DNA replication-dependent induction of gene proximity by androgen

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The male hormone androgen, working through the androgen receptor (AR), plays a major role in physiological process and disease development. Previous studies of AR mainly focus on its transcriptional activity. Here, we found that androgen-induced TMPRSS2 and ERG gene proximity is mediated by AR control of DNA replication rather than gene transcription. We demonstrate that, in both AR transactivation-positive and -negative prostate cells, androgen regulates DNA replication and androgen-induced gene proximity relies on both DNA replication-licensing and actual DNA replication activity. Androgen stimulation advances DNA replication timing of certain genomic regions, which may potentially increase gene proximity through sharing the same replication factory at a similar time. Therefore, we have revealed novel mechanisms of AR biological function, which will stimulate new research directions.

INTRODUCTION

Steroid hormones play a critical role in regulating development and physiological functions. They are also involved in the development of human diseases and malignancies, such as prostate and breast cancers, which are the most common male and female malignancies in Western countries, respectively (1). The male hormone androgen and its receptor [androgen receptor (AR)] play a major role in male character development, bone mineral density, neuronal remodeling and behavior, and cardiovascular remodeling (2–4). A full dissection of the biological function of AR is critical for understanding its role both in normal physiological development and disease pathogenesis, particularly in prostate cancer, where AR is central to disease development, treatment and therapeutic resistance (5–8). The mechanisms of AR action both under physiological and disease conditions have been extensively investigated, but previous work is mainly focused on its role as a transcription factor (its transactivation) (3,9,10).

We and other researchers have recently shown that androgen, though AR, is able to stimulate spatial gene proximity and can consequently induce TMPRSS2:ERG, the prostate cancer-specific and most common fusion gene in human cancers (11–13). Sharing transcription machinery has been reported as a mechanism leading to chromatin movement and gene co-localization (14,15). Hormone receptors, including AR and estrogen receptor, have been reported to recruit genes to specific nuclear bodies for transcription and post-transcriptional editing (16), suggesting the association of androgen-induced gene proximity with AR transactivation. However, some data in those studies suggest that androgen-induced TMPRSS2 and ERG gene proximity may be independent of such transactivation of AR. First, although TMPRSS2 expression is androgen-regulated, the fusion partner gene ERG is not transcriptionally regulated by this hormone and it is generally not expressed in prostate epithelial cells. More importantly, the TMPRSS2 and ERG proximity was induced by dihydrotestosterone (DHT), the highly active form of androgen, in prostate epithelial primary-cultured cells (PrECs) and in the immortalized cell lines PNT1a and PNT2 (11–13), where AR transactivation is absent and AR protein is undetectable by standard western blotting (10,17,18).

In this study, we evaluated the influence of AR, its transactivation and DNA replication on androgen-induced TMPRSS2 and ERG proximity and investigated the implication of androgen-signaling in DNA replication and gene replication timing. We found that androgen-induced TMPRSS2 and ERG proximity is mediated by the function of AR in controlling DNA replication rather than gene transcription. This spatial co-localization relies...
RESULTS

Trace levels of AR, which is not sufficient to regulate down-stream gene transcription, can mediate DHT-induced TMPRSS2 and ERG proximity

As AR expression status in the nonmalignant prostate epithelial cells PNT1a and PNT2 is debatable, we evaluated AR expression in our PNT1a and PNT2 cells in comparison with other prostate cell lines. We detected AR expression at mRNA level in both PNT1a and PNT2 cells at far lower levels than that in LNCaP and VCaP androgen-sensitive cells (Supplementary Material, Fig. S1A). However, we did not detect AR at the protein level by standard western blotting in PNT1a and PNT2 cells (Supplementary Material, Fig. S1B). We then applied a more sensitive approach to detect the AR protein expression in these cells. We immunoprecipitated AR from a large amount (1.5 × 10^7) of cells using an AR-polyclonal antibody, and the precipitated proteins were then evaluated by western blotting analysis using an AR-monomoclonal antibody. We detected the expression of this nuclear receptor in both PNT1a and PNT2 cells (Supplementary Material, Fig. S1C), demonstrating that these cells express AR, although at very low levels.

