The phosphatidylinositol 3-kinase (PI3K)/Akt pathway plays important roles for prostate cancer cell survival, and the androgen receptor (AR) plays essential roles for prostate cancer cell proliferation. Here, we provide the first linkage by the identification of Forkhead transcription factor FOXO3a, the PI3K/Akt downstream substrate, as a positive regulator for the induction of AR gene expression. Both Western blot and real-time PCR assays demonstrate that FOXO3a can induce AR expression at the protein and mRNA levels, and gel shift and chromatin immunoprecipitation assays further demonstrate that FOXO3a can induce 5α-AR promoter activity via binding to the consensus DNA-binding sequence in the AR 5′ promoter −1290 to −1297 (5′-TTGGTTTCA-3′). Under normal growth conditions, blocking PI3K/Akt signals by LY294002 causes LNCaP cell arrest in G1 phase rather than apoptosis. However, further blocking of AR functions by AR small interfering RNA leads to dramatic LNCaP cell death, suggesting that AR may play important protective roles when the PI3K/Akt signal pathway is blocked by LY294002. Together, our data provide the first model to explain how PI3K/Akt and AR can cooperate to control LNCaP cell growth and death under normal conditions.

Prostate cancer (PCa) is one of the most frequently diagnosed malignancies and is the second leading cause of cancer deaths among American men (1). Androgen and the androgen receptor (AR) (2–4) play important roles in this malignancy, and androgen ablation has been the main therapeutic option for the treatment of locally advanced or metastatic PCs. The AR is a member of the nuclear receptor superfamily that is composed of a variable NH2-terminal domain, a highly conserved DNA-binding domain, a hinge domain, and a ligand-binding domain. Although the functional significance of the androgen-AR signaling pathway in PCa progression is well studied, regulation of AR gene expression and its potential linkage to PCa progression remains largely unknown.

The FOXO subfamily of Forkhead transcription factors, FOXO1 (FKHR), FOXO3a (FKHR1), and FOXO4 (AFX), is regulated by the PI3K/Akt pathway. Direct phosphorylation of FOXO proteins by Akt results in cytoplasmic retention and the inactivation of FOXOs, inhibiting the expression of FOXO-regulated genes, which control diverse functions, including cell differentiation, proliferation, cell death, metabolism, and longevity (5). In tumor cells, in vitro studies found that the induction of apoptosis and cell cycle arrest by FOXOs through regulating target genes like p27kip1, cyclin D, Fasl, and BIM depends on cell types and physiological conditions (6, 7). A study found that FOXO3a induces apoptosis in LAPC4 prostate cancer cells via the up-regulation of its direct target tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (8). Also, FOXOs play a complex role in tumorigenesis (7).

In tumor cells Akt is often constitutively activated either through deletions or mutations in phosphatase and tensin homologue on chromosome 10 (PTEN) or through the activity of autocrine growth factors (6). For example, in prostate cancer LNCaP cells the loss of PTEN activity results in constitutively active Akt and leads to the inactivation of FOXO factors. In the present study, we demonstrated that activated FOXO3a could enhance AR gene expression and AR transactivation via binding to the AR 5′ promoter region. This suggests that inhibition of the PI3K/Akt pathway, the dominant survival factor in prostate cancer LNCaP cells, may result in the activation of the FOXO3a transcription factor, which may then induce the AR gene expression to protect cells from apoptosis caused by inhibition of PI3K/Akt pathway. This important finding implies that inhibition of both PI3K/Akt and AR signaling may become a powerful approach to treat the prostate cancer.

EXPERIMENTAL PROCEDURES

Materials—FOXO3a-wild type (FOXO3a-WT) and FOXO3a-TM were gifts from Dr. M. Greenberg (Harvard Medical School, Boston). pU6/BS was a gift from Dr. Y. Shi (Harvard Medical School, Boston). LY294002 was purchased from Calbiochem, and dihydrotestosterone (DHT) was from Sigma. The plasmids pCMV-AR, pSG5-AR, promoter-luciferase reporters containing four copies of androgen-response elements (ARE)4-luc, and pRL-TK have been described previously (9, 10). AR 5′ promoter-luciferase reporter was provided by Dr. C. Y. Young (Mayo Graduate School, Mayo Foundation, Rochester, MN). The anti-AR polyclonal antibody, NH27, was
produced as described previously (11). The anti-FLAG M2 monoclonal antibody was from Sigma, and anti-FKHL1(FOXO3a) (number 06951) was from the Upstate Group. The anti-phospho-FOXO3a, and the alkaline phosphatase-conjugated or horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies were from Santa Cruz Biotechnology. The AR small interfering RNA (siRNA) expression vector was constructed as described previously (12).

