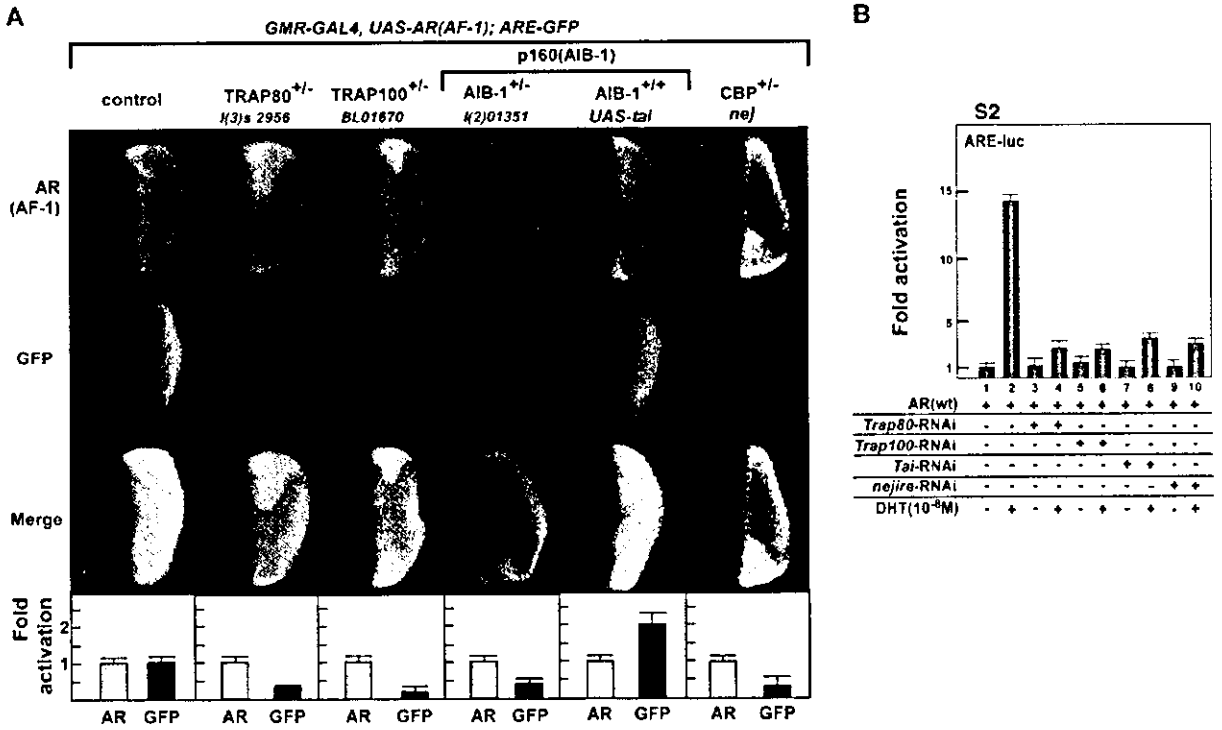

Fig. 3. AR (AF-1) Transactivation in *Drosophila* Is Induced by Its Endogenous Co-activators.

A. AR (AF-1) transactivation in the eye disc is reduced in mutants deficient in homologs of mammalian co-activators. Genotypes are *GMR-GAL4, UAS-hAR(AF-1); ARE-GFP, in trans* to TRAP80 mutant [*l(3)s2956*], TRAP100 mutant (*BG01670*), AIB-1 mutant (*tai01351*), overexpressed AIB-1 (*UAS-tai*), or CBP mutant (*nej*). *GMR-GAL4* expressed AR (AF-1) was detected with anti-hAR N-20 antibody in the third instar eye imaginal discs (upper panel). The transactivation function of AR (AF-1) was evaluated by levels of GFP expression and green fluorescence (middle panel) and merged images are shown in the lower panel. The relative abundance of GFP activities is corrected by anti-AR antibody-stained AR protein levels using NIH images and indicated as mean ± for at least three samples from different eye discs. B. AR transactivates *via* endogenious co-activators in the *Drosophila* Schneider's cell line 2. S2 cells were transfected with the expression vectors of an ARE-luciferase reporter plasmid. 0.1 μg of AR (wt) expression plasmid and 0.2 μg of dsRNAi constructs [*Trap80-RNAi, Trap100-RNAi, AIB-1 (taiman)-RNAi*, and *CBP (nejire)-RNAi*] were transfected as indicated in the images in the absence or presence of DHT (1×10^{-8} M). The data presented are from a representative experiment out of the five independent experiments performed. Luciferase reporter activity of AR transactivation in the absence of DHT is presented as fold induction. Values are mean ± SD.

their AF-2 domain. hAR (AF-1) expression targeted by *GMR-Gal4* was observed in the third instar larva eye disc (Fig. 3). Ligand-independent ARE-GFP transactivation by hAR (AF-1) was clearly evident in these flies, while no enhancement of ligand-dependent transactivation in AR (AF-1) was detected (data not shown). We then crossed these flies expressing hAR (AF-1) with *Drosophila* deficiency mutants of the *dTRAP80, dTRAP100,*

Tai (p160), and *nej(CBP)*, whose mammalian counterparts are known to be recruited by the nuclear receptor AF-2 domain[17,18,31,32] but their interaction with the AF-1 domain has not been demonstrated. The transactivation by AR (AF-1) in the eye discs of resulting hybrids was significantly reduced, while hAR (AF-1) expression levels estimated by anti-hAR antibody were unaffected. On the contrary, an overexpression of *tai* (p160) driven by *GMR-Gal4* markedly enhanced the AR (AF-1) transcriptional activity in the eye disc. Finally, we attempted to determine by transient expression assay in S2 cells whether the AR (wt) transactivation function reduces with dsRNAi constructs which repress endogenous expression of the co-activators Trap80, Trap100, AIB-1, and CBP. The DHT-induced transactivation function of AR (wt) was assessed 72 h after as RNAi transfection and was severely attenuated nearly to basal transcription levels (Fig. 3B, compare lane 2 with lanes 4, 6, 8, and 10). A similar effect on AR (AF-1) transactivation was observed both in mammalian cells and S2 cells (data not shown). Thus, by employing the well characterized and defined *Drosophila* genetic mutant system, we were able to demonstrate that the hAR (AF-1) domain functionally associates with co-activators previously known only to interact with the AF-2 domain of nuclear receptors.

The genetic approach in animals is a powerful technique that offers the advantage of being able to detect endogenous transcription co-regulators associated with a given transcription factor *in vivo* under different physiological conditions or different genetic backgrounds. In this study, we established a *Drosophila* model system by ectopically expressing functional hAR and its mutants. The application of genetic screening using these fly lines may help us to identify novel hAR (AF-1) co-regulators and determine fine mechanisms and processes through which hAR (AF-1) regulates gene expression.

Although the findings here indicate that both the p160/CBP HAT co-activator and the TRAP/Mediator are important in hAR-mediated gene expression, supporting the previous observations *in vitro*, several recent studies suggest a requirement for chromatin remodeling complexes in gene regulations by nuclear receptors in addition to their association with co-activators. For example, the ATP-dependent ISWI chromatin remodeling complex is required for retinoic acid receptor/RXR binding to chromatin templates.[33] Furthermore, the SWI/SNF chromatin remodeling complexes, including WINAC, have been shown to play a critical role in vitamin D receptor/RXR and several nuclear receptor-mediated transactivation processes.[34–36] *Drosophila* has been also shown to possess chromatin remodeling factors that appear to be functionally conserved from yeast to mammalians,[37] and transcriptional controls by hAR in flies are supposed to require such chromatin remodeling complexes. While studies in yeast implicate a temporal role for the SWI/SNF complex in gene regulation preceding the recruitment of HAT coactivators, experiments with nuclear receptors and other activators in mammalians suggest that the SWI/SNF complexes function only after the recruitment of HAT co-activator complexes.[33,38] Future genetic studies are required to reveal a stage or temporal role for each of the co-activator complexes in the context of recruitment of chromatin remodeling factors on nuclear receptor target promoters, and further to identify a novel molecule bridging between a co-activator complex and a chromatin remodeling complex.

