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Glioma pathogenesis-related protein 1 induces prostate cancer cell death through Hsc70-mediated suppression of AURKA and TPX2

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ABSTRACT

In this study we report that expression of glioma pathogenesis-related protein 1 (GLIPR1) regulated numerous apoptotic, cell cycle, and spindle/centrosome assembly-related genes, including *AURKA* and *TPX2*, and induced apoptosis and/or mitotic catastrophe (MC) in prostate cancer (PCa) cells, including p53-mutated/deleted, androgen-insensitive metastatic PCa cells. Mechanistically, GLIPR1 interacts with heat shock cognate protein 70 (Hsc70); this interaction is associated with SP1 and c-Myb destabilization and suppression of SP1- and c-Myb-mediated AURKA and TPX2 transcription. Inhibition of AURKA and TPX2 using siRNA mimicked enforced GLIPR1 expression in the induction of apoptosis and MC. Recombinant GLIPR1-ΔTM protein inhibited AURKA and TPX2 expression, induced apoptosis and MC, and suppressed orthotopic xenograft tumor growth. Our results define a novel GLIPR1-regulated signaling pathway that controls apoptosis and/or mitotic catastrophe in PCa cells and establishes the potential of this pathway for targeted therapies.

Abbreviations: GLIPR, glioma pathogenesis-related protein 1; MC, mitotic catastrophe; PCa, prostate cancer; Hsc70, heat shock cognate protein 70; MEF, mouse embryonic fibroblasts; IP, immunoprecipitation.

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1. Introduction

Human glioma pathogenesis-related protein 1 (*GLIPR1*) is a p53 target gene that is downregulated in prostate cancer (PCa) (Ren et al., 2002, 2004). Enforced GLIPR1 expression in PCa cells led to growth suppression and/or apoptosis (Li et al., 2008; Ren et al., 2002, 2004; Satoh et al., 2003), whereas inactivation of *Glipr1*, predisposed mice to tumorigenesis (Li et al., 2008). Intraprostatic administration of the *GLIPR1* gene expressed by an adenoviral vector to PCa patients was found to induce tumor cell apoptosis and stimulate host immune response (Sonpavde et al., 2011). Molecular evidence of inactivation of GLIPR1 tumor suppressor activities was also found in several other malignancies (Aytekin et al., 2010; Tam et al., 2010; Xiao et al., 2011). Some mechanisms underlying GLIPR1-mediated cytotoxicity have been identified, including ROS-JNK signaling in R24, a TSU-Pr1-derived subline (Li et al., 2008), and CK1α–mediated β-catenin and c-Myc protein destruction in multiple prostate cancer cell lines (Li et al., 2011). Interestingly, our cDNA microarray data showed remarkable changes in mitotic (M)-phase genes (Figure 1A) that are not typical JNK or c-Myc targets, suggesting that additional signaling pathway may be involved in GLIPR1-induced cell cycle arrest and cell death.

Heat-shock cognate protein 70 (Hsc70; gene symbol, *HSPA8*) is a member of the HSP 70 family. It is constitutively expressed in most tissues and is involved in various housekeeping and chaperone functions [reviewed in (Bukau et al., 2006; Daugaard et al., 2007; Khalil et al., 2011; McMahon and Boucrot, 2011)]. Hsc70 can also promote cell survival (Florin et al., 2004). Hsc70 knockdown abolished VEGF-induced expression of phosphatidylinositol 3-kinase and Akt