**Cell Culture and Transfections**—The AR-positive human prostate cancer LNCaP cells with a passage number less than 35 for all the experiments were purchased from ATCC. LNCaP cells and CWR22R cells (a gift from Dr. C. Kao) were maintained in 10% FBS, RPMI 1640. Transfection was performed by using either SuperFect™ (Qiagen) or electroporation (Bio-Rad, gene pulser). For electroporation, the voltage setting was 280 V and capacitance 950 microfarads; the sample volumes were 400 μL, and the total DNA amounts were 10 μg.

**Luciferase Reporter Assays**—The cells were transfected with plasmids in 10% CD-FBS media for 16 h and then treated with ethanol or 10 nM DHT for 24 h, or without any treatment if the cells were cultured in normal medium. After washing with PBS and harvesting, the cell lysates were prepared and used for luciferase assay according to the manufacturer’s instructions (Promega). The results were obtained from at least three sets of transfection and are presented as the mean ± S.D.

**Immunofluorescence and Microscopy**—Briefly, LNCaP cells were plated on slide chambers, incubated overnight, transfected with FOXO3a-WT and FOXO3a-TM for 24 h, and then treated with 20 μM LY294002 or EtOH for another 24 h. The cells were fixed with 4% paraformaldehyde for 20 min and permeabilized with 100% methanol for 15 min at 4 °C. The slides were rinsed with PBS twice and incubated in 5% BSA for 30 min. The primary antibody against FOXO3a was added for 1 h. The secondary antibodies were added for 1 h, followed by application of the counter medium containing 4′,6-diamidino-2-phenylindole. Slides were examined by microscope. A FITC-conjugating anti-rabbit antibody was used as secondary antibody.

**Northern Blot Analysis**—LNCaP cells were cultured following the methods described previously and treated with or without 10 nM DHT for 24 h. Total RNA was isolated, and Northern blot analysis was performed as described previously (10).

**Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts and EMSA were carried out as described previously (13). Briefly, nuclear extract proteins were prepared from EtOH or 20 μM LY294002-treated LNCaP cells, and 8 μg of nuclear extract protein was incubated in a reaction solution containing 20 mM Tris-HCl/pH 7.9, 2 mM MgCl₂, 1 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, and 2 μg of poly(dI-dC). After 20 min, the 32P-end-labeled duplex oligonucleotide (2 × 10⁶ cpm) was added, and the reaction was incubated for another 20 min in ice. For antibody supershift analysis, 2 μg of anti-FOXO3a antibody (Upstate Group) or normal rabbit IgG was added to the reaction mixtures and preincubated in ice for 20 min. The sequence of the probes used is 5′-GAACAAATTATGTTCTTTGTTTCACGGCTTGGTACATC-3′.

**RNA Interference**—The pSuperior.retro.puro vector (OligoEngine) was used for the expression of siRNA in LNCaP cells. FOXO3a siRNA vector was generated by a gene-specific insert (GTGGAGCTGGACCCGAGT) to target FOXO3a. A scrambled control vector was constructed using an insert (GTGCTGTAGGAGTCATCC) with no significant homology to any mammalian gene sequence.

**Detection of Apoptosis and Cell Cycle Using Flow Cytometry Assay**—LNCaP cells grown in 100-mm dishes were treated with either ethanol or 20 μM LY294002 for 24 h, or transfected with AR siRNA or empty vector, and then treated with either ethanol or 20 μM LY294002 for 24 h. The cells were digested with trypsin/EDTA. Approximately 1 × 10⁶ cells were fixed by 70% EtOH overnight at 4 °C. Harvested, and then stained with propidium iodine for 20 min at room temperature. The cells were washed twice with PBS, and the fluorescence of cells was measured by flow cytometry as reported previously (14).