In addition to genetic studies, functional screening for ligands with desired biological activities is also applicable by means of ligand-dependency in AR transactivation. Indeed, we showed that HF and BIC, major androgen antagonists, did not induce AR mediated transactivation *in vivo* and in S2 cells, suggesting that the structural alteration of the ligand binding domain in hAR with androgen antagonists causes an association with endogenous co-repressors. Recently, it has been reported that an EcR co-regulator, SMRTER, a *Drosophila* homolog of the mammalian co-repressor SMRT, associates with endogenous Sin3A similar to its vertebrate counterpart. These findings perhaps indicate that the basic mechanism of transrepression by NRs is conserved between vertebrates and insects.[39] Since mammalian HDAC co-repressor factors including SMRT are recruited to an antagonist-bound AR on the mammalian androgen-responsive gene promoters,[40] it is conceivable that antagonist-bound hAR in flies also recruits SMRTER for transrepression. To test this hypothesis and to determine the molecular basis of antagonist-induced transrepression by AR, identification of the antagonist-bound hAR-SMRTER or other functionally similar complexes is of interest.

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Function of androgen receptor in gene regulations☆

Shigeaki Kato[a,b,*], Takahiro Matsumoto[a], Hirotaka Kawano[a], Takashi Sato[a], Ken-ichi Takeyama[a,b]

[a] *Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan*
[b] *SORST, Japan Science and Technology Corporation, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan*

Abstract

Most of the androgen actions are considered to be mediated by the androgen receptor (AR) of the target genes. The AR is composed of a fairly large molecule because of the long A/B domains of its N-terminal. However, the independent roles of the AR as well as those of the estrogen receptors largely remained unknown mainly due to the lack of the AR knockout (ARKO) mice line. We have succeeded in generating the ARKO mouse by means of a conditional targeting using the Cre/loxP system. The ARKO males grew healthily although they showed a typical feature of the testicular feminization mutation (Tfm) and the hormonal assay revealed significantly lower serum androgen and higher LH levels in comparison with those of the wild type (WT) males. The serum estrogen levels were, however, comparable between both the ARKO and the WT. Another hallmark of the ARKO males was a state of high bone turnover osteopenia, in which the acceleration in the bone resorption clearly exceeded the bone formation. Male-typical behaviors were disrupted in male ARKO mice. Aiming at a quick differentiation of an androgen-dependent polyQ disease such as Kennedy's disease, the authors also developed the Drosophila fly-eye model in which the wild type and the polyQ-expanded human AR (hAR) was induced in the eyes of Drosophila. When androgen was administered to the flies induced with the polyQ-expanded hAR, their optical nerves were devastated.

Keywords: Androgen; Androgen receptor; KO mice; Transgenic fly

1. Introduction

The androgen receptor (AR), a member of the steroid hormone receptors superfamily, is composed of a fairly large protein in comparison with thyroid hormone receptors (TR), Vitamin D receptors (VDR), retinoid receptors (RXR) as well as estrogen receptors [1–3]. It is because the A/B domains of the N-terminal of the AR that include a polyQ repeat are much longer than those of other receptors [4–6]. Androgen controls the expression of genes via the AR, in which the AR positively or negatively regulates the expression of the target genes acting as androgen-dependent transcription factors, under the existence of co-activators [5–7]. When the AR functions on the DNA of the genes, the complex of the co-activators interact as a trigger with the basal transcription factor and the AR for initiating the transcription.

Recent studies of two subtypes of estrogen receptors, ERα and ERβ, found that, especially in the knockout mouse, a clear phenotypes such as osteoporosis were not manifested perhaps because the plasma level of androgen had been extremely elevated [8]. This may be explained by the fact that androgen is the precursor of estrogen in the female mouse. It has been also reported that in the aromatase knockout female mouse, the circulating testosterone levels are markedly elevated [9]. Such being the case, there was a demand in developing the androgen receptor knockout (ARKO) mouse to investigate the actions of sexual steroid hormones individually. Androgen is required for the genital organs as well as sexual behavior not only in males but also in females. And in the clinical aspect, it is well known that some prostatic cancer can be androgen-dependently aggravated. A clarification of these issues was also expected with the development of the ARKO mouse.

2. Phenotypes of androgen knockout mouse

There were basic and technical difficulties in generating an ARKO mouse. When the AR gene is mutated in the male mouse, the mouse turns out phenotypic female without having a normal female or male genitalia and is infertile [10,11]. Moreover, as the AR gene is located only

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* Corresponding author. Tel.: +81-3-5841-8478; fax: +81-3-5841-8477.
E-mail address: uskato@mail.ecc.u-tokyo.ac.jp (S. Kato).

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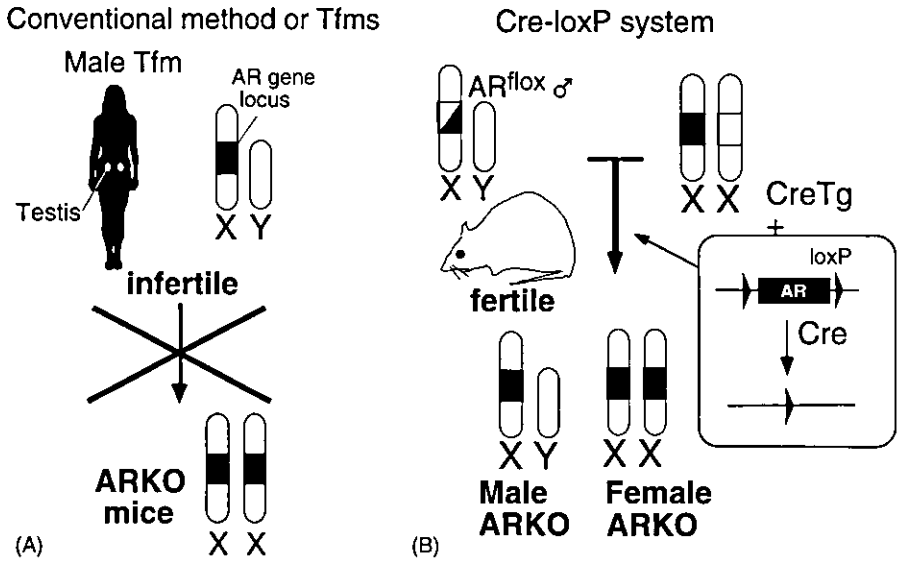

Fig. 1. Strategy for generating ARKO mice line when the male ARflox mouse with a partially modified AR gene induced by lox P and the female transgenic mouse ($CreTg^{+}$) generated by applying recombinase Cre were mated, all the AR genes were disrupted during the embryogenesis; thus, an ARKO mice line was obtained.

on the X chromosome, there is no male hetrozygoute of the AR gene-dirsrupted animals to transfer the mutated AR gene. It is thus impossible to obtain a female homozygote by either naturally occurring genetic mutaions or conventional targeted gene disruption method. Thus, the animal which has a recessive genotypic change in the AR gene can not be generated by means of the usual methods.

Such being the case, we planned to introduce the recombinase Cre/Lox-P base sequence (Cre-lox P system) into the mouse AR gene locus (Fig. 1) to generate ARKO mice line [12]. To begin with, we generated a potential AR knockout (ARKO) mouse (floxed ARf) by introducing the lox P, a capsid of a DNA breaking enzyme, in the AR gene by homologous recombination in ES cells. Three lox P sites were successively introduced in the first intron of the mouse AR gene. The male floxed AR mice are completely fertile/normal so far, and showed a normal expression and function of the AR, nevertheless, under the partially modified AR gene. On the other hand, a female transgenic mouse was generated by applying the recombinase Cre, which induces a recombination at the site between the two lox P