To test whether the low-level expression of AR in PNT1a and PNT2 cells harbors AR transactivation, we analyzed the androgen-mediated expression of two known AR target genes, the prostate-specific antigen (PSA) and the TMPRSS2 gene, at mRNA level. PNT1a, PNT2 and LNCaP cells were cultured in androgen-stripped medium and then stimulated with DHT at different time points (0.5, 3, 6, 12 and 24 h). As expected, quantitative RT-PCR analysis showed that DHT treatment increased the expression levels of both genes over this treatment period in the highly AR-expressing LNCaP cells (Supplementary Material, Fig. S2). Consistent with previous reports, PNT1a and PNT2 cells showed very low expression of PSA and TMPRSS2 mRNA, and their levels were not stimulated upon DHT treatment, indicating that these cells lack AR transactivation.

We then investigated whether androgen-induced TMPRSS2 and ERG gene proximity is actually mediated by the low AR expression in these cell lines by using a loss-of-function approach. We pharmacologically inhibited (with bicalutamide) or knocked-down AR (by siRNA) (Supplementary Material, Fig. S3) in PNT1a and LNCaP cells prior to stimulation with DHT. As shown in Figure 1, DHT-induced TMPRSS2 and ERG proximity was significantly decreased after both knock-down and inhibition of AR in PNT1a cells, indicating that very low levels of AR expression mediate the DHT-induced co-localization of these two genes. As expected, inhibition of AR in LNCaP cells also significantly reduced the DHT-induced TMPRSS2 and ERG proximity (Fig. 1C).

To confirm that the DHT-induced spatial proximity was specific for DHT stimulation, BAC probes specific for two genomic regions of chromosome 6q15-16.1 (Supplementary Material, Fig. S4A), which are separated to each other by 3 Mb and do not present AR-binding sites, were used as negative control for androgen-induced co-localization analysis. For this, LNCaP, PNT1a and DU145 cells treated with or without DHT (100 nM) for 3 h were fixed on slides and co-localization of the two regions on chromosome 6 was evaluated by fluorescence in situ hybridization (FISH). The co-localization rate between these two genomic regions was not increased in any of the three evaluated cell lines upon DHT treatment (Supplementary Material, Fig. S4B and C).

Androgen influences DNA replication in prostate cells through AR

Given that AR has been previously reported to act as a DNA-licensing factor and to stimulate DNA replication (19–21), we investigated the influence of androgen and AR on DNA replication. We quantified the incorporation rates of the nucleoside analog BrdU in PNT1a, PNT2 and LNCaP cells in the presence or absence of DHT and bicalutamide. The AR-negative prostate on both DNA replication licensing and actual DNA duplication and may be caused by androgen-stimulated advancement of DNA replication timing of certain genomic regions. Therefore, we revealed novel insights into the role of AR in other fundamental biological processes, including regulating DNA replication timing and directing movement of specific genes.
cancer cell line DU145 was used as a negative control. DHT stimulation increased the proportion of cells with high DNA replication activity in all the three AR expression-positive cell lines (Fig. 2). In PNT1a and PNT2 cells, inhibition of AR by bicalutamide significantly decreased the percentage of cells with high DNA replication activity (Fig. 2A and B). Surprisingly, LNCaP cells showed the most prominent response, where DNA replication was completely blocked by high-dose bicalutamide treatment (Fig. 2C). As expected, levels of DNA replication in AR-negative DU145 cells remained invariant upon AR stimulation or inhibition (Fig. 2D).