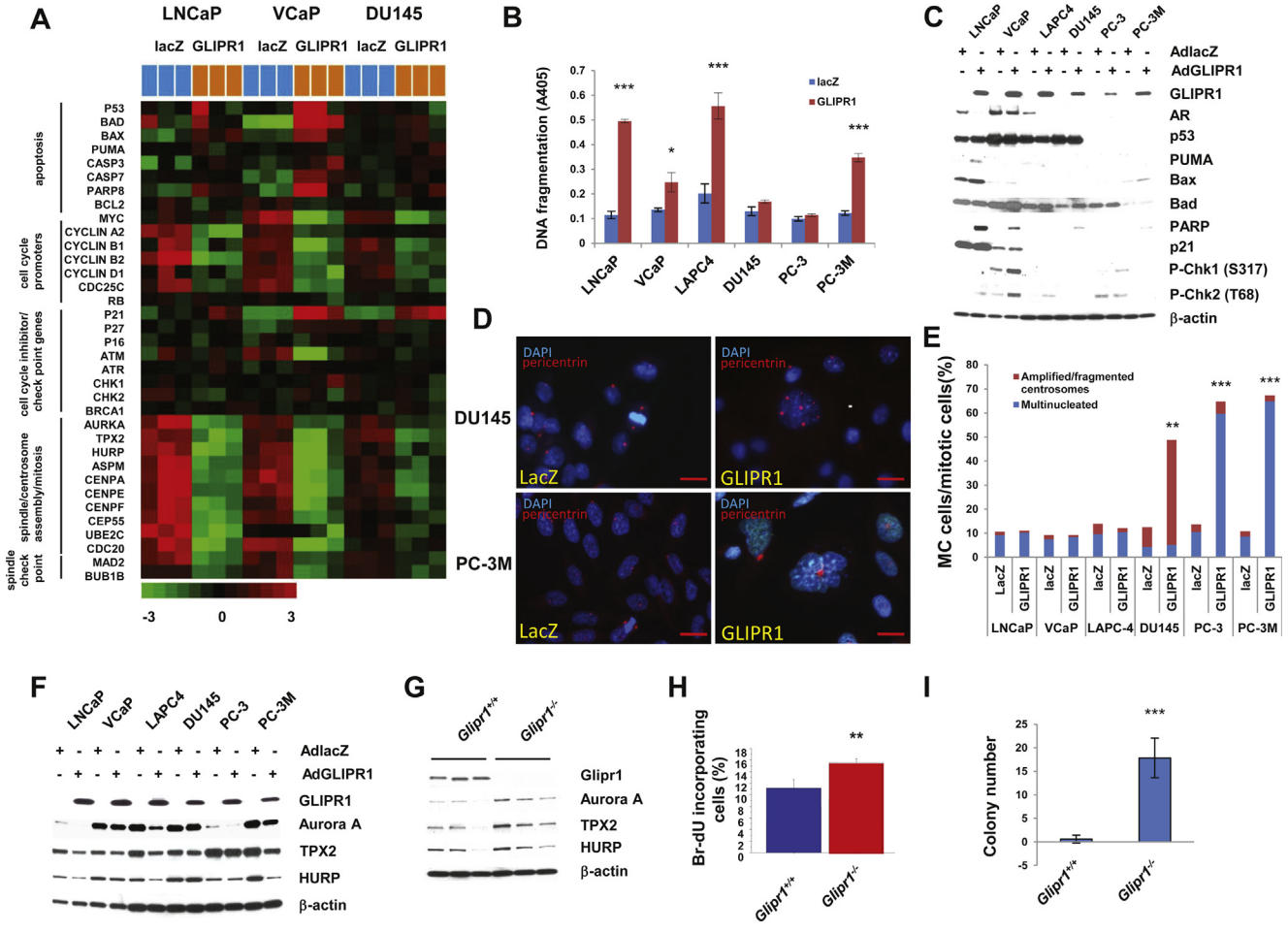

Figure 1 – GLIPR1 expression induces apoptosis and mitotic catastrophe (MC) in PCa cells in a context-dependent manner. (A–F) PCa cells were transduced with AdGLIPR1 or control AdlacZ for 48 h (A) cDNA microarray heat map summarizes the effect of GLIPR1 expression on apoptosis-, cell cycle-, and spindle/centrosome assembly-related genes in PCa cells. (B) DNA fragmentation assay. Error bars indicate SD. (C) Western blotting shows states of AR, p53, and proapoptotic proteins and cell cycle checkpoints. (D–E) Forty-eight hours after gene transduction, PCa cells were treated with 100 nM bortezomib for 2 h to arrest mitotic cells at the metaphase-to-anaphase transition and then labeled with pericentrin, a centrosomal marker, and DAPI. (D) Increased mitotic cells with multipolar/fragmented centrosomes (top right) and giant multinucleated cells (bottom right) were observed. Scale bars = 15 μm. (E) Quantitation of MC frequency. (F) Western blotting shows GLIPR1 downregulation of Aurora A, TPX2, and HURP, a substrate of Aurora A. (G–I) Analysis of *Glipr1*$^{+/+}$ and *Glipr1*$^{-/-}$MEFs. Loss of *Glipr1* in *Glipr1*$^{-/-}$MEFs results in higher expression of Aurora A, TPX2, and HURP (G) and in significantly increased cell proliferation as measured by BrdU incorporation assay (H) and colony formation (I). Error bars indicate SD. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ in all panels.

phosphorylation (Shiota et al., 2010) and resulted in marked cell death in various cell types (Rohde et al., 2005).

Aurora A (gene symbol, *AURKA*) is a serine/threonine protein kinase that plays a critical role in spindle and centrosome assembly. Aurora A deregulation has been found in a variety of cancer types [reviewed in (Asteriti et al., 2010; Gautschi et al., 2008; Saeki et al., 2009; Vader and Lens, 2008)]. Its overexpression overrides the mitotic spindle assembly checkpoint, yields polyploid cells with supernumerary centrosomes, and leads to genetic instability and resistance to cancer therapeutic treatments (Anand et al., 2003; Dutertre and Prigent, 2003; Jiang et al., 2003; Meraldi et al., 2002). The kinase activity of Aurora A requires autophosphorylation of the Thr288 in the catalytic domain, and it is counteracted by PP1 phosphatase. Binding of targeting protein for Xenopus kinase-like protein 2 (TPX2) to Aurora A induces a conformation change that makes Thr288 inaccessible to the phosphatase, which stabilizes Aurora A in the active conformation (Bayliss et al., 2003; Giubettini et al., 2011; Tsai et al., 2003). Amplification of chromosome 20 q, where both Aurora A and TPX2 reside, often occurs in tumors (Asteriti et al., 2010; Gautschi et al., 2008; Vader and Lens, 2008).

In addition to their roles in mitosis, Aurora A and TPX2 are implicated in antiapoptotic functions and resistance to cancer therapeutic agents (Dar et al., 2008; Evans et al., 2008; Moss et al., 2009; Scharer et al., 2008; Sumi et al., 2011; Zhang et al., 2008). Specific inhibitors targeting the Aurora A–TPX2 complex are being aggressively developed and tested alone or in combination with other cancer therapeutic agents [reviewed in (Andrews, 2005; Asteriti et al., 2010; Cheung et al., 2011; Dar et al., 2010; Gautschi et al., 2008; Kelly et al., 2011; Perez Fidalgo et al., 2009)].

Here we report that GLIPR1 interacts with Hsc70 and that this interaction is associated with disruption of Hsc70's chaperone protection function for SP1 and c-Myb, resulting in suppression of *AURKA* and *TPX2* transcription and induction of cell context-dependent apoptosis and/or mitotic catastrophe (MC), a mechanism of cell death during aberrant mitosis (Portugal et al., 2010; Singh et al., 2010; Vakifahmetoglu et al., 2008) in PCa cells. Administration of recombinant GLIPR1-ΔTM protein inhibited AURKA and TPX2 expression, induced apoptosis and MC, and suppressed growth of human xenograft prostate tumors.