**Chromatin Immunoprecipitation Assay (ChIP)**—The ChIP assays were performed essentially as described previously (13) but with some modifications. Cell lysates were preclarified sequentially with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology) and protein A-agarose. Anti-FOXO3a antibody (2.0 μg) was added to the lysate and incubated at 4 °C overnight. For the negative control, no antibody was added in the sample. The following primer pairs, which span the region –1412 to –1181 of the AR promoter, were used for the amplification of PCR product (including real time PCR): forward primer, 5′-ACCTGTCTCCCAAATGACAATG-3′; reverse primer, 5′-CAACAGGGGACATCATAGG-3′.

**Real Time PCR Analysis**—For AR expression assay, total RNAs from 5 × 10⁶ LNCaP cells that were transfected with 10 μg of FOXXO3a-WT, FOXXO3a-TM, or empty vector were isolated for 24 h after transfection by using TRIzol following the manufacturer’s protocol. 5 μg of RNA were reverse-transcribed by Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. cDNA was subjected to real time PCR using the SYBR Green PCR Reagents kit (Bio-Rad). Experiments were performed in triplicate for each data point. PCR primers were as follows: AR forward, 5′-CCTGGGTCCGCAACTTACAC-3′; AR reverse, 5′-GCACGTTGTGACATCGGCTACTC-3′.

For the ChIP assay, real time PCR was performed essentially as described previously (13). The same primer pair used for the conventional PCR was used for real time PCR.

**RESULTS**

**Blockade of PI3K/Akt Enhances AR Expression and AR Transactivation**—Early reports indicated that PI3K/Akt and AR may cooperate with each other to promote prostate cancer cell survival and growth. For example, AR transactivation can be induced by LY294002, a selective PI3K inhibitor, to play a dominant role in cell proliferation to compensate for the loss of PI3K/Akt signaling (9–11, 15). Here we further demonstrate that addition of LY294002 in LNCaP cells results in the increase of prostate-specific antigen (PSA) mRNA expression with DHT treatment (Fig. 1A). PSA is an androgen-induced target gene that...
has been used as a marker to monitor the progress of prostate cancer. This increase suggests that blockade of PI3K/Akt signaling pathway will enhance AR transactivation. To dissect the potential mechanism of how PI3K/Akt modulates AR transactivation, we treated LNCaP cells with LY294002 and applied Western blot analyses to detect the protein level of phosphorylated Akt (pAkt), PSA, and AR. As shown in Fig. 1B, pAkt was inhibited by LY294002 treatment, but PSA and AR protein levels were increased significantly, suggesting that blockade of PI3K/Akt pathway may enhance AR transactivation via the increase of AR protein expression.

**FOXO3a Induces Androgen Receptor Gene Expression**

In our previous report we demonstrated that overexpression of Akt is able to phosphorylate AR and cause AR ubiquitination and degradation (16). However, this does not rule out the possibility that additional mechanisms exist between Akt and AR when the PI3K/Akt pathway is inhibited. To find out how inhibition of PI3K/Akt increases AR protein expression, we hypothesized that PI3K/Akt might also go through its downstream target gene(s) to modulate AR gene expression. The FOXO family of Forkhead factors, FOXO1, FOXO3a, and FOXO4,
are direct downstream targets for Akt, and phosphorylation of the FOXO by Akt can result in the cytoplasmic retention with inactivation (7). Early studies also showed LNCaP cells express these FOXO factors with the FOXO3a as the predominant one (8). In LNCaP cells, Akt is constitutively active, resulting in FOXO3a being constitutively phosphorylated and inactivated (6, 17). FOXO3a-TM is a constitutively active form of FOXO3a, with all three Akt phosphorylation sites (Thr-32, Ser-252, and Ser-314) mutated to alanine. Here we first transfected LNCaP cells with FOXO3a-WT or FOXO3a-TM and assayed for their influence on AR expression. As shown in Fig. 2A, FOXO3a-TM was located in the nucleus and FOXO3a-WT in the cytosol. Treatment with LY294002 caused FOXO3a-WT to translocate from the cytosol into the nucleus. Next we tested the effects of FOXO3a on AR expression. As shown in Fig. 2B and C, both AR protein expression (by Western blot analysis) and AR mRNA expression (by real time PCR quantitation) were enhanced by transfected FOXO3a-TM, but not FOXO3a-WT, suggesting that the Akt downstream target FOXO3a might be able to modulate AR expression at both the mRNA and protein levels. The expression and phosphorylation of FOXO3a are shown by Western blot analysis in Fig. 2D.