DNA replication is required for androgen-induced TMPRSS2 and ERG proximity

Given that AR has been previously reported to interact with certain licensing factors (19–21), we investigated whether this interaction affects DHT-induced TMPRSS2 and ERG proximity. We first confirmed that AR directly interacts with the licensing factor subunit MCM7 in LNCaP cells by immunoprecipitation analysis (Supplementary Material, Fig. S5). We then knocked-down MCM7 with siRNA (Supplementary Material, Fig. S6A) and detected a blockage of the DHT-induced TMPRSS2 and ERG proximity in PNT1a cells (Fig. 3A). Knock-down of MCM7 in the AR transactivation-positive LNCaP cells (Supplementary Material, Fig. S6B) also completely blocked DHT-induced TMPRSS2 and ERG proximity (Fig. 3B). These data demonstrate that androgen-induced TMPRSS2 and ERG proximity is associated with AR-DNA replication-licensing function in both AR transactivation-negative and -positive cells.

We further investigated whether androgen-induced TMPRSS2 and ERG proximity takes place during the DNA replication process. We enriched PNT1a, PNT2 and LNCaP cells in the S-phase using the DNA polymerase-α inhibitor aphidicolin before DHT treatment (Supplementary Material, Fig. S7A). S-phase cell enrichment significantly increased DHT-induced TMPRSS2 and ERG proximity rates in all three cell lines (Fig. 3C–F).

We then assessed the effect of inhibiting DNA elongation on androgen-induced TMPRSS2 and ERG proximity, assuming that those genomic loci will be recruited to the replication site but cannot be replicated. For this, LNCaP cells were treated with DHT whereas DNA synthesis was inhibited with aphidicolin (Supplementary Material, Fig. S7B). Surprisingly, we observed a complete blockage of the DHT-induced TMPRSS2 and ERG co-localization (Fig. 3E). We also confirmed that androgen-induced TMPRSS2 and ERG proximity relies on active DNA synthesis by knocking-down DNA polymerase-α with siRNA (Supplementary Material, Fig. S8). Abrogation of this enzyme also blocked the androgen-stimulated gene proximity rates in both cell lines (Fig. 3G and H). Therefore, androgen-induced gene proximity depends on active DNA replication.

As TMPRSS2 and ERG are located on the same chromosome, we investigated whether androgen-induced inter-chromosomal proximity, such as the previously reported TMPRSS2 and ETV1 in LNCaP cells (12,13), also takes place during the DNA replication process. Upon blockage of PNT1a cells for DNA replication following the same procedure as mentioned earlier (Supplementary Material, Fig. S7B), we observed that, although DHT treatment increases the rates of TMPRSS2 and ETV1 proximity, DNA replication blockage with aphidicolin prevented the androgen-induced spatial proximity between these two genomic loci (Supplementary Material, Fig. S9). These data suggest that androgen-induced inter-chromosomal proximity also depends on DNA replication.

In order to verify that the androgen-induced gene proximity occurs during DNA replication, we used FISH analysis to evaluate the TMPRSS2 and ERG proximity in PNT1a and LNCaP cells where the nascent DNA was labeled with BrdU. We observed that most of the co-localized signals occur in cells undergoing replication (Fig. 3I and J). This finding further supports our above-mentioned observation that androgen-induced TMPRSS2 and ERG proximity is driven by DNA replication.
We also determined the impact of gene transcription on DHT-induced gene proximity in LNCaP cells. For this, we inhibited cellular transcription with the RNA polymerase inhibitor α-amanitin in combination with DHT treatment (Supplementary Material, Fig. S7C). The inhibition of transcription reduced, but did not fully block, the DHT-induced TMPRSS2 and ERG proximity (Supplementary Material, Fig. S10), suggesting that gene transcription activity is not essential for androgen-induced gene proximity.

**Androgen advances TMPRSS2 and ERG replication timing**

Spatial proximity of two genomic loci occurs when these two loci are found in the same location and at the same time. We investigated whether androgen may influence DNA replication timing and consequently co-coordinate the replication of certain genes, increasing their chance of sharing replication loci and co-localize. We first determined whether TMPRSS2 and ERG replicate at the same stage of the S-phase by precipitating BrdU pulse-labeled DNA from different flow-sorted (early, middle and late) S-phase fractions in PNT1a and PNT2 cells (Supplementary Material, Fig. S11A). Both TMPRSS2 and ERG were detected using PCR in BrdU-labeled DNA, precipitated from the early S-phase cells (Fig. 4A and Supplementary Material, Fig. S11B), indicating the early replication timing of both genes.