2. Materials and methods

2.1. Cell lines

LNCaP, VCaP, DU145, and PC-3 (ATCC), LAPC-4 (from Dr. Charles Sawyers of the University of California at Los Angeles), and PC-3M (from Dr. Isaiah J. Fidler of MD Anderson Cancer Center) were validated by STR DNA fingerprinting with the AmpFℓSTR Identifiler kit (Applied Biosystems) in MD Anderson's Characterized Cell Line Core.

2.2. cDNA microarray analysis

Total RNA was isolated from LNCaP, VCaP, and DU145 PCa cells transduced with AdGLIPR1 or control AdlacZ. cDNA microarray analysis was performed, and the data were normalized and statistical analysis performed as previously described (Kim et al., 2010). Microarray data were deposited in the GEO database (accession number GSE32367).

2.3. Quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis

Quantitative RT-PCR was performed as described previously (Li et al., 2008). Specific Taqman probes and primers for Q-RT-PCR: HSPA8: forward: 5′CCGAACCACTCCAAGCTATG3′, reverse: 5′CATCAAATCTGCGTCCAATGAG3′, probe: 56-FAM /CCGATCAACCGTTCAGTGTCCGTAAA/3IABlk_FQ; AURKA: forward: 5′ACACCCAAAAGAGCAAGC3′, reverse: 5′AGAGGGC GACCAATTTCA3′, probe: 56-FAM/AAAGTCTTCCAAAGCC-CACTGCC/3IABlk_FQ; TPX2: forward: 5′TGCAGAAAGG GGAGGTG3′, reverse: 5′GTCAGTCTCCAAGCAGAAAG3′, probe: 56-FAM/ATGGTGTCAAAATGAGGCAAGGGAAG/3IABlk_FQ.

2.4. Western blotting analysis

Antibodies against GLIPR1 were as described previously (Ren et al., 2002). AR (sc-816), p53 (sc-126), c-Myc (sc-40), TPX2 (sc-81413), and SP1 (sc-420) were all from Santa Cruz Biotechnology; Hsc70 (ab51052) and fibrillarin (ab4566) were from Abcam; Bax (#5023), Bad (#9292), p21 (#2947), cleaved PARP (#9541), P-Chk1 (S317, #2344), P-Chk2 (T68, 2661), TCF1(#2203), active β-catenin (#4270) and NuMA (#8967) were all from Cell Signaling; Aurora A (A1231), β-actin (A1978), and DLGAP5 (HURP, SAB4503702) were from Sigma; and c-Myb (#05–175) was from Millipore.

2.5. Immunofluorescence

The immunofluorescence of the target proteins was evaluated by using a Nikon Eclipse 90i image analysis system with NIS-Elements AR software (version 3.0). Hsc70 and GLIPR1 colocalization was analyzed by using deconvoluted digital images generated by AutoQuant software (Media Cybernetics). Antibodies: GLIPR1, as described above, Hsc70 (Abcam, ab 19136), γ-tubulin (Abcam, ab11316), pericentrin (ab4448), γ-tubulin (ab11316), Abcam; Numa (#8967), phosphor-histone 3 (Ser10, #9706) and caspase 3 (#9664), Cell Signaling; α-tubulin (11H10) Sigma.

2.6. Quantitation of mitotic catastrophe and apoptosis

To evaluate the morphological features of mitotic catastrophe, PCa cells were cultured on coverslips placed in 24-well culture plates. The multinucleated cells and mitotic cells with multiple/fragmented centrosomes were counted on at least 6 separate coverslips for each cell lines under given experimental condition after DAPI nuclear staining and pericentrin immunofluorescence staining. From each coverslip, thirty to 50 images were acquired automatically using the Nikon eclipse 90i image analysis system via two fluorochrome channels for the DAPI and pericentrin immunofluorescence staining under a 20x microscopic objective. Measurement of the total cells was performed automatically on DAPI images using the NIS Element software (version 3.0). Counting of total mitotic cells including bipolar mitotic cells, mitotic cells with multiple/fragmented centrosomes and multinucleated cells (considered as mitotic cells with unfinished cytokenesis) were performed manually on each images in which both

DAPI and pericentrin staining were merged. The percentages of the mitotic cells with multiple/fragmented centrosomes or with multiple nuclei in the total mitotic cell population as well as the percentages of apoptotic cells in the total cell population were recorded separately.

2.7. ChIP assays

ChIP assays were performed by using a Millipore ChIP kit. The input and immunoprecipitated DNAs were subjected to PCR using primers corresponding to the −103 to +181 base pairs of the AURKA promoter (forward: 5′CCGTTGGCTCCACCACT T3′; reverse: 5′CCTCGTCCGCCACTGAGA3′) and primers corresponding to the −244 to +162 base pairs of the TPX2 promoter (forward: 5′ATGCAGGCGACTCCATGTTA3′; reverse: 5′ GCCTA GTCGAATGCACCAAT3′). Antibodies (ChIP grade): trimethyl-histone H3K4 (ab8580), c-Myb (ab45150), Abcam; trimethyl-histone H3K27 (#07–360), acetyl-histone H3K27 (#07–449), Millipore; RNA polymerase II (059K0604), Sigma; SP1 (sc-14027) and normal rabbit IgG, Santa Cruz.