**FIGURE 3.** FOXO3a-TM enhanced AR promoter reporter gene activity. A, AR-positive LNCaP cells were transfected with 150 ng of AR 5’ promoter-luciferase, 2.5 ng of pRL-TK, and 450 ng of FOXO3a-WT, FOXO3a-TM, or empty vector for 24 h and then subjected to 20 μM LY294002 treatment for another 24 h. Cells then were harvested for luciferase assay. Duplicate samples were analyzed for each single data point. B, AR-positive LNCaP and CWR22R cells were transfected with 150 ng of (ARE)4-luc, 5 ng of pRL-TK, and 450 ng of FOXO3a-TM, FOXO3a-WT, or empty vector. Transfected cells were treated with 16 h with 10 nM DHT or ethanol as vehicle controls. Duplicate samples were analyzed for each data point. The results shown represent the averages of results of at least two independent experiments. C, FOXO3a-siRNA and control plasmids were transfected into LNCaP cells by electroporation. After 48 h, Western blot was applied to detect FOXO3a expression. D, FOXO3a-siRNA and control plasmids transfected LNCaP cells were cultured in 10% FBS of growth medium for 24 h and then treated with vehicle or 20 μM LY294002. After another 24 h, cells were harvested for Western blot analysis with indicated antibodies. At least two independent experiments were done for each result.
ence of FOXO3a-TM on AR expression at the transcriptional level. As shown in Fig. 3A, in LNCaP cells, FOXO3a-TM, but not FOXO3a-WT, significantly enhanced AR 5′/H11032 promoter activity. Moreover, LY294002 treatment further enhanced AR 5′ promoter activity with or without transfection of FOXO3a-WT. To test if the enhanced AR expression by FOXO3a-TM also resulted in the enhancement of AR transactivation, we assayed its potential effects on ARE reporter activity. As shown in Fig. 3B, lower panel, FOXO3a-TM, but not FOXO3a-WT, enhanced AR transactivation by increasing the ARE reporter activity in LNCaP cells. Similarly enhanced effects were also observed, as shown in Fig. 3B, upper panel, when we replaced LNCaP cells with CWR22R, another prostate cancer cell line with active Akt (18). Although FOXO3a induces AR expression and enhances AR transactivation, it is still not clear whether FOXO3a is necessary for the LY294002-mediated effect on AR expression and transcription regulation. We constructed a FOXO3a siRNA plasmid and found it inhibited endogenous FOXO3a expression efficiently in LNCaP cells (Fig. 3C). However, LY294002 treated LNCaP cells, which had been transfected with FOXO3a-siRNA, still showed slightly increased expression of AR (Fig. 3D), indicating that FOXO3a is not the only mechanism for LY294002-mediated AR expression and activity. Previously we reported that Akt can phosphorylate AR to induce AR degradation. The inhibition of Akt stabilizes the AR protein (16). Therefore, other mechanisms, such as AR stability, may contribute to the observation that knocking down FOXO3a cannot completely abolish the LY294002 effect on AR expression.

Sequence analysis of the AR 5′/H11032 promoter region found that it contains one putative FOXO3a consensus binding site, which is −1290 to −1297 (5′-TTGTCTTCA-3′). We first performed EMSA to determine the DNA binding capacity of FOXO3a. The 35-bp double-stranded DNA oligonucleotide probe was added to nuclear extracts from LNCaP cells treated with EtOH or 20 μM LY294002 for 24 h. Complexes were resolved on 4.5% polyacrylamide gels. The specific complex is indicated by the arrowhead. LNCaP nuclear extracts were also incubated with FOXO3a antibody (Ab) (lanes 3 and 4) or normal IgG (lanes 5 and 6). LNCaP cells were treated with 20 μM LY294002. After 24 h, the DNA proteins were cross-linked with formaldehyde and subjected to chromatin immunoprecipitation assay using an anti-FOXO3a antibody or without antibody (negative control) and the indicated primers. Reaction products were resolved by electrophoresis. C, reaction products were quantified by real time PCR. After analysis, a melting curve was performed to confirm the amplification of a single product.