To investigate more precisely the replication time of TMPRSS2 and ERG and determine whether androgen induces changes in the replication timing of those genomic regions, we performed FISH analysis of TMPRSS2 and ERG together with an early-replication control gene, PYGM. Relative replication timing of these genes was established based on the number of doublet (replicated chromatins) and singlet (un-replicated chromatins) signals of TMPRSS2 or ERG compared with PYGM. Without DHT treatment, ERG replicated at a similar time to PYGM in LNCaP cells, but later in PNT1a cells; and TMPRSS2 replicated later than PYGM in both cell lines. After DHT treatment, the replication time for both ERG and TMPRSS2 was advanced so that both genes replicated earlier than PYGM in both cell lines (Fig. 4B and C and Supplementary Material, Fig. S12).

**DISCUSSION**

It is important to understand the underlying mechanisms causing fusion genes. As it has been reported that sharing transcription factory is a mechanism resulting in fusion genes (22) and hormone receptors are generally known as transcription factors, it is assumed that the androgen-induced TMPRSS2 and ERG spatial proximity is driven by the activity of AR in controlling gene transcription (12,13,23). However, in this study, we demonstrate that androgen-induced TMPRSS2 and ERG proximity is mediated by the role of AR in controlling DNA
replication rather than its transactivation, revealing novel insights both into the role of the biological function of androgen and mechanisms of genomic movement influenced by DNA replication.

In our previous study, we induced TMPRSS2 and ERG spatial proximity in both PNT1a and PNT2 cells (11), where AR transactivation has not been detected (9,10,17,18). However, AR expression in PNT1a and PNT2 cells is controversial. Whereas some studies report these cells as AR expression negative (10,17,18), AR protein has been detected in both cell lines (17,24). In this study, using immunoprecipitation analysis, we revealed that PNT1a and PNT2 cells harbor trace levels of AR but lack AR transactivation. We also demonstrate the requirement of AR activity in androgen-induced TMPRSS2 and ERG proximity in these cells, suggesting that trace levels of AR, which are insufficient for AR transactivation, may mediate androgen-induced gene proximity through an AR-mediated non-transactivation function.

AR has been reported as a DNA replication-licensing factor in prostate cancer cells (20,21) and is responsible for induction of S-phase entry through stimulation of the Src/Raf/Erk pathway (25). Here, we have confirmed the previously reported direct interaction between AR and pre-replication complex protein MCM7 (21), we provide further data to clarify the influence of androgen and AR on DNA replication. Low levels of AR expression, like those in PNT1a and PNT2 cells, showed a modest impact on DNA replication. In androgen-sensitive cells with abundant AR protein expression like LNCaP, AR seems to be essential for DNA replication to the point that AR inhibition completely blocked DNA replication. The observed levels of DNA replication activity in LNCaP cells cultured in medium with androgen-stripped serum may be explained either by low-level androgen remaining in the charcoal-stripped serum or the AR functional stimulating effect of phenol red in the culture medium (26).

The paradox between AR dependence and AR transactivation independence of the androgen-induced TMPRSS2 and ERG proximity may therefore be explained by the role of AR-associated DNA replication activity, which remains in PNT1a and PNT2 cells. Our data on the prevention of the androgen-mediated TMPRSS2 and ERG proximity by inhibiting DNA replication with aphidicolin or knock-down of MCM7 or DNA polymerase-α with siRNA not only support this notion but also indicate that androgen-induced genomic proximity depends on both the recruitment of the pre-replication complex and active DNA replication process. AR has been reported to co-localize with DNA replication loci (27), and recent immunoprecipitation analysis identified the direct interaction of AR and DNA polymerase-α (28). Taken together, these data suggest the involvement of AR in the DNA replication process and a critical role of AR in mediating androgen-induced genomic proximity through controlling DNA replication. The finding that transcription inhibition only partially reduced gene proximity further supports the notion that gene transcription activity may not be essential for androgen-induced gene proximity. As gene transcription is associated with DNA replication activity, the reduction of TMPRSS2 and ERG co-localization rate upon transcription inhibition may be caused by indirectly affecting the DNA replication.