2.8. Promoter construction and luciferase assay

The AURKA-luc was purchased from SwitchGear Genomics. TPX2 promoter was amplified by PCR using genomic DNA from normal prostate tissue and primers: forward: 5′ACTCATCGCCTCAAGTGATCT3′, reverse: 5′CCCTAGCTGCTC CCTCTTTT3′. The purified PCR product was phosphorylated and then cloned to pGL4-Basic vector (Promega) using the Asp718 site to generate TPX2-luc. The construct was confirmed by DNA sequencing.

2.9. Protein stability analysis

PC-3M cells were transfected with pcDNA-GLIPR1 or control pcDNA empty vector or with HSPA8 siRNA or negative control siRNA. Cells were grown in complete medium for 48 h and then treated with cycloheximide (100 μg/ml) in serum-free medium for the indicated time. After Western blotting, SP1 and c-Myb protein bands were measured by using computer-assisted densitometry, and the half-life was determined by linear fitting of the densitometry data.

2.10. Generation of recombinant GLIPR1-ΔTM protein

GLIPR1-ΔTM coding sequence was obtained from normal prostate tissue by RT-PCR using specific primers (forward: 5′CCCAAGCTTGCAAATATTTTGCCAGAT3′, reverse: 5′ATAGT TTAGCGGCCGCTCTGTTACGTGGATATAT3′). The PCR product was digested with HinIII and NotI and inserted into pSectag/Hygro2 HinIII and NotI sites to generate pSec-GLIPR1-ΔTM. Conditioned medium from 293 Freestyle cells transfected with pSec–GLIPR1-ΔTM was collected and centrifuged, and GLIPR1-ΔTM was purified using Ni-NTA agarose.

2.11. GLIPR1-ΔTM protein uptake

The Alexa Fluor 488–conjugated GLIPR1-ΔTM was applied to cultured PCa cells for 24 h in RPMI 1640 containing 0.5% fetal bovine serum. After that treatment, cells were rinsed with PBS and fixed in 4% formaldehyde for 5 min at room temperature. Afterward, they were counterstained with DAPI, followed by confocal microscopic analysis.

2.12. Orthotopic PCa model

The human PCa cells PC-3 and VCaP were transduced with lentivirus stably expressing luciferase (Luc). The in vivo experiments were conducted with an institution approved aminal protocol (IACUC protocol #: 10-07-12032). Eight-nine week-old nude mice (Taconic Farms, Inc.) were randomly divided into three groups. Each group contained 9-14 mice. Mice were housed in compliance with institutional and governmental requirements and approved by M.D. Anderson Cancer Center's Animal Care and Use Committee. Aliquots of 1.5×10^6 PC-3–Luc cells and 3.0×10^6 VCaP–Luc cells in 25 μl PBS were injected directly into the right lobe of the dorsolateral prostate to induce orthotopic tumors. The tumors were allowed to grow for 7 days for PC-3–Luc and 14 days for VCaP–Luc before treatment. The control group was treated with PBS, and the experimental groups with different concentrations of GLIPR-ΔTM protein (40 or 80 μg/dose) intravenously (IV) through the tail vein for VCaP model and intraperitoneally (IP) for PC-3 model three times per week for 2 weeks. Tumor growth was monitored by the measurement of luminescence signal using Xenogen IVIS200 (Caliper Life Sciences) and by the measurement of tumor wet weight. Data presented are the average of at least two independent experiments.

2.13. Statistical analysis

Unless specifically indicated, unpaired *t* testing was used in the experiments in which probability was determined.

2.13.1. Theory

There is an urgent need to define new "druggable" molecular pathways and to develop therapeutic approaches for apoptosis-resistant cancer cells. Previous studies have demonstrated that GLIPR1 can suppress growth and induce apoptosis in PCa cells in vitro and can inhibit tumor growth and metastasis in vivo, however, the mechanisms underlying its tumor suppressor function have not been fully illustrated. Its relationship with Hsc70, AURKA and TPX2 is unknown. This study revealed that GLIPR1 can induce apoptosis and/or MC in PCa cells, including p53-mutated/deleted, androgen-insensitive metastatic prostate cancer (PCa) cells, a category of therapy-resistant PCa. Our results further showed that GLIPR1's binding to chaperone protein Hsc70 is associated with disruption of an oncogenic signaling pathway that involves an interaction of Hsc70 with SP1 and c-Myb and activation of AURKA and TPX2 transcription. AURKA and TPX2 are two important therapeutic targets for cancer and other malignancies, including PCa. Therapeutic testing of GLIPR1-ΔTM in cultured PCa cells and orthotopic PCa xenografts validated this mechanism of action and demonstrated the therapeutic potential of GLIPR1-based protein therapy.

3. Results

3.1. *GLIPR1 inhibits AURKA and TPX2 and induces apoptosis and/or MC in human PCa cells*

From cDNA microarray analysis using samples from the PCa cell lines LNCaP, VCaP, and DU145 transduced with adenoviral vector expressing GLIPR1 or control lacZ, we found that overexpression of GLIPR1 in PCa cells led to upregulation of multiple proapoptotic genes and downregulation of the prosurvival gene *BCL2* and cell cycle-promoting genes (Figure 1A), supporting the results of previous studies (Li et al., 2008, 2011). Remarkably, multiple spindle/centrosome assembly-related genes, including *AURKA* and *TPX2*, were downregulated (Figure 1A).