**FIGURE 4. Identification of the FOXO3a binding to the AR promoter through EMSA supershift analysis and ChIP.** A, The probe was incubated with 8 μg of LNCaP nuclear extracts, which were treated with EtOH or 20 μM LY294002 for 24 h. Complexes were resolved on 4.5% polyacrylamide gels. The specific complex is indicated by the arrowhead. LNCaP nuclear extracts were also incubated with FOXO3a antibody (Ab) (lanes 3 and 4) or normal IgG (lanes 5 and 6). B, LNCaP cells were treated with 20 μM LY294002. After 24 h, the DNA proteins were cross-linked with formaldehyde and subjected to chromatin immunoprecipitation assay using an anti-FOXO3a antibody or without antibody (negative control) and the indicated primers. Reaction products were resolved by electrophoresis. C, reaction products were quantified by real time PCR. After analysis, a melting curve was performed to confirm the amplification of a single product.
showed the existence of a protein-DNA binding complex, suggesting other trans-factors may also bind to this region.

Because the chromatin environment modulates transcription factor binding, an issue that cannot be addressed by EMSA, ChIP assays were undertaken to determine whether FOXO3a was bound to the endogenous AR promoter when PI3K/Akt was blocked. LNCaP cells were treated with 20 μM LY294002 for 24 h. DNA-protein complexes in the LNCaP cells were cross-linked in situ, and the chromatin was isolated and sheared. Subsequently, the chromatin was immunoprecipitated with the anti-FOXO3a antibody, and the DNA was purified and sub-

FIGURE 5. Flow cytometric analysis of LNCaP cells. A, effects of AR siRNA on AR expression in LNCaP cells. LNCaP cells were transfected with AR siRNA or empty vector by electroporation. After 24 h, cell lysates were applied to the Western blot and probed with anti-AR or β-actin antibody. B, effects of AR and LY294002 on cell cycle and apoptosis induction in LNCaP cells. LNCaP cells were transfected with AR siRNA or empty vector. After 24 h, cells were treated with 20 μM LY294002 (LY) for 24 h. The cells then were stained with propidium iodide to detect cell cycle and apoptosis as described under “Experimental Procedures.” C, histograms of the cell cycle analysis. D, histograms of the sub-G1 population.
suggesting that this transcription factor indeed is bound by the anti-FOXO3a antibody caused more than 3-fold up-regulation (Fig. 4B). By using quantitative real time PCR, we found that the predicted size. By using quantitative real time PCR, we found that the anti-FOXO3a antibody caused more than 3-fold up-regulation (Fig. 4C), suggesting that this transcription factor indeed is bound in vivo to the regulatory portion of the AR gene. A single amplification product was evident in the melting curve following the real time PCR, again confirming specificity of the assay for the AR promoter. Taken together, these findings suggest that LY294002 treatment caused the recruitment of FOXO3a to the AR promoter consensus sites, suggesting FOXO3a induces AR expression by binding directly to the AR promoter region. Figs. 3 and 4 both demonstrated that the Akt downstream target substrate, FOXO3a, is able to enhance AR expression at the transcriptional level via binding to the AR promoter region at a specific DNA binding consensus site.

