Human endogenous retrovirus protein Rec interacts with the testicular zinc-finger protein and androgen receptor

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More than 2000 human endogenous retrovirus (HERV) sequences are present in the human genome, yet only a few are intact and able to produce proteins. The normal functions of these, if any, are unknown, but some HERV proteins have been implicated in cancers, in particular germ-cell cancers. For instance, it has been documented that (i) patients with germ-cell tumours frequently produce antibodies against HERV proteins; (ii) transgenic mice expressing HERV-K (HML-2) rec are prone to testicular carcinoma in situ; and (iii) Rec can bind and suppress a guardian of germline stem-cell pluripotency, the promyelocytic leukaemia zinc-finger protein (PLZF). This study identified the PLZF-related testicular zinc-finger protein (TZFP) as a binding partner of HERV-K (HML-2) Rec. Interactions occurred via the N- and C-terminal domains of Rec and the C-terminal DNA-binding zinc-finger domain of TZFP (aa 375–450). Not much is known about the function of TZFP. The protein is expressed predominantly in the testis, where it functions as a transcriptional repressor that is active during specific stages of spermatogenesis. The most intensely studied function of TZFP is that of a co-repressor of the activated androgen receptor (AR). Here, it was shown that Rec can form a trimeric complex with TZFP and AR, and can relieve the TZFP-mediated repression of AR-induced transactivation. In addition, Rec was able to overcome the direct transcriptional repression by TZFP of the c-myc gene promoter in reporter assays. Thus, HERV-K (HML-2) Rec may function as an oncprotein by de-repressing oncogenic transcription factors such as AR.

INTRODUCTION

Approximately 2000 proviral DNA sequences (copies of retroviral RNA genomes) and more than 30 000 sequences related to regulatory long-terminal repeats (LTRs) of retroviruses are dispersed throughout the human genome and account for 8% of a cell’s DNA (Lander et al., 2001). These human endogenous retroviruses (HERVs), subdivided into about 30 families, originate from ancient exogenous predecessors that entered the germline 40 million years ago and have since been propagated through mechanisms of infection, reinfection, amplification and retrotransposition (Barbulescu et al., 1999). The vast majority of these sequences have accumulated inactivating mutations; however, conserved and thus potentially functional open reading frames (ORFs) for the viral proteins Gag (group-specific antigens), Prt (protease), Pol (reverse transcriptase) and Env (envelope) are found in up to 50 proviral copies per genome (Mayer et al., 1999). Among the HERV sequences with known important functions for the host is the HERV-W env gene on chromosome 7q21.2. This gene encodes syncytin-1, a surface protein expressed in the placenta that is crucial for the efficient fusion of trophoblast cells to syncytiotrophoblasts (Blond et al., 2000; Mallet et al., 2004). Apart from their roles in normal physiology, HERV proteins have also been implicated in disease, in particular in the development and progression of cancers (reviewed by Ruprecht et al., 2008). Not only are specific RNA transcripts from the various HERV families detected in many primary tumours and tumour cell lines, but patients with germ-cell...
tumours often harbour serum antibodies directed against proteins encoded by the HERV-K (HML-2) family, and the expression of HERV-K (HML-2) Rec (Fig. 1a), a 14.5 kDa relative of the regulatory Rev/Rex proteins of human immunodeficiency virus (HIV) and human T-lymphotropic virus (HTLV) that is produced by the env gene through alternative splicing, can provoke testicular carcinoma in situ in transgenic mice (Galli et al., 2005; Sauter et al., 1995, 1996). Moreover, we have documented previously that HERV-K (HML-2) Np9 protein, expressed instead of Rec as the result of a 292 bp deletion in an HERV-K (HML-2) subtype env reading frame, can affect the cancer-relevant Notch signalling pathway through interaction with the ligand of Numb protein X (LNX; Armbruester et al., 2004). Finally, Rec and Np9 are able to stimulate c-Myc oncoprotein expression by binding and inhibition of the c-myc gene repressor promyelocytic leukaemia zinc-finger protein (PLZF; Denne et al., 2007).