The increase of androgen-induced gene co-localization in S-phase-enriched cell populations and decrease in cells with a disrupted DNA replication process also suggest that androgen-induced gene proximity may occur at specific nuclear loci in association with DNA replication. It is known that DNA replication occurs in nuclear loci-DNA replication factories and a number of replicons share one replication factory for simultaneous DNA synthesis (29–32); thus, temporal co-localization of genomic regions, which are routinely located apart, may be induced by sharing replication factories. DNA replication-mediated gene proximity requires not only the recruitment of
different genomic regions to the same replication factories but also at a similar time point. It has been reported that plant hormones can change DNA replication timing in certain plant tissues (33). Here, we report for the first time that a hormone also at a similar time point. It has been reported that plant hor-

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Based on our findings in this study, we propose a model for androgen-induced \textit{TMPRSS2} and \textit{ERG} proximity through re-programming replication timing. As DNA replicates at normal rate, unaffected by androgen stimulation in AR null cells, \textit{ERG} and \textit{TMPRSS2}, located 3 Mb apart, are generally replicated using different replication factories. However, owing to their close spatial location, they may occasionally share the same replication factory, thus generating a limited number of replication-associated co-localized FISH signals, irrespective of the presence of androgen (Fig. 5A). When androgen-sensitive cells are grown in an androgen-depleted environment, the limited rate and lack of AR-regulated cooperation of DNA replication rarely induces replication-associated \textit{TMPRSS2} and \textit{ERG} gene proximity in addition to those occurring by random chance. This may explain the observed higher co-localization rate in the AR null DU145 compared with PNT1a, PNT2 and LNCaP cells cultured in androgen-free medium (11). When cells with AR are stimulated by androgen, AR binds to the pre-replication complex (loaded onto replication origins in G1-phase) located at specific genomic regions, including \textit{TMPRSS2} and \textit{ERG}, to fire those origins at very early S-phase for replication. As many origins located between \textit{TMPRSS2} and \textit{ERG} are AR-independent and not fired for replication at that stage, \textit{TMPRSS2} and \textit{ERG} have a greater chance of sharing the same replication factory (co-localized) (Fig. 5B). Apart from the replication timing change, AR may also bring those genomic loci to specific replication factories by other mechanisms. Therefore, DHT-stimulated cells have a high co-localization rate for genomic loci, whose replication is regulated by AR.

In summary, using the androgen-induced \textit{TMPRSS2}:\textit{ERG} fusion as a model system, we have demonstrated that AR controls DNA replication, including replication timing, and chromatin movement in prostate cells, even in those with a very low level of AR and without AR-mediated transcriptional activity. This novel AR function may occur in other cell types with low AR activity, such as renal cells. As we showed that certain cellular processes or features, previously thought to be associated with AR transactivation, were the result of DNA-replication regulation function of AR, reevaluation of the previously established cellular functions of AR and the associated mechanisms is required. As androgen and AR play important cellular and physiological roles, this new initiative of AR research may not only enhance our basic biological knowledge but also improve human health. There is increasing evidence that the DNA replication program has a critical impact on genetic inheritance and stability (34,35) and, in cancer cells, genomic breakpoints are correlated with replication timing (36). Androgen and AR-induced genomic changes, through controlling DNA replication, should be extensive-ly investigated. This study also encourages investigating whether and how other steroid hormones control DNA replication.

\textbf{MATERIALS AND METHODS}

\textbf{Cell lines and culture conditions}

Cell lines, including the SV40-immortalized prostate epithelial cell lines PNT1a and PNT2 (obtained from Norman Maitland and Colin Cooper, respectively) and prostate cancer cell lines LNCaP, DU145 and VCaP (ATCC), were routinely cultured in Dul-becco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum. Normal prostate epithelial cells (PrEC) (Lonza) were cultured in Clonetics PreBM medium (Lonza).