We analyzed the effects of GLIPR1 on apoptosis and MC using p53 wild type, androgen-sensitive LNCaP cells; p53-mutated, androgen-sensitive VCaP and LAPC4 cells; and p53-mutated or deleted, androgen-insensitive DU145, PC-3, and PC-3M cells. Interestingly, GLIPR1 induced DNA fragmentation, an indication of apoptosis, in all androgen-sensitive and PC-3M PCa cell lines but had limited apoptotic effect on DU145 and PC-3 cells (Figure 1B). Of note, GLIPR1-induced activation of cell cycle checkpoints (indicated by increased p21, P-Chk1, and P-Chk2) and proapoptotic genes (indicated by higher PUMA, Bax, Bad, and p21 expression) predominantly occurred in androgen-sensitive LNCaP, VCaP, and LAPC4 cells, whereas the levels of these genes were substantially reduced in control or GLIPR1-transduced androgen-insensitive DU145, PC-3, and PC-3M cells (Figure 1C). Thus, with the exception of PC-3M cells, GLIPR1-stimulated apoptosis correlated with GLIPR1 induction of proapoptic genetic pathways in androgen-sensitive PCa cells.

Immunofluorescence analysis showed that GLIPR1 expression led to increased mitotic cells with multiple and/or fragmented centrosomes and multinucleated cells—morphological

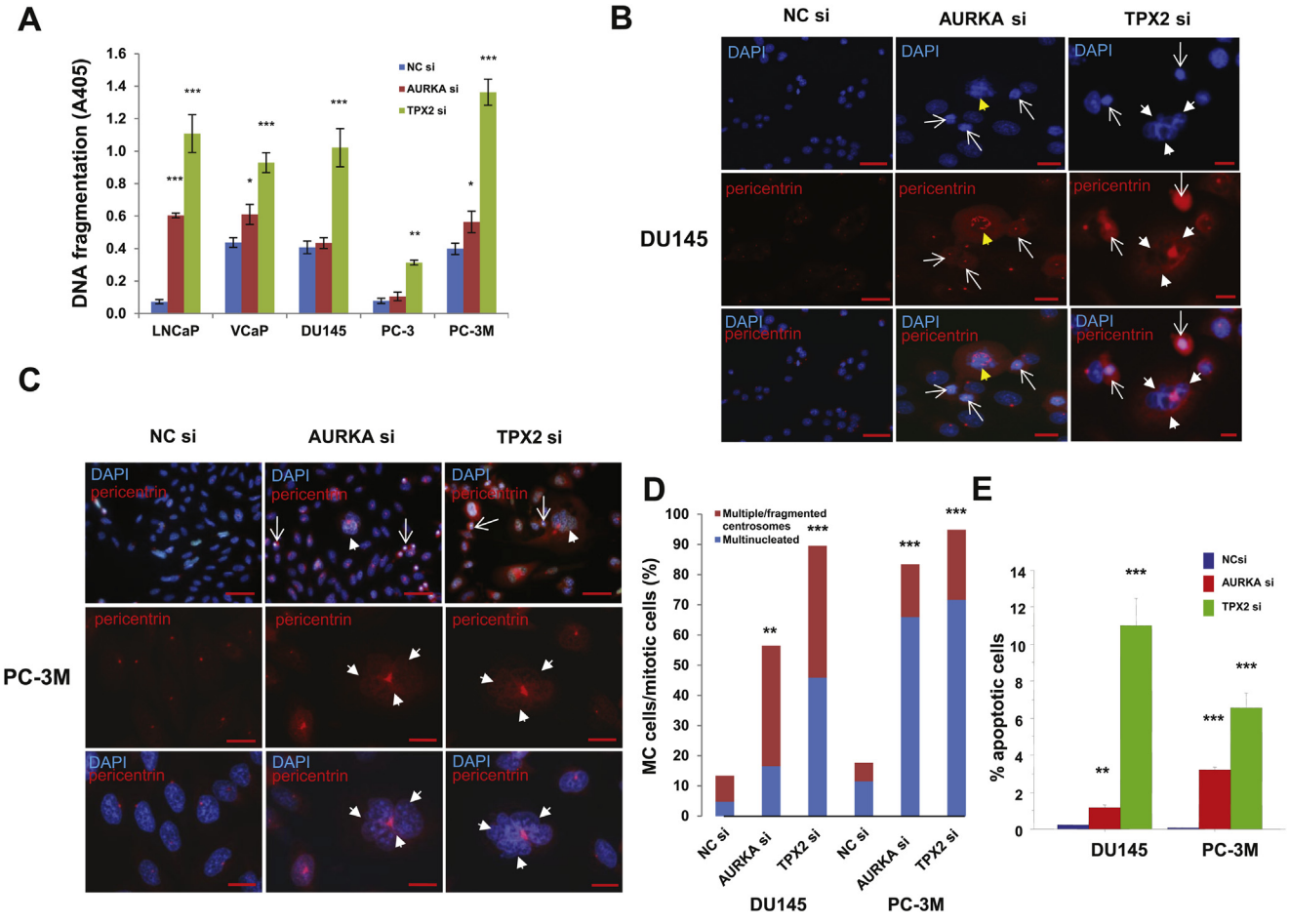

Figure 2 – Inhibition of AURKA or TPX2 induces apoptosis and/or MC in PCa cells. (A) DNA fragmentation assay shows that knockdown of *AURKA* induced apoptosis in LNCaP, VCaP, and PC-3M cells and that knockdown of *TPX2* induced extensive apoptosis in all PCa cell lines tested. Error bars indicate SD. (B–C) DAPI nuclear staining (blue) and pericentrin immunofluoresence (red) demonstrate induction of apoptosis (arrows) and MC (yellow arrowheads for mitotic cells with multiple/fragmented centrosomes and white arrowheads for multinucleated cells) by AURKA or TPX2 siRNA in DU145 (B) and PC-3M (C) cells. Scale bars = 100 μm (left columns in B and top row in C) and bars = 12 μm (middle and right columns in B and middle and bottom rows in c). (D–E) quantitative comparison of MC frequency (D) and the fraction of monopolar apoptotic cells (E), which demonstrate condensed nuclei with irregular centrosomal pole as identified by DAPI and pericentrin labeling (arrows in B and C). *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ in all panels when compared with control NC si.