Testicular zinc-finger protein (TZFP) is a relative of PLZF that contains an N-terminal POZ (poxvirus zinc finger) domain plus three C3H2 zinc fingers in its C terminus that bind to the DNA motif TGTACAGTGT, with the C at position 5 and G at position 7 being critical (Fig. 1b; Lin et al., 1999; Tang et al., 2001). TZFP is expressed predominantly in the testis where it is active during specific stages of spermatogenesis and is localized in discrete dots in the nucleoplasm (Inoue et al., 2000). The protein can recruit histone deacetylase 2 (HDAC2) to promoters and, like PLZF, seems to function primarily as a transcriptional repressor of genes (Tao et al., 2006). However, the target genes and the biological function of TZFP are largely elusive. The so far best characterized function of TZFP is that of a direct interaction partner and co-repressor of the androgen receptor (AR) (Ishizuka et al., 2005). The central portion of TZFP can associate with agonist-bound (i.e. active as a transcription factor) AR and repress AR-mediated transactivation through the recruitment of HDAC2 and, possibly, dissociation of co-activator TIF2 (Tao et al., 2006).

AR is a member of the steroid receptor superfamily, composed of an N-terminal transactivation domain, a DNA-binding domain, a hinge region and a C-terminal ligand-binding domain (Fig. 1c; Cheng et al., 2002; Mangelsdorf et al., 1995). In the absence of ligand (androgens), AR is bound and sequestered in the cytoplasm by heat-shock proteins, whereas, upon ligand binding, AR translocates into the nucleus where it associates with DNA of the consensus motif GG(A/T)ACAnnnTGTTCT and – with dependence on the recruitment of co-repressors or co-activators – engages in the repression or activation of genes. Importantly, apart from its many functions in normal physiological processes, AR can act as an oncogenic protein, for instance by supporting cell cycle progression (Balk & Knudsen, 2008) and, consequently,

![Fig. 1. Schematic diagrams of the three interacting proteins. (a) HERV-K (HML-2) subtype 2 sequences can give rise to a 315 bp rec transcript by alternative splicing of the env ORF. The Rec protein of 105 aa appears as a 14.5 kDa signal in Western blots upon transfection of cells with control plasmid (C) or Rec expression plasmid (Rec), detected with our polyclonal anti-Rec antibody K3086 (diluted 1:100). Rec contains a nuclear-localization signal (NLS), a nuclear-export signal (NES) and a dimerization domain. The RNA-binding domain overlaps the NLS. gag, Group-specific antigen gene; prt, protease gene; pol, reverse transcriptase gene; env, envelope gene. (b) TZFP consists of 487 aa (~55 kDa), an N-terminal POZ domain and a C-terminal zinc-finger DNA-binding domain. (c) Human AR is a transcription factor of 919 aa (~94 kDa) with three major protein domains.](http://vir.sgmjournals.org)
AR-associating inhibitors such as TZFP might be viewed as tumour suppressors, whilst antagonists of this interaction again might act oncogenically. Here, we report that TZFP primarily active in spermatogenesis is bound and regulated by Rec, a human endogenous retrovirus protein implicated in germ-cell cancers.

**RESULTS**

In an attempt to identify further Rec interaction partners, yeast two-hybrid screens with full-length Rec fused with the DNA-binding domain of the LexA transactivating complex (pEG202-Rec) as the bait were carried out. An initial search identified TZFP as an interacting protein. *In vitro* glutathione S-transferase (GST) pull-down assays showed that full-length TZFP bound to GST–Rec but not to GST alone, and, moreover, that the interaction required the C-terminal zinc fingers (aa 375–450) but not the N-terminal POZ domain of TZFP (Fig. 2a). In turn and in accord with results for PLZF (Boese et al., 2000; Denne et al., 2007), TZFP associated with domains in both the N and C terminus of Rec (Fig. 2b). Screening of a human testis cDNA library identified two splice variants of TZFP, one giving rise to a truncated protein consisting of the N terminus up to aa 196 (variant α) and a second lacking a portion of exon 1 but containing the C terminus with the zinc-finger domain (variant β; Fig. 2c). In agreement with the results of the interaction mappings, variant β but not variant α was able to associate with GST–Rec in pull-down assays (Fig. 2c). The *in vitro* interaction between TZFP and Rec was confirmed *in vivo* by co-immunoprecipitation (Fig. 2d). Thus, human endogenous retrovirus protein Rec binds to the C-terminal zinc-finger domain of TZFP.