AR Plays Important Roles in LY294002-induced LNCaP Cell Apoptosis—What are the potential consequences after the Akt downstream target substrate, FOXO3a, exerts its effects on the increase of AR expression and AR transactivation? One possibility is that induced AR expression and AR transactivation might be able to protect LNCaP cells from apoptosis because of the loss of P13K/Akt activity. Early reports showed that the PI3K/Akt pathway was a dominant survival factor in LNCaP cells (19). Inhibition of PI3K activity by LY294002 would lead to the induction of apoptosis in androgen-deprived LNCaP cells, but the addition of androgen would rescue the cells from LY294002-induced apoptosis. It is therefore conceivable that the inhibition of PI3K/Akt by LY294002 might lead to the induction of apoptosis as well as the activation of the Akt downstream target, FOXO3a. The activated, nonphosphorylated form of FOXO3a would then translocate into the nucleus and be able to bind to the AR promoter, to enhance AR expression and AR transactivation after the cells were supplied with androgens. This could be one of the reasons that the addition of androgen can rescue LY294002-induced apoptosis. To test the above hypothesis, assuming AR can play protective roles in LNCaP cell apoptosis (20), we applied RNA interference strategy to reduce AR protein amounts, and we examined the subsequent effects on the LY294002-induced cell apoptosis. AR siRNA plasmid was constructed previously (12). As shown in Fig. 5A, Western blot analysis showed that AR siRNA significantly suppressed AR protein expression. By using flow cytometric assay with propidium iodide staining (Fig. 5B), we found that under normal growth medium culture conditions, the addition of LY294002 leads to cell arrest but not to cell death. It was shown that LY294002 increased cell G1 arrest from 56.3% (EtOH treatment control) to 88.0% (Fig. 5C). Most interestingly, knocking down AR by siRNA caused 51.0% of the cells to enter sub-G1 phase, and also caused an increase in G2 arrest in the remaining cells. Co-transfection of AR siRNA plus LY294002 treatment further induced cell apoptosis. As shown in Fig. 5D, 65.7% of cells underwent apoptosis, suggesting that AR existence in the cells plays an important protective role for LY294002-induced cell apoptosis.

DISCUSSION

Although the relationship between AR and Akt remains controversial, a variety of mechanisms has been suggested to account for how Akt influences AR signaling pathways. These include the following: 1) Akt directly interacts with AR and then suppresses AR activity (9–11, 21); 2) Akt directly interacts with AR and then enhances AR activity in a ligand-independent manner (22); 3) Akt indirectly enhances AR transactivation via the inhibition of GSK3β, the downstream substrate of PI3K/Akt, protein kinase A, and mitogen-activated protein kinase; GSK3β was found to suppress directly AR transactivation (23); and 4) Akt indirectly enhances AR transactivation by inhibiting GSK3β to positively regulate β-catenin (24). β-Catenin has been reported as a ligand-dependent AR co-activator (25). With different ligand requirements to influence AR activity, GSK3β and β-catenin may cooperate but also may act independently to modulate AR activity at different levels (23). How Akt influences AR may depend on cell physiological conditions. Recently, we found Akt either suppresses or enhances AR activity, depending on the LNCaP cell passage number (26). In this study, by using LNCaP cells with a passage number less than 35 for all the experiments, we confirmed that the PI3K/Akt inhibitor, LY294002, strongly enhanced AR expression and activity. It is unlikely that GSK3β and β-catenin play the dominant role; otherwise, LY294002 should reduce AR expression. In addition to our finding that Akt can directly suppress AR function, our current studies suggest that FOXO3a, a substrate of Akt, might be one of the mediators linking PI3K/Akt and AR. The FOXO3a binding consensus sequence has been reported as TGTGTTAC (27). Most interestingly, we found that the AR promoter contains a putative FOXO3a-responsive element located from −1290 to −1297 (5′-TGTTTTCG-3′). In this study, both EMSA and ChIP assays demonstrated that FOXO3a could directly bind to the AR promoter through this consensus sequence. This finding strongly supports our hypothesis that PI3K/Akt can communicate with AR via FOXO3a.

LNCaP is a PTEN-null prostate cancer cell line that contains constitutively active Akt. Accordingly, FOXO3a could be constitutively phosphorylated and inactivated as a result of PI3K/Akt signaling (6, 17). PI3K/Akt has been reported as a dominant growth factor-activated cell survival pathway in LNCaP cells (19). It is suggested that the inhibition of PI3K/Akt should result in cell apoptosis. Indeed, under serum-free medium culture conditions, LY294002 induced LNCaP cell apoptosis (19). However, androgen can rescue LNCaP from LY294002-induced apoptosis (19, 28). Here we found that AR plays a critical role for this...
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...their reagents. We thank Karen Wolf and Hector Avila for helpful reading of the manuscript. We also thank the members in Dr. Chang's laboratory for technical support and insightful discussion.

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J. Biol. Chem. 2005, 280:33558-33565.
doi: 10.1074/jbc.M504461200 originally published online August 1, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M504461200

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