\textbf{Pharmacological treatments of cells}

All pharmacological treatments of cells were carried out in 10% charcoal-stripped serum containing media.

AR activity was stimulated with DHT (Sigma–Aldrich), whereas AR blockage was induced with bicalutamide (Sigma–Aldrich). For co-localization analysis, cells were treated with vehicle (no drug) or 10 \(\mu\)M bicalutamide for 2 h before treatment with or without DHT (100 nM) for 3 more hours. For DNA replication rate analysis, cells cultured in androgen-free medium for 18 h were treated with vehicle, 100 nM DHT in combination with 0, 5 or 20 \(\mu\)M of bicalutamide for 3 h prior to BrdU pulse-labeling (10 \(\mu\)M, 30 min).

To increase the number of S-phase cells, cultured cells were treated with 6 \(\mu\)M aphidicolin (Sigma–Aldrich) for 2 h and then released for 1 h by washing three times with PBS and cultured in androgen-free medium. After 1 h of recovery, cells were treated with vehicle or DHT (100 nM) for 3 more hours (Supplementary Material, Fig. S7A). For inhibition of DNA synthesis, cells were treated with 6 \(\mu\)M aphidicolin for 2 h and then vehicle or DHT (100 nM) was added to the medium for a further 3 h whereas aphidicolin was present (Supplementary Material, Fig. S7B).

To inhibit RNA transcription, cells were treated with 2.2 \(\mu\)M of \(\alpha\)-amanitin (Fluka) for 2 h and then vehicle or DHT (100 nM) was added to the medium for further 3 h while \(\alpha\)-amanitin was present (Supplementary Material, Fig. S7C).

\textbf{Immunoprecipitation and co-immunoprecipitation analysis}

Immunoprecipitation of AR in the PNT1a and PNT2 cell lines and co-immunoprecipitation of AR with MCM7 in LNCaP cells were performed using the same protocol. Cells were harvested with lysis buffer (25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5% NP-40) containing protease inhibitors. Cleared extracts (1 mg) were incubated with 2 \(\mu\)g of rabbit polyclonal anti-AR [N20] (Santa Cruz Biotechnology) was used as immunoprecipitation-generating antibody (Santa Cruz Biotechnology) was used as immunoprecipitation-negative control. The immunoprecipitated samples were separated by SDS–PAGE and transferred to nitrocellulose membranes. Western blotting was performed as described later. This experiment was done with three biological replicates.

\textbf{Fluorescence in situ hybridization}

Co-localization analysis was performed using standard two-color FISH protocol. The BAC clones RP11-476D17 (\textit{ERG}),
RP11-124L22 (ETV1) and RP1-44N23 (6q15) were labeled with the fluorescein 12-dUTP (green), and the BAC clones RP11-535H11 (TMPRSS2) and RP1-23E21 (6q16.1) were labeled with the tetramethyl-chodamine-5-dUTP (red). All these BAC clones were obtained from the Institute of Cancer Research, Sutton, UK.

Replication timing analysis was performed using standard two-color FISH protocol with simultaneous detection of S-phase nuclei by bromodeoxyuridine (BrdU)-labeling of newly synthesized DNA. We used the tetramethyl-chodamine-5-dUTP-labeled (green) BAC clones RP11-535H11 for TMPRSS2 and RP1-476D17 for ERG, and the fluorescein 12-dUTP-labeled (red) BAC clone RP11-869B15 (Invitrogen) for PYGM.

Induced genomic proximity was quantified and represented as the percentage of co-localized signal pairs. For each probe pair, inter-signal distances between the closest allele pairs were measured in micrometers using ImageJ software. Co-localization was defined positive for paired signals with inter-signal distances lower than 0.2 μm. Around 1000 signal pairs were evaluated per sample. Replication timing was determined by comparing the number of doublet and singlet signals of TMPRSS2 or ERG with the early replication gene PYGM in each cell. Each FISH replication timing analysis experiment was performed in biological duplicates, and a minimum of 100 nuclei were counted per sample.