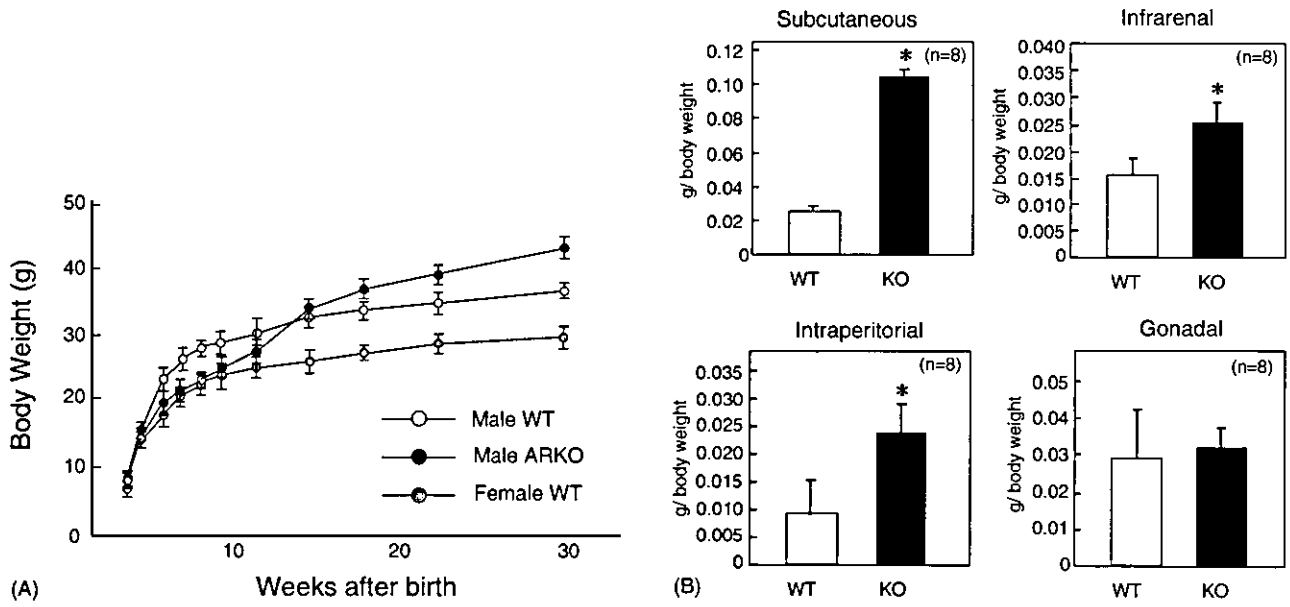

Fig. 2. Obesity in adult male ARKO mice: (A) growth curve of ARKO mice; (B) wet tissue weight of adipose tissue of male ARKO mice (Sato et. al [13]).

sequences in the same direction. Thus, in the Cre transgenic female mouse ($CreTg^+$), one of the two AR gene has been disrupted to generate the female $CreTg^+$ mice with heterozygous disruption of the AR gene. When the male floxed AR mice and these female $CreTg^+$ mice were mated, the AR gene was disrupted by expressed Cre under the CMV strong promoter during the embryogenesis.

The male ARKO which looks like a complete female had the small testes and cecum-like vagina but had no uterus and ovaries; and showed a similarity with the clinical Tfm. [13] The histological findings such as the hypertrophic Leydig cells suggested impaired spermatogenesis. The growth curves for 56 days after the birth of the female ARKO mouse (Fig. 2) were completely comparable with those of the WT female but those of the male ARKO were clearly retarded in comparison with those of the WT male and were rather similar to those of the females.

Estimation of plasma hormone levels in the male ARKO revealed markedly lowered androgens as well as a luteinizing hormone, but there was no difference in the estradiol level in comparison with that of the wild type (WT). These suggest that we can investigate the effect of androgens independently by using the ARKO mouse in that only the AR is disrupted while the estrogen receptors remain intact.

The bone densitometry showed a marked osteopenia, and the 3D-CT indicated that both of the trabecular bone and cortical bone volumes were remarkably reduced in the ARKO male mouse in comparison with that of the WT littermate male mouse at 6–16 weeks of age. Since the bone volumes result from bone remodeling which is the coupling of the formation/resorption of the bone, we compared bone formation and resorption on the proximal tibia in the ARKO and WT male by means of an histomorphometric analysis. Unexpectedly, the bone formation in the ARKO male exceeded that of the WT male by 15–20% (Fig. 3). On the

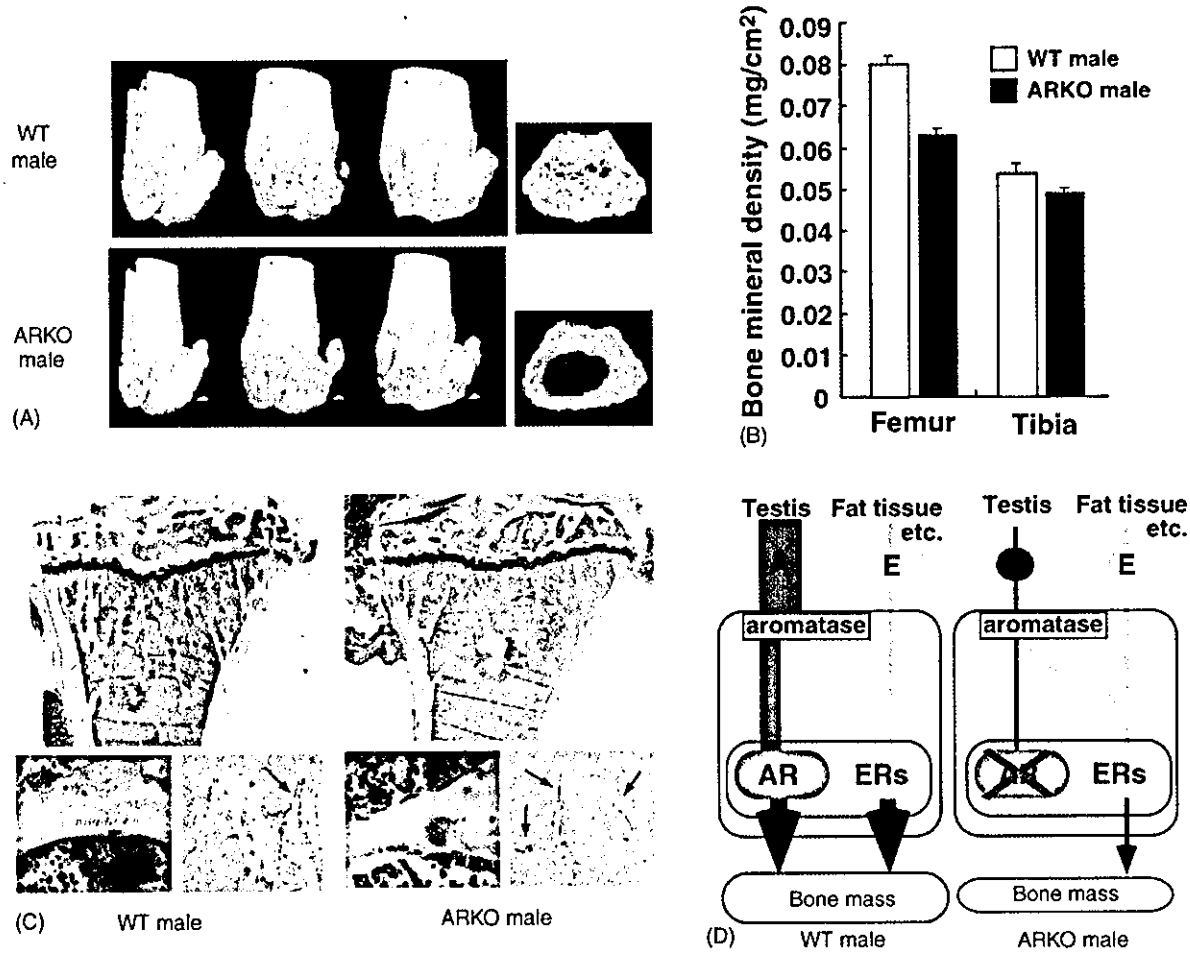

Fig. 3. Osteopenia in male ARKO mice: (A) three-dimensional CT images of distal femora and axial sections of distal metaphyses of male ARKO mice; (B) bone mineral density of male ARKO femur and tibia; (C) high turnover of male ARKO bone. Histological features and histomorphometry of the proximal tibiae from 8-week-old male ARKO and WT mice. For Villanueva-Goldner staining of sections from representative ARKO and WT male littermates, mineralized bone is stained green; (D) schema of skeletal sex hormone action. In male WT mice, skeletal sex hormone activities are mediated by both AR and ER. In female WT mice, skeletal function of ER is likely to dominate over that of AR as serum levels of AR ligands in females are quite low. In male ARKO mice, testicular testosterone production is severely impaired by hypoplasia of the testes, leading to a lack of skeletal sex hormone actions. In contrast, female ARKO mice may not be greatly affected by disruption of AR signaling.