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**Fig. 2.** Mapping of the interaction of Rec and TZFP. (a) *In vitro*-translated 35 S-labelled TZFP is retained by GST–Rec but not GST alone (top). Among the TZFP variants of full-length TZFP, TZFP ΔZn (aa 1–369), TZFP ΔPOZ (aa 112–487) and TZFP ΔPOZ/Zn (variants a–d, respectively), only the constructs containing the C-terminal zinc-finger domain bound to GST–Rec (right panels). (b) *In vitro*-translated 35 S-labelled TZFP associates with N- and C-terminally truncated Rec proteins: Rec exon 1 (10 kDa), Rec aa 47–105 (6 kDa), Rec–Pes (8 kDa) and Rec aa 47–89 (5 kDa) (proteins a–d, respectively). (c) Screening of a testis cDNA library by RT-PCR for TZFP splice variants (upper gel). The lower panel shows labelling of the TZFP variant α (aa 1–196) and TZFP variant β (aa 1–190 and 294–383, lacking 104 aa in the centre) splice variants, and employment in GST pull-down assays. (d) Co-immunoprecipitation of Rec and HA–TZFP. 293T cells were transfected with equal quantities of Rec and HA–TZFP expression plasmids for 48 h. Cell lysates and immunoprecipitation (IP) were performed as described in Methods. Precipitates were loaded onto SDS–polyacrylamide gels. Immunoblotting and detection of the proteins was performed as described by Boese et al. (2000).
One of the few known interaction partners of TZFP in the mouse is AR (Ishizuka et al., 2005). To test whether human TZFP could also associate with human AR (Fig. 1c), GST pull-down assays were again employed, this time with full-length AR as the input and GST–TZFP as the fusion protein. Specific binding could be demonstrated (Fig. 3a). Again, the in vitro binding could be confirmed in vivo by co-immunoprecipitation (Fig. 3b). Finally, we asked whether Rec, TZFP and AR could be found in a trimeric complex in vivo. Co-immunoprecipitation showed that this was indeed the case (Fig. 3c). Combined, all binding assays thus indicated that human endogenous retrovirus protein Rec, TZFP and human AR can associate in vitro and in vivo. Furthermore, immunofluorescence studies revealed that all proteins localized to the nucleus, as expected (not shown).

TZFP represses androgen-activated AR-mediated transactivation through the recruitment of HDAC2 (Tao et al., 2006). To begin to explore the potential role of Rec as a regulator of TZFP/AR-controlled genes, we resorted to transient co-expression studies in the presence of appropriate reporter plasmids. Whilst Rec alone had no significant effect on the expression of luciferase from a plasmid driven by a wild-type c-myc gene promoter fragment carrying the PLZF (TZFP) recognition motif, expression of TZFP was able to repress luciferase production significantly (Fig. 4a; \( P = 0.0005 \)). Notably, simultaneous expression of Rec was able to relieve the repression by TZFP (\( P = 0.002 \)). These effects were dependent upon the TZFP (PLZF)-binding site in the c-myc gene promoter as luciferase expression from a plasmid construct with a mutated TZFP (PLZF) recognition motif was not affected (Fig. 4a). In a further, similar assay, a reporter consisting of the AR-responsive mouse mammary tumour virus (MMTV) LTR promoter upstream of a luc reporter gene (McConnell et al., 2003) (lower graph). (b) MMTVluc carries an AR-responsive promoter upstream of a luc reporter gene. Results are shown as means ± SD from at least three experiments, and \( P \) values show significance levels.
gene was employed. Expression and ligand activation of AR resulted in a 2.5-fold stimulation of luc expression, and co-expression of TZFP eliminated this transactivation (Fig. 4b; \( P=0.00001 \) and \( P=0.0009 \)), indicating that both mouse TZFP (Ishizuka et al., 2005) and human TZFP can regulate AR-mediated transactivation. Inclusion of Rec into this assay again revealed its activity as a de-repressor of TZFP-mediated repression of transactivation (\( P=0.003 \)). By contrast, Rec alone had no significant effect on the basal and transactivator-induced luc expression. Similar results were obtained with a reporter consisting of the human AR-responsive prostate-specific antigen gene promoter driving luc (PSAluc) (not shown).

Next, we asked whether the splice variants of TZFP could affect AR-mediated transactivation in this assay and whether Rec could interfere with this function. As shown in Fig. 2(c), TZFP and variant \( \beta \) but not variant \( \alpha \) associate with Rec, and we found that only TZFP and TZFP \( \beta \) were also able to bind to AR (Fig. 5a). Thus, interaction of TZFP with Rec and AR is dependent on the presence of the C-terminal zinc-finger domain. In complete accord with these binding profiles, only TZFP and variant \( \beta \) repressed the AR-responsive PSA promoter, and Rec exerted an appreciable effect of de-repression only on these protein combinations (Fig. 5b, c), whilst TZFP variant \( \alpha \) was essentially non-functional in this assay (Fig. 5d). In summary, these data strongly indicated that HERV-K (HML-2) Rec can (i) physically associate with TZFP with an intact zinc-finger domain; (ii) be precipitated as a trimeric complex with TZFP and AR; and (iii) function as a de-repressor of transcriptional transactivation by inhibiting the repressor activity of TZFP.