BrdU incorporation and detection of BrdU-labeled DNA

For PCR detection of BrdU-labeled DNA, cells were stimulated with vehicle or DHT (100 nM) for 3 h and pulse-labeled with 10 μM BrdU for 30 min. Cells were then FACS sorted into G1, and three fractions of S-phase (S1, S2 and S3) according to the DNA content on a FACScalibur flow cytometer (Becton Dickinson). Immunoprecipitation of nascent gDNA was performed as previously described (37).

For immunofluorescent detection of newly synthesized DNA using BrdU labeling, cells were pulse-labeled with 10 μM BrdU for 30 min. BrdU was stained by incubation with mouse anti-BrdU (1:500) (BD Bioscience) for 1 h, followed by incubation with the secondary antibody Alexafluor 488 donkey anti-mouse IgG (1:500) (Invitrogen) for 1 h. Florescent images were scanned using the Arial system with seven Z stacks, and percentage of positive cells in the whole population was counted with consideration of the area and density of BrdU-labeled signals.

siRNA and knock-down studies

Transfection of cultured cells with siRNA was performed using DharmaFECT Transfection Reagents (Dharmacon) for 48 h. The commercially available siRNAs used in this study are listed in Supplementary Material, Table S1. The knock-down levels were tested using quantitative RT–PCR (qRT–PCR) assay and western blotting analysis.

Quantitative reverse-transcription PCR (qRT–PCR)

Relative mRNA levels were determined using predesigned TaqMan gene expression probes (Life Technologies) on an ABI Prism 7700 Sequence detector (Life Technologies). All qRT–PCR reactions were performed using TaqMan Master Mix (Life Technologies) and predesigned TaqMan gene expression probes. Exponential amplification of each target gene was evaluated using Sequence Detection Software version 1.3 (Applied Biosystems) with a total 50 cycle program. All probes used in this study are listed in Supplementary Material, Table S2.
PCR for BrdU-precipitated DNA

After precipitating the BrdU-labeled DNA from the cell cycle sorted cells, PCR analysis to determine early and late replication genes was performed using the AmpliTaq Polymerase kit (Applied Biosystems by Life Technologies) according to the manufacturer’s recommendations. For each PCR reaction, 1000 cell equivalents were used. PCR products were observed by running a 1.2% agarose gel in 1×TBE solution. Uniform recovery of the BrdU-enriched DNA in the different fractions was examined by amplifying mitochondrial (mt) DNA, which is continuously replicated throughout the cell cycle and independently of nuclear DNA in mammalian cells. For early replication control, a region at the gene glycogen phosphorylase (PYGM) was targeted as it has been reported to replicate at early stages of the S-phase (37). All primers were purchased from Sigma–Aldrich, and respective primer sequences are listed in Supplementary Material, Table S3.

Western blotting

Western blotting was performed on cell lysates containing 105 cells. Mouse monoclonal β-actin and mouse monoclonal GAPDH antibodies were used at 1: 10000 dilution. All other primary antibodies were used at 1: 1000 dilution in 1% BSA/PBS-T. Immunoblotted proteins were detected with horseradish peroxidase-conjugated secondary antibodies (Thermo Scientific). All antibodies used in this study are listed in Supplementary Material, Table S4.

Data analysis and statistics

Relative mRNA expression levels were normalized to GAPDH, and samples without detectable expression after 40 cycles of amplification are indicated as undetectable (Und). Results are reported as mean ± SEM for three independent experiments. Comparisons were performed with a two-tailed paired Student’s t-test. Differences in the frequency of co-localization signals and BrdU replication rates between groups were compared using a two-tailed χ² test. P-values <0.05 were considered statistically significant.

AUTHORS’ CONTRIBUTIONS

Y.-J.L., N.C.B. and D.S. designed the study. N.C.B. performed the experiments and analyzed the data. X.M. performed the AR-immunoprecipitation analysis and helped with FISH quantification. B.D.Y. and D.S. provided conceptual advice. Y.-J.L. and N.C.B. wrote the paper with contributions from all the other authors.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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