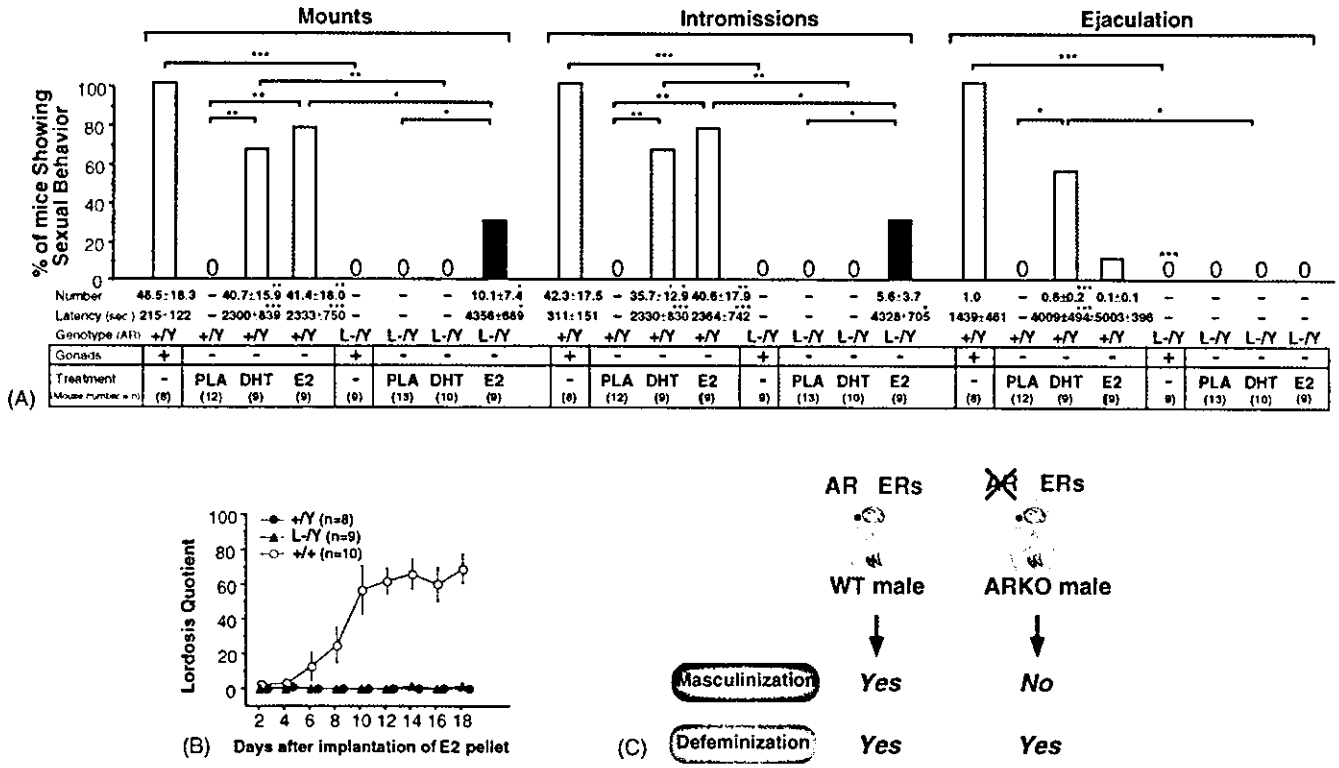

Fig. 4. Male-typical behaviors were impaired in male ARKO mice: (A) impaired male sexual behaviors in ARKO mice were partially recovered by estrogen, but not by androgen; (B) no female sexual behavior (lordosis) in male ARKO mice; (C) brain masculinization requires AR function.

other hand, the bone resorption in the ARKO male was more remarkable and exceeded that of the WT male by 40–50%. In view of these results, we concluded that the reduction in the bone found in the ARKO male was based on the high bone turnover osteopenia [14].

A characteristic change was seen in the body fat composition [13]. More than 10 weeks after the birth ARKO male became fat and the weight exceeded the normal growth curve; and the accumulation of white fat which almost doubled in comparison with the WT male was recognized under celiotomy (Fig. 2). Since there were no clear differences in serum lipids, especially in total cholesterol and free fatty acid, the AR might have suppressed the differentiation of the adipose cells. On the other hand, the sexual behavior of the ARKO mouse either as male or female was found not to be normal; nevertheless the normal gonadal differentiation was found in the ARKO female. Thus, it was considered that abnormal sexual behavior resulted in lowered number of offspring by about half of that of the WT female.

Male-typical behaviors were abolished in male ARKO mice, however, these mice showed no female sexual behavior. Estrogen treatment was effective to recover the impaired male sexual behaviors except ejaculation, suggesting that both of androgen and estrogen signalings mediated their nuclear receptors are essential for expression and maintenance of male sexual behaviors (T. Sato and T. Matsumoto, unpublished result) (Fig. 4).

3. Functional analyses of polyQ-expanded AR mutant in drosophila fly-eye model

An important disease group other than the testicular feminization mutation (Tfm) and androgen insensitivity syndrome (AIS) that is related to the mutation of the AR gene is the triplet repeat disease, or so-called polyQ expansion, in which the poly Q repetitions of the A/B domain of the N-terminal are expanded [4,5]. Spinobulbar muscular atrophy (SBMA) is one of the polyQ diseases and also named as Kennedy's disease. Other polyQ diseases such as Huntington's disease, spino-cerebellar ataxis (SCA1), and Machado-Joseph disease are seen both in males and females [15,16], while manifestation of SBMA can not be seen in the female, even if she is a carrier. Since the AF-1 functions of the A/B domain are androgen-dependent, the reason that the disease occurs only in the male was considered to be dependent on the concentration of androgen.

Aiming at proving this theory, we tried to use the Drosophila fly-eye model [17]. As the lifespan of the fly is short, we thought we could quickly obtain the assay results. The fly possesses nuclear receptors [18]. For example, it has the receptors for ecdysone, metamorphotic hormone, and its partner gene, the ultrabithorax gene. The latter is identical to the human retinoid receptor (RXR). Since the ecdysone receptor of the fly functions as a heterodimer, its DNA binding site is considered to be a direct repeat sequence; on the contrary, the DNA binding site of the human steroid

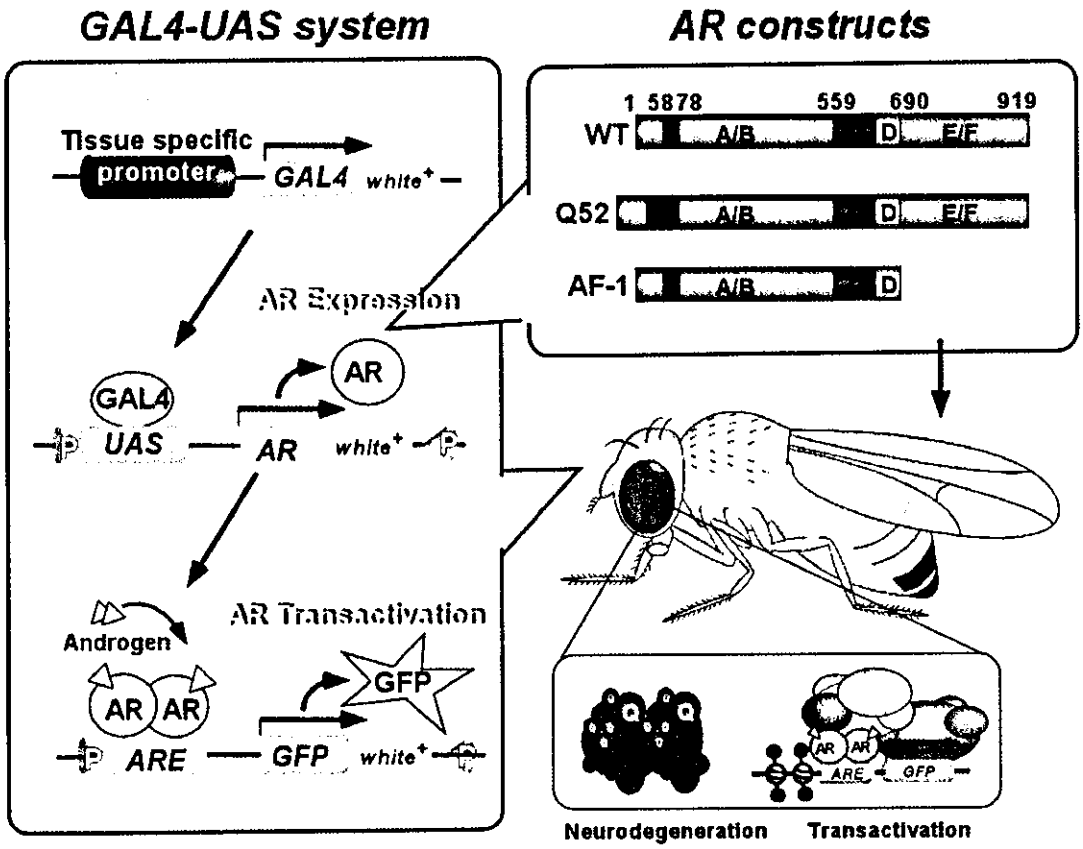

Fig. 5. Inducing hARs to the Drosophila eye, the human ARs, wild type, and polyQ-expanded, were induced in the Drosophila eyes using GAL4 UAS, then the reporter genes were bound to the GFP.

hormone receptor that functions as the homodimer is of a palindrome sequence. Such being the case, we expressed the human AR (hAR) in the fly-eye, tissue/stage specifically, using GAL4 UAS, a conditional gene expression system [19], under the expectation that this AR expression would not impair the functions of the intrinsic receptors in the fly. Then, the reporter gene, a DNA sequence, which can bind to the marker green fluorescent protein (GFP), was bound to the GFP (Fig. 5). In such a fly-eye model, the AR expression can be detected as red by staining it with the antibody; and the transcription function can be recognized as green fluorescent.