**DISCUSSION**

The HERV-K (HML-2) protein Rec, a homologue of the regulatory Rev/Rex RNA transporters of exogenous HIV and HTLV, and produced by an alternative transcript from the env ORF, has been implicated in the development and/or progression of cancer, primarily germ-cell cancer, in several studies over the past decade (reviewed by Ruprecht et al., 2008). For example, as a sequel to work that showed that a very high percentage of patients with germ-cell tumours produce antibodies directed against HERV-K (HML-2) proteins, investigation of the various HERV proteins revealed that Rec (but not Gag or Env) is able to transform immortal fibroblasts to allow tumour growth in nude mice (Boese et al., 2000). Moreover, Rec inducibly produced in the testis of transgenic mice resulted in the development, by 19 months of age, of carcinoma \textit{in situ} reminiscent of the precursor lesions observed in human testicular germ-cell tumours (Galli et al., 2005).

In the meantime, the systematic search for protein interaction partners eventually led to the identification of several key proteins from well-known tumour/differentiation/cell cycle control pathways as being Rec-associated and modulated, and thereby allowed for the first time informed guesses on the molecular basis of Rec’s function in tumorigenesis. Among the most intriguing binding
partners appears to be PLZF (Boese et al., 2000; Denne et al., 2007), as PLZF was eventually documented not only to have a role in leukaemogenesis but also to be important for the regulation of self-renewal of spermatogonial stem cells (Barna et al., 2000; Buas et al., 2004; Costoya et al., 2004). PLZF is a transcriptional repressor, and a large part of its effects may be exerted indirectly through inhibition of transcription of the oncogenic c-myc gene (McConnell et al., 2003). We have shown here that the apparently also spermatogenesis-regulating, PLZF-related TZFP can also act as a repressor of c-myc promoter sequences that contain the PLZF (TZFP) binding motif. Rec was able to partially counteract this inhibition in both cases (Denne et al., 2007 and this work), and it is tempting to speculate that the resulting stimulation of c-myc expression in the presence of PLZF (Denne et al., 2007) or TZFP contributes to the oncogenicity of Rec.

The association of TZFP with Rec, in addition to the interaction of PLFZ with Rec, adds a further level of complexity to the potential effects of Rec in testicular germ cells, as (mouse) TZFP has been shown to interact with and regulate AR (Ishizuka et al., 2005). Thus, whilst it was perhaps not surprising to learn that Rec can also bind to TZFP and thereby regulate the c-myc gene promoter, it was unexpected that human AR, TZFP and Rec are able to form a trimeric complex in vivo and that Rec can partially overcome the repression by TZFP of activated AR on AR-responsive promoters. The formation of a stable trimeric complex suggests that the observed de-repression of AR by Rec in the presence of TZFP is unlikely to be due to a mechanism involving disruption of the AR/TZFP complex by competitive binding of Rec and AR to TZFP. Whether the recruitment of Rec into the AR/TZFP complex leads to the disruption or dysfunction of the co-repressor complex on TZFP, consisting of nuclear receptor co-repressor (NCoR), the silencing mediator of retinoid and thyroid hormone receptors (SMRT) and HDAC2 (Tao et al., 2006), awaits further study.

So could Rec affect germ-cell function through the AR/TZFP axis? Recent work employing tissue-specific AR knockout mice has revealed that absence of AR in mouse germ cells fails to affect spermatogenesis and male fertility; however, AR disruption in the spermatogenesis-supporting Sertoli–Leydig cells clearly suppressed this process (Wang et al., 2009), pointing to the possibility that Rec may influence spermatogenesis through AR indirectly. In addition, Rec may of course affect spermatogenesis through TZFP in an AR-independent manner. TZFP seems to serve functions at the pachytene stage of meiotic prophase, as well as in round and elongated spermatids (Inoue et al., 2000; Ishizuka et al., 2003). With regard to AR, it is important to note that this transcription factor, which is essential for expression of the male phenotype, is also a critical player in prostate cancer (Balk & Knudsen, 2000). Whether Rec and TZFP or PLZF are also present in (a subset of) prostate cancers is currently unknown. If this were indeed the case, these proteins could be associated with lower-grade (AR functional; TZFP/PLZF either absent or present together with Rec) or higher-grade (functional AR lacking, or functional AR present plus the presence of TZFP/PLZF) tumour stages.