features that have been associated with MC (Vakifahmetoglu et al., 2008) in the androgen-insensitive cell lines (Figure 1D). The mitotic cells with multiple/fragmented centrosomes contained irregularly disseminated chromosomes (Figure S1); invariably disorganized mitotic spindles (Figure S2) and mismatched spindle poles and centrosomes (Figure S3). Some of these cells expressed activated caspase 3 (Figure S4), indicating their commitment to mitotic death. The multinucleated cells were characterized by the presence of cluster of micronuclei and enlarged single, multiple or fragmented centrosomes (Figure S5). Quantitatively, p53-mutated or deleted and androgen-insensitive prostate cell lines DU145, PC-3, and PC-3M had significantly higher MC frequency in response to enforced GLIPR1 expression than did the androgen-sensitive LNCaP, VCaP, and LAPC4 cells (Figure 1E). To probe the association between GLIPR1 expression and downregulation of spindle/centrosome assembly-related genes, we initially confirmed the downregulation of AURKA and TPX2 at the mRNA (Figure S6) and protein (Figure 1F) levels. Further examination of *GLIPR1*$^{+/+}$ and *GLIPR1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) showed higher Aurora A and TPX2 protein levels (Figure 1G); a higher proliferation rate, indicated by Br-dU incorporation (Figure 1H); and greater colony formation in *GLIPR1*$^{-/-}$MEFs (Figure 1I).

3.2. *Knockdown of AURKA or TPX2 – mimics GLIPR1 overexpression in the induction of apoptosis and MC in PCa cells*

To evaluate the role of AURKA and TPX2 in PCa, we knocked down *AURKA* or *TPX2* expression by transfection of either AURKA or TPX2 siRNA into PCa cells, followed by apoptosis and MC analysis. Consistent with previous studies in other cancer types that showed AURKA or TPX2 inhibition induced apoptosis and spindle assembly disruption (Chowdhury et al., 2012; Evans et al., 2008; Warner et al., 2009), knockdown of AURKA or TPX2 induced apoptosis and MC in PCa cells, mimicking GLIPR1 overexpression. As shown in Figure 2, AURKA inhibition induced moderate levels of apoptosis in LNCaP, VCaP, and PC-3M but had minor effect on DU145 and PC-3 cells (Figure 2A), a pattern mimicking apoptosis induced by enforced GLIPR1 expression (Figure 1B). Remarkably, TPX2 inhibition caused extensive apoptosis in all five tested PCa cell lines (Figure 2A), indicating that in addition to regulation of AURKA, TPX2 may have AURKA-independent pro-survival activities. Moreover, inhibition of AURKA or TPX2 induced marked MC in p53-mutated or deleted, androgen-insensitive DU145 and PC-3M PCa cell