Naturally, the human AR has about 20 polyQ repetitions but when we induce too many repeat in the AR, the transcription ability is reduced and also the in vitro protein biosynthesis becomes suppressed. Consequently, we judged around 52 repetitions would be optimal for monitoring the transcription activity and the nerval death. When androgen is fed to the fly that had expressed a wild type AR (ARwt), a green fluorescent is shown in the eye without any abnormal changes. But when the polyQ repeat AR is expressed, the optical nerves (photo-receptor neurons) of the fly are devastated unless the androgen feeding is discontinued; which means the nervous system disorders are androgen-dependent. When cartinostatic agents for prostatic cancer such as hydroxy flutamide and bicalutamide are administered concomitantly, the nerve disorders of the fly were rather worsened. The results justify the development of a new-type anti-androgen for the treatment of prostatic cancer. As the AR is expressed in the nuclear and disrupts the optical nerves while keeping the transactivations, it was clarified that the disorder is based on an intranuclear event; and we recognized an androgen-dependent apoptosis was concurrently taking place.

Fig. 6 illustrates a speculation on the ligand-dependent structural alterations of the polyQ-expanded hAR [17]. The hAR that is inactive in the transactivitiy without ligand (androgen) gains transactivities under the existence of androgen by its structual alterations and also by recruiting co-activators [7,20]; while, the polyQ repeat induce apoptosis by their aggregating property. Since the plasma testosterone is much lower in the female patients (1/20–1/30), in comparison with those of the male patients, the polyQ aggregation may be difficult to occur. On the other hand, most androgen antagonists inhibit the transactivity of the AR by inhibiting recruitment of the co-activators; but they may not induce a structural alteration of the AR that deprive the aggregation by polyQ repeat. Adding finally, most of the polyQ diseases including Kennedy's disease are of late onset; and the disorders in the gonadal function and skeletal muscles appear after middle age. And on the other hand, the sensitivity of the fly-eye in expressing the polyQ repeat AR slightly changes depending on the stage.

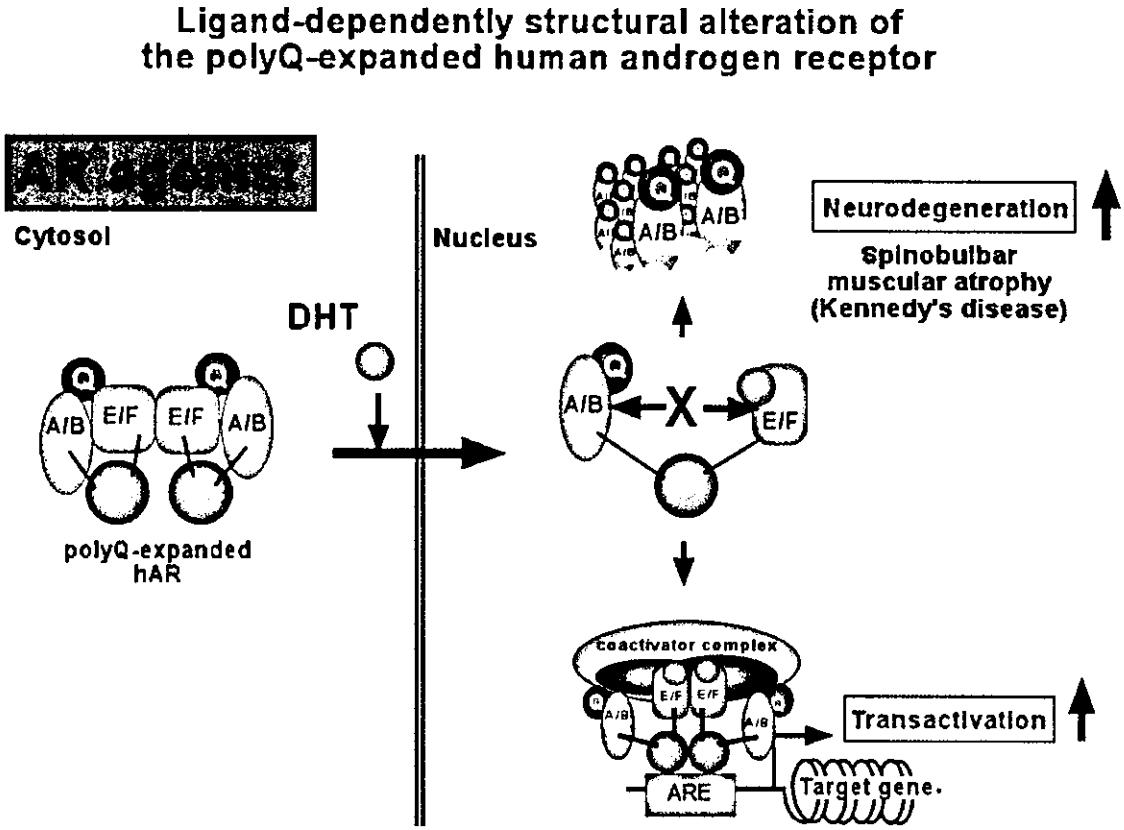

Fig. 6. Androgen-dependent structural alteration by the polyQ-expanded hAR. It is considered that the polyQ-expanded AR is inactive in the transactivation without the agonists (androgens); but under the existence of the agonists, it alters the molecular structure and also recruits the co-activators, while the polyQ repeat induces apoptosis by their aggregation.

In view of these results, we consider that for the management of Kennedy's disease, an anti-androgen treatment, such as an orchidectomy or the development of a new ligand that induces a structural alteration of the polyQ-expansion, may be required.

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In vivo potentiation of human oestrogen receptor α by Cdk7-mediated phosphorylation

Saya Ito[1], Ken-ichi Takeyama[1,2], Ayako Yamamoto[1], Shun Sawatsubashi[1], Yuko Shirode[1,2], Alexander Kouzmenko[1,2], Tetsuya Tabata[1] and Shigeaki Kato[1,2,*]

[1]*The Institute of Molecular and Cellular Biosciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan*
[2]*SORST, Japan Science and Technology, Kawaguchi, Saitama, Japan*

Phosphorylation of the Ser[118] residue in the N-terminal A/B domain of the human oestrogen receptor α (hERα) by mitogen-activated protein kinase (MAPK), stimulated via growth factor signalling pathways, is known to potentiate ERα ligand-induced transactivation function. Besides MAPK, cyclin dependent kinase 7 (Cdk7) in the TFIIH complex has also been found to potentiate hERα transactivation *in vitro* through Ser[118] phosphorylation. To investigate an impact of Cdk7 on hERα transactivation *in vivo*, we assessed activity of hERα in a wild-type and *cdk7* inactive mutant *Drosophila* that ectopically expressed hERα in the eye disc. Ectopic expression of the wild-type or mutant receptors, together with a green fluorescent protein (GFP) reporter gene, allowed us to demonstrate that hERα expressed in the fly tissues was transcriptionally functional and adequately responded to hERα ligands in the patterns similar to those observed in mammalian cells. Replacement of Ser[118] with alanine in hERα (S118A mutant) significantly reduced the ligand-induced hERα transactivation function. Importantly, while in *cdk7* inactive mutant *Drosophila* the wild-type hERα exhibited reduced response to the ligand; levels of transactivation by the hERα S118A mutant were not affected in these inactive *cdk7* mutant flies. Furthermore, phosphorylation of hERα at Ser[118] has been observed *in vitro* by both human and *Drosophila* Cdk7. Our findings demonstrate that Cdk7 is involved in regulation of the ligand-induced transactivation function of hERα *in vivo* via Ser[118] phosphorylation.