When considering an oncogenic function of Rec in cancers, the possibility of cooperation or synergy between Rec and other potentially oncogenic HERV proteins should not be overlooked, as these proteins are usually co-expressed. The rec gene is an alternatively spliced transcript derived from the full-length env ORF of HERV-K (HML-2) subtype 2, and env expression itself has, for instance, been correlated with breast cancer (Wang-Johanning et al., 2003). The highly conserved transmembrane domain of Env proteins has been documented to act immunosuppressively in vitro and in vivo, and thereby to subvert tumour immune surveillance (reviewed by Ruprecht et al., 2008). Another factor of interest in this context is np9, a transcript spliced from the env ORF of the HERV-K (HML-2) subtype 1 sequence carrying a 292 bp deletion within the env gene. The resulting 74 aa, 9 kDa nuclear Np9 protein seems to be expressed exclusively to appreciable levels in malignant but not normal cells (most notably breast cancers, leukemias and germ-cell cancers (Armbruester et al., 2002), shares its N-terminal 14 aa with Rec, and, like Rec, is able to associate with PLZF (Denne et al., 2007) as well as with TZFP (our unpublished observations). In addition, and unlike Rec, Np9 can interact with the RING-type E3 ubiquitin ligase LNX (Armbruester et al., 2004) that targets and helps degrade Numb, the antagonist of the oncogenic transcription factor Notch, which is part of the pro-proliferative Ras pathway (Weijzen et al., 2002). Apart from having a role in numerous cancers, Notch signalling has also been implicated in rat and human spermatogenesis (Hayashi et al., 2001). Finally, Notch is a transcriptional activator of c-myc, but in some experimental settings, its transactivating capacity proved insufficient to promote tumour growth (Hsu et al., 2008; Liao et al., 2007), also pointing to opportunities for synergy. Future investigations will address whether the Np9/LNX/Numb/Notch, the Np9/TZFP pathway (perhaps plus Env) can cooperate to promote germ-cell cancers.

**METHODS**

**Plasmids.** TZFP was identified by screening a human testis cDNA library in a yeast two-hybrid system (MATCHMAKER LexA Libraries, Clontech) using the HERV-K (HML-2) Rec protein as bait as described by Boese et al. (2000). The screen identified the C-terminal 347 aa residues of TZFP as a binding partner. Using appropriate primer pairs and human testis cDNA as template, the coding sequence of TZFP (GenBank accession no. NM_014383) was completed by PCR. All plasmid constructs were prepared using standard methods. pSG5HA-TZF9 was constructed by amplification of TZFP with primers A (5'-GCCGgaattcCATGTCCCTGCCCCCCATAAG-3') and B (5'-GGTGG-TagatcGGGTTGGGAGGAGGAA-3') (EcoRI and BglII sites, respectively, in lower case), which was then inserted in pSG5HA. The vector pSG5HA was generated by inserting the linker 5'-GAATTAGA-TCTCGAGCTCAAGCTTCGAATTCTGCAGGATCCTATCCTTATG-

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This text is a transcription of the scientific content from a page of a document, focusing on the interaction of Rec and TZFP with PLZF, their roles in spermatogenesis and prostate cancer, and the implications for tumour stages. The methods for identifying TZFP through screening a human testis cDNA library and constructing plasmid constructs are also detailed.
AGTTTCCGTTATGGCTTACGTGACCGCGGCGGCGGTACCG-ATC-3' into the 5' EcoRI and the 3' BgIII sites of the vector pSG5 (Stratagene), which were destroyed by this insertion. The linker encodes various restriction enzyme sites preceding the haemagglutinin (HA) tag in front of a stop codon. The vector thus encodes a protein with a C-terminal HA tag. pSG5HA-TZFP ΔZn was constructed by amplification of TZFP with primers A and C (5'-CCCCCAGACT-CCAGGCAGCAGGGG-3') (EcoRI and BglII), pSG5HA-TZFP ΔPOZ was constructed by amplification of TZFP with primers D (5'-CGCCCGGCAacacctGGATGCGTCCAGCGGACAGG-3') and B (HindIII and BglII). pSG5HA-TZFP ΔZn/POZ was constructed by amplification of TZFP with primers D and C (HindIII and BglII). TZFP variant z and TZFP variant β were constructed by amplification of a human pC4-5 testis cDNA library (Matchmaker LexA, human testis cDNA library; Clontech) with primers E (5'-AGCGCAAGGCACAATTGC-3') and F (5'-GTCAGGTTGGAGGAAAGGA-3') and inserted in the vector pGEM (Promega). pSG5HA-TZFP variant x was constructed by amplification of pGEM-TZFP variant x with primers A and G (5'-CGCCCGGCAacacctGGATGCGTCCAGCGGACAGG-3') inserted in vector pCMV-β-gal (Promega). pSG5HA-TZFP variant z was constructed by amplification of pGEM-TZFP variant z with primers A and G (5'-CGCCCGGCAacacctGGATGCGTCCAGCGGACAGG-3').