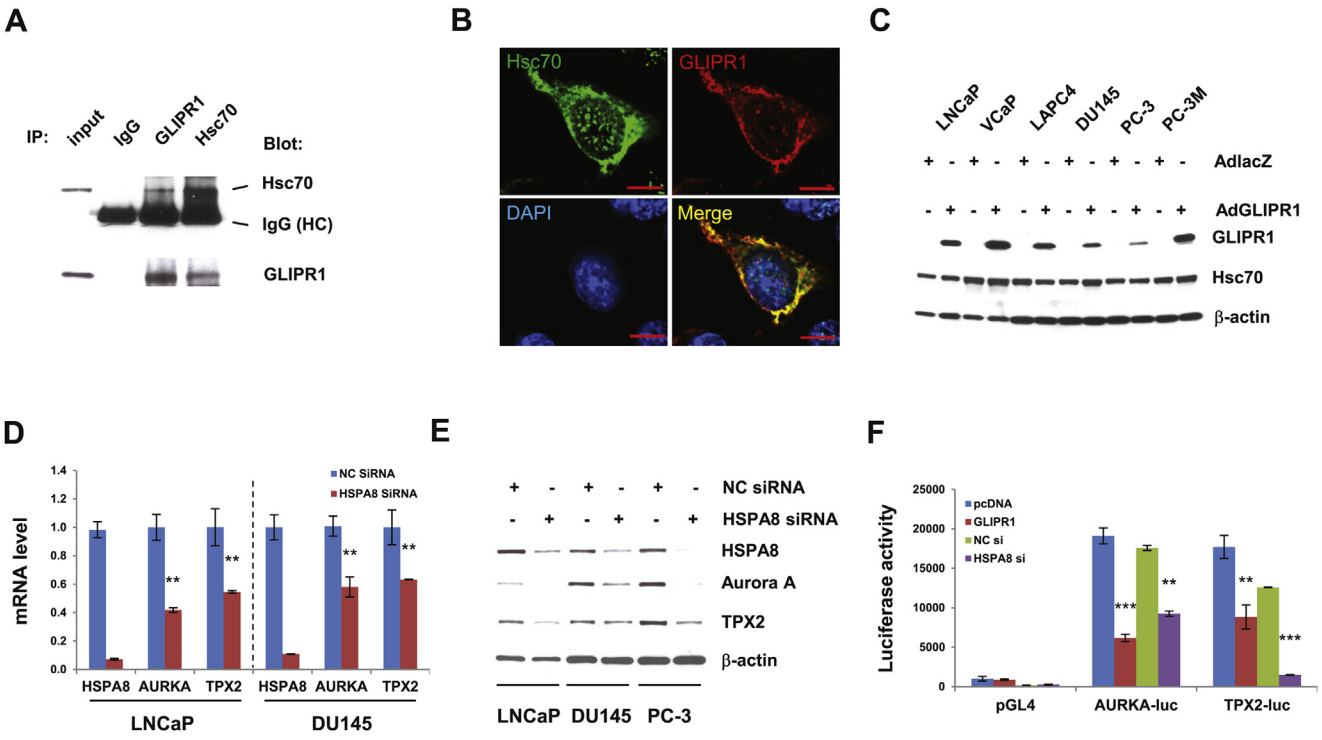

Figure 3 – GLIPR1 suppresses AURKA and TPX2 transcription through interaction with heat shock cognate 71-kD protein (Hsc70). (A) DU145 cells were transduced with AdGLIPR1 for 48 h. Immunoprecipitation (IP) was performed using anti-GLIPR1 or Hsc70 antibodie or with control normal IgG. (B) Deconvoluted immunofluorescence images show co-localization of GLIPR1 (red) and Hsc70 (green) in DU145 cells 48 h after AdGLIPR1 transduction. Scale bars = 5 μm (C) Hsc70 protein levels in the presence or absence of enforced GLIPR1 expression. (D) Real-time RT-PCR results show decreased AURKA and TPX2 mRNA levels in HSPA8 siRNA-transfected PCa cells. (E) Western blots show reduced Aurora A and TPX2 proteins in HSPA8 siRNA-transfected PCa cells. (F) Luciferase reporter assay results show significantly reduced AURKA and TPX2 promoter activities by either enforced GLIPR1 expression or Hsc70 knockdown in LNCaP cells. Error bars in (D) and (F) indicate SD. $^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$ in all panels when compared with the corresponding control.

lines (Figure 2B–D). Monopolar apoptotic cells were also observed in AURKA or TPX2 siRNA-treated DU145 and PC-3M cells (Figure 2E), supporting the results from DNA fragmentation analysis. These data demonstrate that AURKA and TPX2 are crucial regulators of both cancer cell survival and mitosis.

3.3. *GLIPR1 interacts with Hsc70, and GLIPR1 overexpression or Hsc70 knockdown leads to transcriptional suppression of AURKA and TPX2*

To determine the mechanism underlying GLIPR1-induced downregulation of AURKA and TPX2, we conducted mass spectrometric analysis for GLIPR1 protein binding partners. These experiments identified Hsc70 as one of GLIPR1-binding proteins (Supplemental Table). The interaction of GLIPR1 and Hsc70 was further validated by co-immunoprecipitation (IP) with either GLIPR1 or Hsc70 antibody (Figure 3A) and by subcellular co-localization (Figure 3B). Hsc70 expression was slightly changed in either direction in GLIPR1-overexpressing PCa cells (Figure 3C), however, knockdown of Hsc70 with HSPA8 siRNA effectively inhibited AURKA and TPX2 mRNA and protein expression (Figure 3D–E), a phenomenon mimicking GLIPR1 overexpression. These results established a positive association between Hsc70 expression and Aurora A and TPX2 expression and also suggested that the interaction of GLIPR1 and Hsc70 may have an inhibitory effect with regard to Hsc70-mediated positive regulation of AURKA and TPX2 expression.

Luciferase assays proved that overexpression of GLIPR1 or inhibition of HSPA8 indeed led to significant suppression of AURKA and TPX2 promoter activities (Figure 3F and S7).