Introduction

It is thought that most of the wide variety of oestrogen action is mediated through the transcriptional control of target genes by nuclear oestrogen receptor (ER) (Couse & Korach 1999; Ciana *et al.* 2003). The two subtypes of ER, α and β, belong to the nuclear receptor superfamily and act as ligand-induced transcription factors. As in other nuclear receptor superfamily members, structure of ER proteins is divided into five or six functional domains (designated as A to E/F domains). The highly conserved DNA binding domain is located in the C domain, while the ligand-binding domain (LBD) is mapped to the E/F domain. Transactivation function is present in the N-terminal A/B domain (AF-1) and in the C-terminal LBD (AF-2) (Kumar *et al.* 1987; Tora *et al.* 1989). Although both AF-1 and AF-2 are involved in the ligand-dependent transactivation function of ERs, AF-1 is constitutively active, while AF-2 activity is dependent on ligand binding (Endoh *et al.* 1999; Kobayashi *et al.* 2000; Watanabe *et al.* 2001). AF-1 and AF-2 domains have distinctive properties and their activities may depend on cell type and promoter context (Kumar *et al.* 1987; Tora *et al.* 1989).

ER target gene promoters contain oestrogen-response elements (EREs) that are recognized and directly bound by ER homo- or hetero-dimers followed by chromatin remodelling, presumably by recruited ATP-dependent chromatin remodelling complexes (Belandia & Parker 2003; Kitagawa *et al.* 2003). ERE-bound liganded ERs also induce recruitment of a number of histone acetyltransferase (HAT) and non-HAT cofactors that activate transcription (McKenna & O'Malley 2002). HAT coactivator complexes, CBP/p160 (Onate *et al.* 1995; Kamei *et al.* 1996; Chen *et al.* 1997; Spencer *et al.* 1997) and TRRAP/GCN5 (Yanagisawa *et al.* 2002), and non-HAT DRIP/TRAP complexes (Fondell *et al.* 1996;

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**Correspondence*: Email: uskato@mail.ecc.u-tokyo.ac.jp

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Yuan *et al.* 1998; Naar *et al.* 1999; Rachez *et al.* 1999) are thought to act as common coactivator complexes for ERs as well as for other DNA-binding transcription factors. Therefore, ligand binding leads to structural alteration and switch of ER function from transcriptional repression to transcriptional activation via the recruitment of coactivators (Shiau *et al.* 1998; Freedman 1999; Glass & Rosenfeld 2000; Metivier *et al.* 2003).

It is well known that phosphorylation of ERα modulates the activity of both AF-1 and AF-2 (Ali *et al.* 1993; Le *et al.* 1994; Kato *et al.* 1995; Chen *et al.* 2000). Among sites of potential phosphorylation, Ser^{118} residue (S118) in the hERα AF-1 domain has been particularly intensively studied with regard to the state of its phosphorylation and consequent potentiation of AF-1 activity. We have previously demonstrated that Ser^{118} is phosphorylated by ERK, a MAPK activated by the epidermal growth factor (EGF) or insulin-like growth factor (IGF) signalling, that results in the AF-1 potentiation in cultured cells (Kato *et al.* 1995). More recently, Chen and colleagues have shown that Cdk7 also phosphorylates hERα Ser^{118} in an oestrogen-dependent manner and enhances ERα transactivation in mammalian cells in culture (Chen *et al.* 2000). As Cdk7 is a key subunit of the basal transcription factor TFIIH complex (Frit *et al.* 1999; Egly 2001), it has been suggested that this phosphorylation takes place when TFIIH is recruited adjacent to hERα, presumably in the transcription initiation complex. Therefore, accumulating evidence suggests that phosphorylation of hERα Ser^{118} may play a significant role in regulation of AF-1 activity. However, the physiological role of Ser^{118} phosphorylation and associated kinases in hERα function remain to be established *in vivo*.

In *Drosophila melanogaster*, at least 20 members of the nuclear receptor (NR) family, such as the ecdysone receptor (EcR), have been genetically identified that, similar to the vertebrate NRs, are thought to transcriptionally control expression of target genes (Talbot *et al.* 1993; Baker *et al.* 2003). Recently, we reported that human androgen receptor ectopically expressed in *Drosophila* tissues was transcriptionally active and responsive to AR agonists and antagonists (Takeyama *et al.* 2002). In the present study, to assess an impact of Ser^{118} phosphorylation by Cdk7 and related kinases on hERα activity *in vivo*, we generated transgenic *Drosophila* lines in which hERα was ectopically expressed in specific *Drosophila* tissues using a GAL4/UAS system (Brand & Perrimon 1993). hERα expressed in the fly was transcriptionally functional and responded adequately to ER ligands, as expected from mammalian studies. Apparently, for its transactivation function in these transgenic flies, hERα recruited endogenous co-activators, such as those shown to be homologous to mammalian CBP and AIB1 (Akimaru *et al.* 1997; Bai *et al.* 2000). We found that replacement of S118 with alanine residue (S118A) in hERα resulted in the marked reduction of ligand-induced hERα transactivation in transgenic fly eye disc. Furthermore, in a *cdk7* inactive mutant *Drosophila* (*cdk7*^{ts}) (Larochelle *et al.* 2001), transactivation by the wild-type but not the S118A hERα was significantly reduced. In addition, both human and *Drosophila* recombinant Cdk7 were equally able to phosphorylate hERα at Ser^{118} *in vitro*. We have also shown that Cdk7 acts as a co-activator of hERα transactivation in transfected cells in culture. Therefore, our results provide for the first time genetic evidence that phosphorylation of Ser^{118} potentiates transcriptional activity of hERα and that Cdk7 is involved in regulation of the ligand-induced transactivation function of hERα *in vivo* through Ser^{118} phosphorylation.

Results

hERα in *Drosophila* is transcriptionally functional

Our previous studies showed that human androgen receptor ectopically expressed in *Drosophila* tissues was adequately functional (Takeyama *et al.* 2002). We have utilized the same strategy to generate transgenic *Drosophila* expressing hERα together with ERE-dependent green fluorescent protein (GFP) as a reporter gene. Wild-type hERα (HEG0), AF-1 (HE15) or AF-2 (HE19) domains (as illustrated in Fig. 1A) were ectopically expressed in photoreceptor cells under control of the glass multimer reporter (*GMR*) gene promoter (Moses & Rubin 1991) using the *Drosophila melanogaster GAL4-UAS* system (Brand & Perrimon 1993). The eye disc, one of several larval discs in *Drosophila*, has been shown to be an effective model to assess Cdk7 function as a cell survival signal. Expression of hERα proteins was estimated by staining with immunofluorescent antibody. Levels of GFP reporter expression in respective eye discs were quantified by green fluorescence and normalized against the levels of ERα protein to determine fold of activation.

Dietary administration of 17β-oestradiol (E2) for 5 days from hatching remarkably induced GFP expression (Fig. 1B). The partial oestrogen agonist tamoxifen (TAM) and pure antagonist ICI182.780 exhibited partial oestrogenic and anti-oestrogenic actions, respectively, similar to that observed in mammals (McDonnell *et al.* 1995). E2-dependent (AF-2) and -independent (AF-1) transactivation functions were observed in the C-terminal-LBD and N-terminal A/B domain expressing transgenic flies, respectively, as expected from previous studies (Kumar *et al.* 1987; Tora *et al.* 1989; Kobayashi *et al.* 2000; Watanabe