**Coimmunoprecipitation.** 293T cells were transfected with the pSG5HA or pSG5 construct using NanoFectin I (PAAB Laboratories), according to the manufacturer’s recommendation. At 48 h post-transfection, the cells were harvested. After washing and centrifugation, the cell pellets were resuspended in AB linking buffer (0.02 M Tris/HC1 (pH 7.4), 0.05 M NaCl, 0.001 M EDTA, 0.15% NaCl, 0.5% deoxycholate, 0.5% SDS, protease inhibitors, 0.1 mM PMSF, 10 mM iodoacetamide, 1 μg aprotinin ml⁻¹) and sonicated for 1 min. The cells were incubated on ice for 1 h and cleared by centrifugation. In the next step, the antibody for immunoprecipitation was added (40 μl) and the mix was incubated for 1 h on ice. The protein G-Sepharose beads were added to the mix (80 μl) and incubated for 2 h at 4 °C with gentle shaking. The beads were collected by centrifugation and washed five times in AB linking buffer. The pellets were boiled for 5 min in SDS gel loading buffer. The supernatants were loaded onto SDS–polyacrylamide gels. Immunoblotting was performed as described by Boese et al. (2000). The proteins were separated by 10 and 12.5% SDS-PAGE and transferred to a nitrocellulose membrane (Immobilon-P, Millipore). Non-specific binding was blocked with 5% non-fat milk in PBS for 30 min. Detection was performed by enhanced chemiluminescence (ECL) with ECL Hyperfilm (GE Healthcare).

**Western blotting and antibodies.** Western blotting was carried out as described elsewhere (Boese et al., 2000). Rec was detected with rabbit polyclonal anti-Rec antiserum K3086 (produced in house) diluted 1:100. To detect the HA–TZFP constructs, monoclonal rat anti-HA antiserum (clone 3F10; Roche) was used at a dilution of 1:100. Polyclonal rabbit anti-AR antiserum (clone N-20; Santa Cruz Biotechnology) was used at 1:200. Secondary horseradish peroxidase-coupled antibodies were used at a 1:1000 dilution (Sigma).

**Reporter gene assay and statistical analysis.** To study repression and anti-repression in reporter gene assays, 1600 ng reporter plasmid was transfected in an initial set of experiments with decreasing quantities (500–50 ng) of effector plasmid 1 (producing the repressor protein) to titrate the minimal effector needed to obtain significant repression. Increasing quantities (50–200 ng) of reporter plasmid 2 (producing the anti-repressor) were then used to titrate the minimal level of effector 2 to achieve anti-repression. In each experiment, equal total levels of plasmid DNA were transfected. Mostly, reporter and effector plasmids were used in a 1:1 ratio (400 ng reporter, 1600 ng effector) with 100 ng pEGFP included in each transfection to normalize for transfection efficiency by fluorescence-activated cell sorting. 293T cells were transfected with NanoFectin I (PAAB Laboratories). Expression levels of the effector proteins were monitored by Western blotting. In experiments with MMTVlac and PSAuc, cells were treated with 10⁻⁵ M of the androgens (5-α, 17-β)-17-hydroxy-androstane-3-on (Sigma) at 12 h after transfection. Transfected cells were harvested at 24–42 h time points, and lysates were assayed for luciferase activity with a Dual-Luciferase kit (Promega) as recommended by the manufacturer. The luciferase activity was normalized to the transfection efficiency measured by fluorescence-activated cell sorting. Each experiment was performed at least three times. P values were calculated with Sigma Plot software, version 4.01 (SPSS Inc.).

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