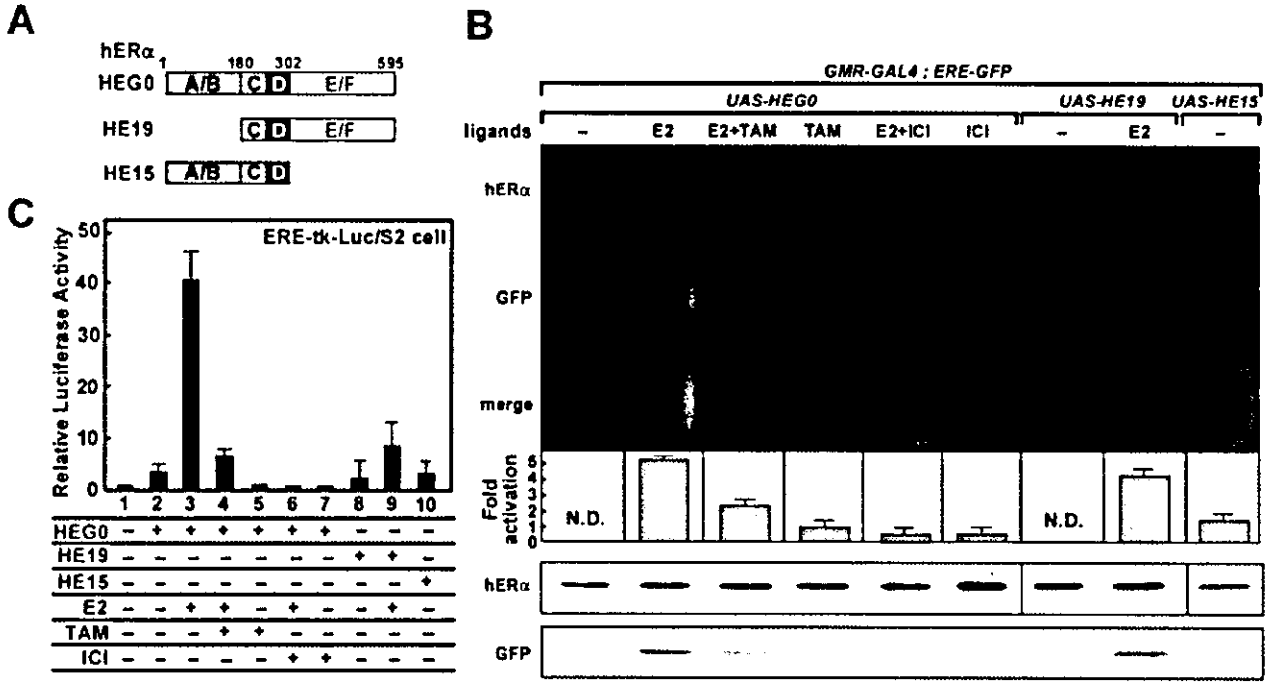

Figure 1 Ligand dependent transactivation of hERα in *Drosophila*. (A) Schematic representation of hERα constructs. The DNA binding domain (DBD) is located in the C domain (grey box). The transactivation function-1 (AF-1) region is located in the N-terminal A/B domain (blue box), while the transactivation function-2 (AF-2) region is located in the C-terminal E/F domain (white box) that also contains the ligand binding domain (LBD). (B) Ligand-dependent transactivation of hERα mutants in eye imaginal discs. Expression of hERα mutants in third instar larva eye discs driven by *GMR-GAL4* was detected with ERα antibodies (B10 or HC-20) (red). Transactivation of hERα mutants was estimated by GFP expression (green). The anterior is to the right. Bottom panels: hERα and GFP expression in four pairs of adult heads as detected by Western blotting. Fold-activation was calculated using hERα expression levels as normalizing factor. Ligands, 10^{-3} M 17β-oestradiol (E2), 10^{-2} M tamoxifen (TAM), and 10^{-2} M ICI 182.780 (ICI), were added in 100 μL of vehicle on top of 10 mL of the medium before hatching. Flies were kept at 25 °C. (C) Measurement of hERα mutants transactivation in Schneider cells. Schneider cells were transfected with hERα mutant expression plasmids, Actin-GAL4 plasmid, ERE-tk-luc reporter plasmid and pRL-CMV internal control plasmid in the presence or absence of 10^{-8} M E2, 10^{-8} M TAM or 10^{-8} M ICI. Firefly luciferase activity (ERE-tk-luc) was measured and normalized against Renilla activity (pRL-CMV-luc) as an internal control. Data are shown as the average and standard deviation of three independent experiments.

et al. 2001). Similar hER ligand effects and hERα AF-1 and AF-2 activities were observed in Schneider (S2) cells derived from *Drosophila* embryos (Fig. 1C). These data indicated that hERα ectopically expressed in *Drosophila* tissues was adequately functional in ligand-induced transactivation, presumably through recruitment of endogenous co-regulators. Therefore, it appears that human steroid receptors ectopically expressed in *Drosophila* retain their transactivation function.

Co-activation of hERα by *Drosophila* CBP and p160 HAT homologues

As hERα was transcriptionally functional in insect cells in culture and in *Drosophila* eye disc cells *in vivo*, ability of endogenous fly co-activators to modulate hERα transactivation was assessed in mutant flies deficient for *Drosophila* homologues of mammalian p160 (*tai*) or CBP (*nej*) (Akimaru *et al.* 1997; Bai *et al.* 2000). The oestrogen-induced transactivation function of hERα was clearly reduced in both of these mutants without affecting levels of hERα expression (Fig. 2). These data suggest that *Drosophila* homologue of the mammalian p160/CBP HAT complex acts as a co-activator of hERα in the fly cells. This was further confirmed by the observation of enhanced hERα transactivation in flies over-expressing TAI, *Drosophila* AIB1 homologue, in the eye disc.

The p160/CBP HAT complex has been shown to activate hERα AF-2 via the direct association of p160 family member proteins with helix 12 of the hERα LBD (Onate *et al.* 1995; Chen *et al.* 1997; Heery *et al.* 1997). However, little is known about the role of the

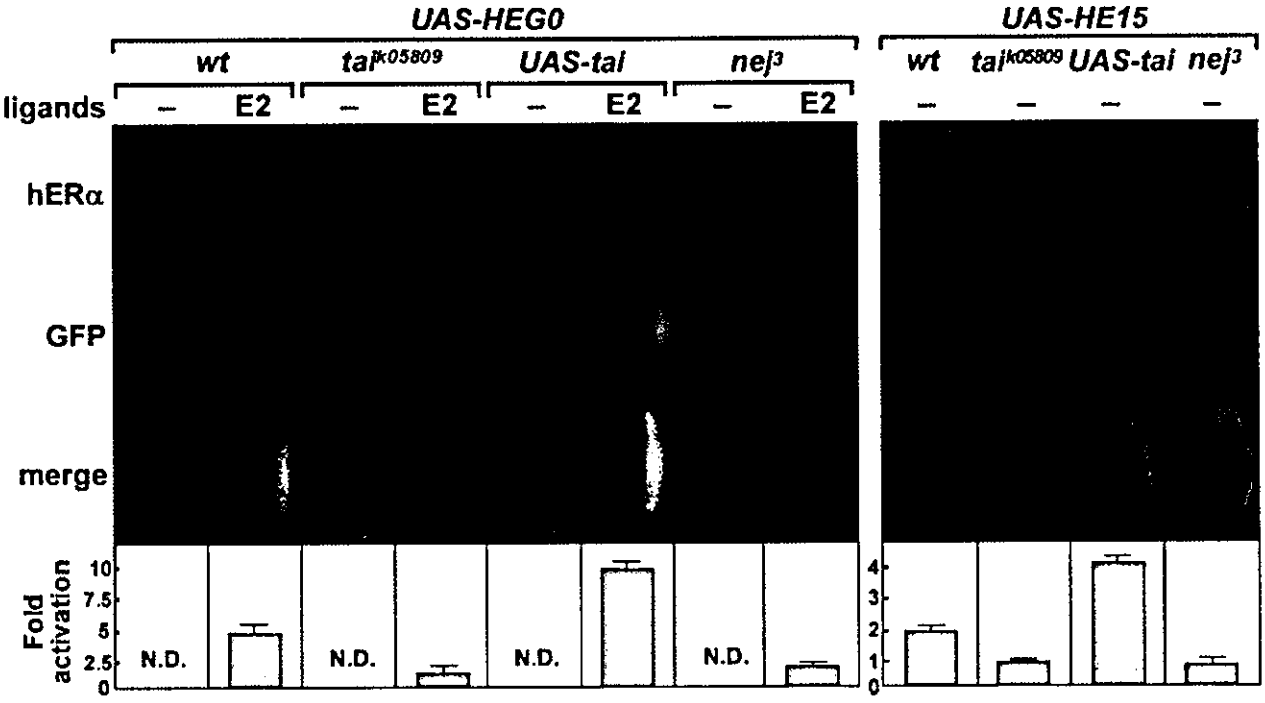

Figure 2 hERα transactivation regulated by *Drosophila* transcriptional co-activators. hERα expression (red) and transactivation (green) were visualized by immunostaining with ERα antibodies (B10 and HC-20) and GFP expression, respectively, in eye imaginal discs. Fly lines contained single copies of *GMR-GAL4*, *UAS-hERα* (*HEG0* or *HE15*) and *ERE-GFP* with or without heterozygous tai^{k05809}, *UAS-tai* or nej^{3}.

p160/CBP complex in modulation of hERα AF-1 activity. Although it is presumed that the complex bridges the AF-1 and AF-2 domains to synergistically enhance hERα transactivation function (Kobayashi *et al.* 2000), the p160/CBP complex was also able to enhance transcriptional activity of the AF-1 domain alone (i.e. the HE15 mutant). Indeed, similar patterns of AF-1 domain (HE15) and full-length hERα (HEG0) transactivation in mutant flies (Fig. 2) suggest that hERα AF-1 activity is modulated *in vivo* by the p160/CBP co-activator complex.

Significant role of Ser^{118} in hERα function *in vivo*

In mammalian cells, the potentiation of hERα AF-1 by phosphorylation of the Ser^{118} residue has been well documented (Kato *et al.* 1995; Chen *et al.* 2000). However, the impact of Ser^{118} phosphorylation in hERα transactivation function has not yet been verified *in vivo*. We tested the significance of hERα Ser^{118} in the insect S2 cells transfected with hERα mutants containing a serine to alanine replacement at position 118 (HE457, HE15/457) (Fig. 3A and 3B). These mutants exhibited decreased transactivation capacities even though levels of the mutant expression appeared to be similar to that of wild-type hERα.

We then examined the role of Ser^{118} in hERα function in transgenic flies (Fig. 3C). Although mutant and wild-type hERα expression levels in third instar larval eye discs were indistinguishable, a clear reduction in GFP induction was observed in the alanine replacement mutants. These findings provided evidence that the Ser^{118} residue played a pivotal role in hERα transactivation *in vivo*.

In vivo potentiation of hERα by Cdk7-mediated phosphorylation at Ser^{118}

As it is likely that the Ser^{118} residue could be phosphorylated by a number of endogenous protein kinases to support hERα transactivation, we studied the ability of dCdk7 to phosphorylate hERα at Ser^{118} *in vitro* and *in vivo*. The serine/threonine kinase Cdk7 is indispensable for transcription initiation by RNA polymerase II as an essential component of the transcription factor TFIIH complex (Frit *et al.* 1999; Egly 2001). $dcdk7^{ts}$ mutant flies express a temperature-sensitive Cdk7 mutant that is inactive at temperatures at or above 30 °C (Larochelle *et al.* 2001). We assessed transactivation function of HEG0 and HE457 in these $dcdk7^{ts}$ mutant flies (Fig. 4, left panel). Oestrogen-induced transactivation of

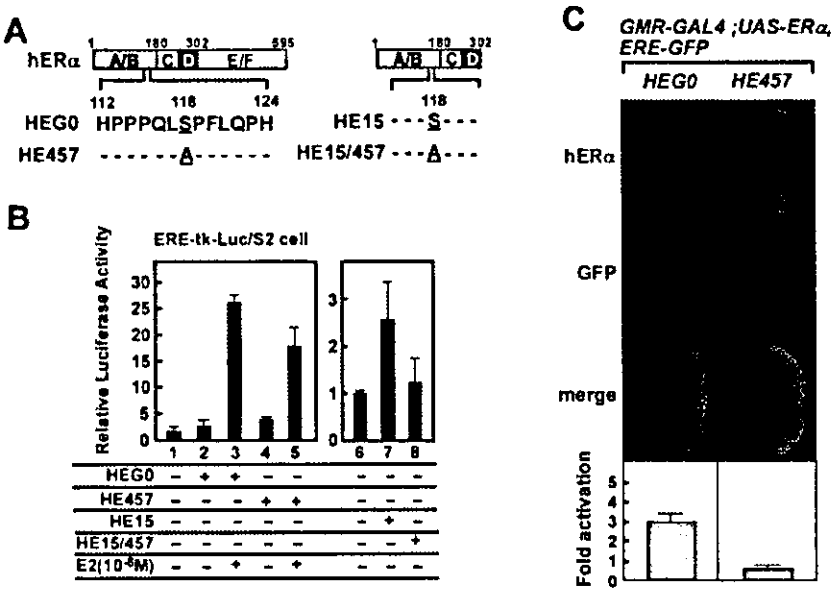

Figure 3 hERα transactivation is regulated by phosphorylation at Ser^{118} in *Drosophila*. (A) Schematic representation of hERα mutant constructs. Ser^{118} residue is the main phosphorylation site. (B) Transactivation of HEG0 and HE15 mutants in Schneider cells. Schneider cells were transfected with ERE-tk-luc reporter plasmid, Actin-GAL4 plasmid and each hERα mutants, and then incubated with or without 10^{-8} M E2. Luciferase activity data are shown as the average and standard deviation of three independent experiments. (C) Expression (red) and transactivation (green) of hERα mutants in eye imaginal discs. Fold-activation is represented as described (Fig. 1 legend). Genotypes are *GMR-GAL4/SM, UAS-hERα, ERE-GFP/TM3*.

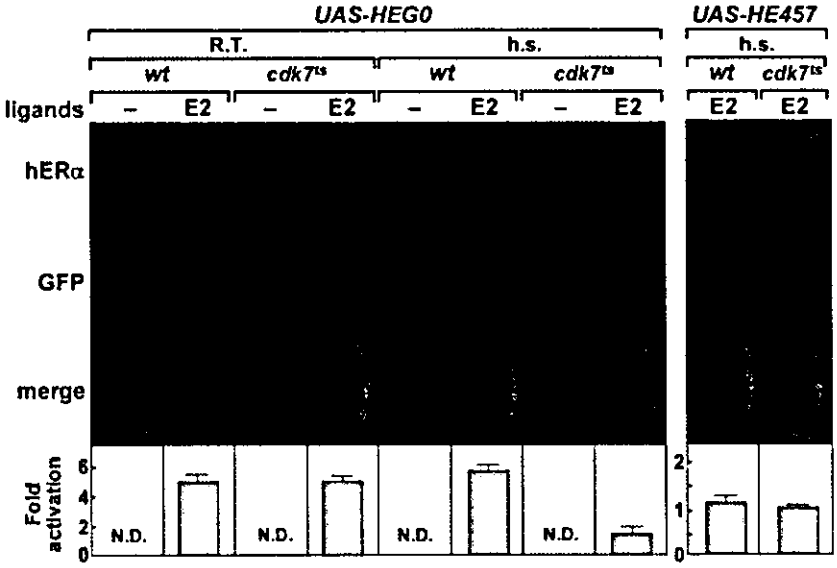

Figure 4 hERα transactivation is enhanced by *Drosophila* Cdk7 through phosphorylation of Ser^{118}. hERα expression (red) and transactivation (green) in eye imaginal discs containing single copies of *GMR-GAL4, ERE-GFP* and *UAS-hERα (HEG0, HE457)* with or without heterozygous *cdk7*^{s}. *cdk7*^{ts}, the temperature-sensitive *cdk7*^{P140S} gene, was introduced into the *Df(1)JB254*-Pw^{+}[*snf*$^{+}$, *dhd*$^{+}$] (*cdk7*deficient) background. Flies were then incubated at 25 °C (room temperature) or 30 °C (h.s.) for 24 h in medium containing E2. GFP expression levels are represented as described.

HEG0 in *dcdk7*^{ts} flies was significantly reduced at 30 °C in comparison with that at room temperature (25 °C). In contrast, HE457 transactivation in *dcdk7*^{ts} flies was not affected by exposure to high temperatures (Fig. 4, right panel). These results indicate that Cdk7 potentiated hERα transactivation *in vivo* through Ser^{118} phosphorylation.

To further confirm this conclusion, we examined whether hERα Ser^{118} is a substrate for dCdk7 *in vitro*. A recombinant GST-fused hERα segment (amino acids 56–180) chimera protein expressed in *E. coli*, and dCdk7 and hCdk7 expressed in 293T cells were used for the *in vitro* phosphorylation assay (Fig. 5A). GST-fused human retinoic acid receptor α1 (hRARα1), a well-characterized substrate for the mammalian Cdk7 (Rochette-Egly *et al.* 1997) was used as a positive control. dCdk7 and hCdk7 were equally capable of phosphorylating hERα and hRARα1. However, the Cdk7 phosphorylation was clearly reduced when the S118A mutant (HE457) was used as a substrate (Fig. 5B).

Activation of the hERα S118A mutant by *Drosophila* AIB1 homologue

Finally, using a fly line with ectopical over-expression of *Drosophila* AIB1 homologue (TAI) in the eye disc, we