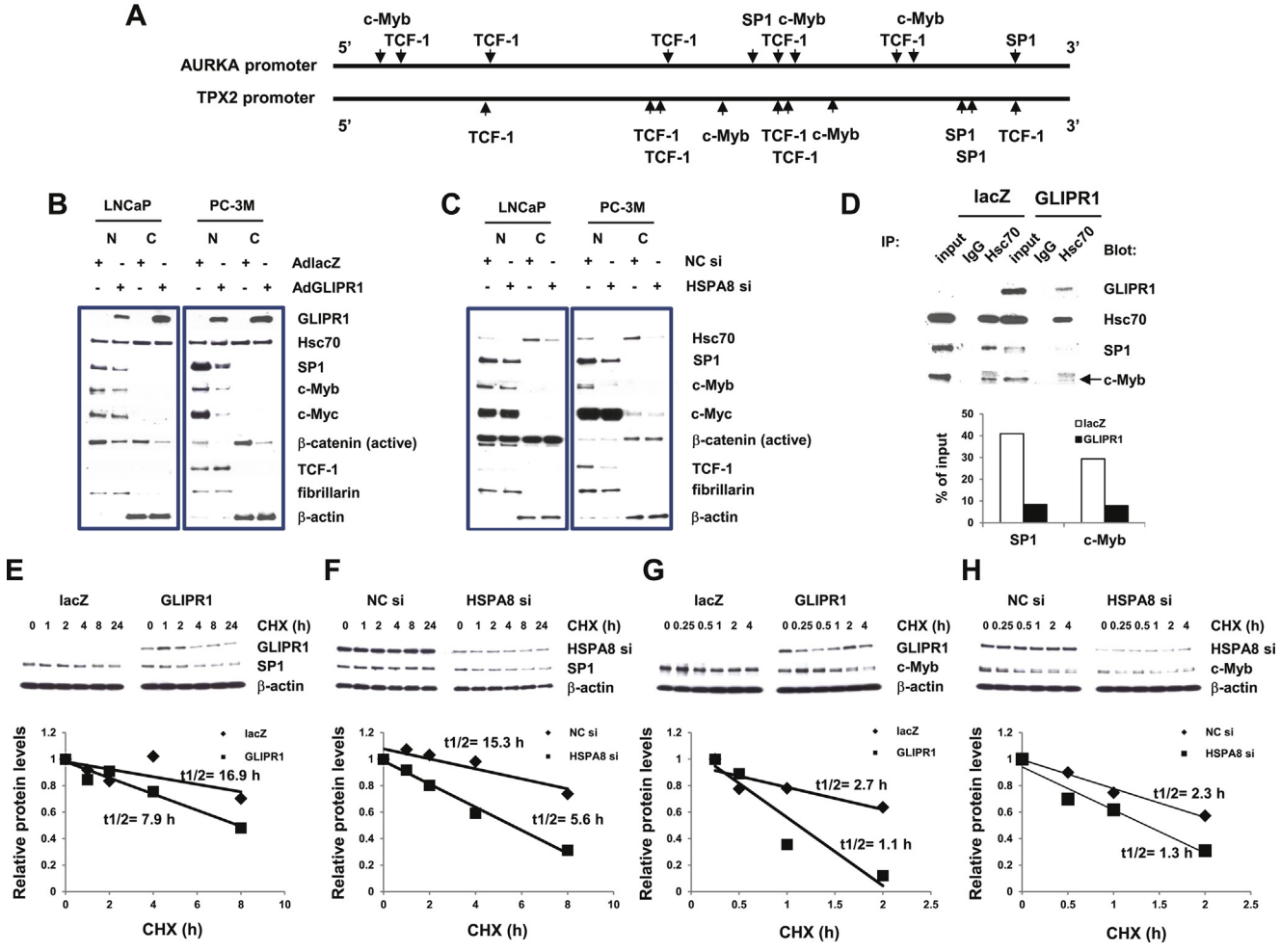

Figure 4 – GLIPR1 expression or Hsc70 inhibition diminishes Hsc70 chaperone protection of SP1 and c-Myb and decreases protein stability of SP1 and c-Myb. (A) Diagram illustrates transcription factor (TF) binding sites that appear in both AURKA and TPX2 promoters. Nuclear and cytosolic fractionation was performed using GLIPR1-transduced (B) or HSPA8 siRNA-transfected (C) PCa cells and a nuclear extract kit (Active Motif). Relative levels of selected TFs were determined by Western blotting analysis. (D) Analysis of Hsc70 IP complex for states of Hsc70 binding to SP1 and c-Myb in the presence and absence of enforced GLIPR1. (E–H) Quantitative analyses were performed to determine the effects of enforced GLIPR1 expression or Hsc70 inhibition on SP1 (E–F) and c-Myb (G–H) protein stability. Top panels are representative gel images, and bottom panels are quantitative data from an average of three experiments.

3.4. *GLIPR1-induced transcriptional suppression of AURKA and TPX2 involves association of SP1 and c-Myb with GLIPR1–Hsc70 complex and regulation of SP1 and c-Myb protein stability*

3.4.1. *GLIPR1 expression or Hsc70 inhibition diminishes Hsc70 chaperone protection of SP1 and c-Myb*

The finding of transcriptional suppression of AURKA and TPX2 by overexpression of *GLIPR1* or knockdown of *HSPA8* led us to hypothesize that Hsc70 may promote AURKA and TPX2 activities by chaperone protection or stabilization of specific transcription factors (TFs) that stimulate AURKA and TPX2 transcription, and also that GLIPR1's interaction with Hsc70 disrupts this protective, stabilizing function. Since knockdown of Hsc70 suppressed AURKA and TXP2 transcription simultaneously, we considered Hsc70 as affecting AURKA and TPX2 through one or more common TFs. A motif search of AURKA and TPX2 promoters using DNASIS MAX (Hitachi Genetic Systems) yielded three common TFs: SP1, c-Myb, and TCF-1. Each has multiple binding sites on both promoters (Figure 4A). Analysis of nuclear and cytoplasmic fractions of GLIPR1-enforced or HSPA8 siRNA-transfected PCa cells revealed that SP1 and c-Myb were downrgulated in both GLIPR1-enforced and HSPA8 siRNA-transfected PCa cells, whereas TCF-1 was downregulated by HSPA8 siRNA but not by GLIPR1 (Figures 4B and 4C). Since our previous study (Li et al., 2011) showed that GLIPR1 suppresses cell cycle activities through targeted destruction of c-Myc and β-catenin, we also included c-Myc and β-catenin in this analysis. The results showed that only GLIPR1 overexpression but not Hsc70 knockdown led to suppression of c-Myc and β-catenin (Figures 4B and 4C). These results led us to consider that SP1 and c-Myb, but not TCF-1, β-catenin and c-Myc, might mediate the transcription of AURKA and TPX2 and be subjected to regulation by association with a GLIPR1–Hsc70 complex. To gain insight into the mechanism for how Hsc70 affects SP1 and c-Myb, we determined: (1) the possibility of direct binding of Hsc70 to SP1 or c-Myb; (2) the effects of GLIPR1 on Hsc70's binding to SP1 and c-Myb; (3) the effects of GLIPR1 and Hsc70 on the protein stability of SP1 and c-Myb.

Consistent with a previous report that identified Hsc70 as an SP1-binding protein by using a yeast two-hybrid system (Gunther et al., 2000), we co-precipitated SP1 with Hsc70 in our Hsc70 IP experiment (Figure 4D); we similarly co-precipitated c-Myb with Hsc70. An important finding is that in the presence of GLIPR1, the ratio of SP1 and c-Myb to Hsc70 in the Hsc70 IP complex were markedly reduced (Figure 4D), suggesting that the binding of GLIPR1 to Hsc70 disrupts Hsc70's binding to SP1 and c-Myb.

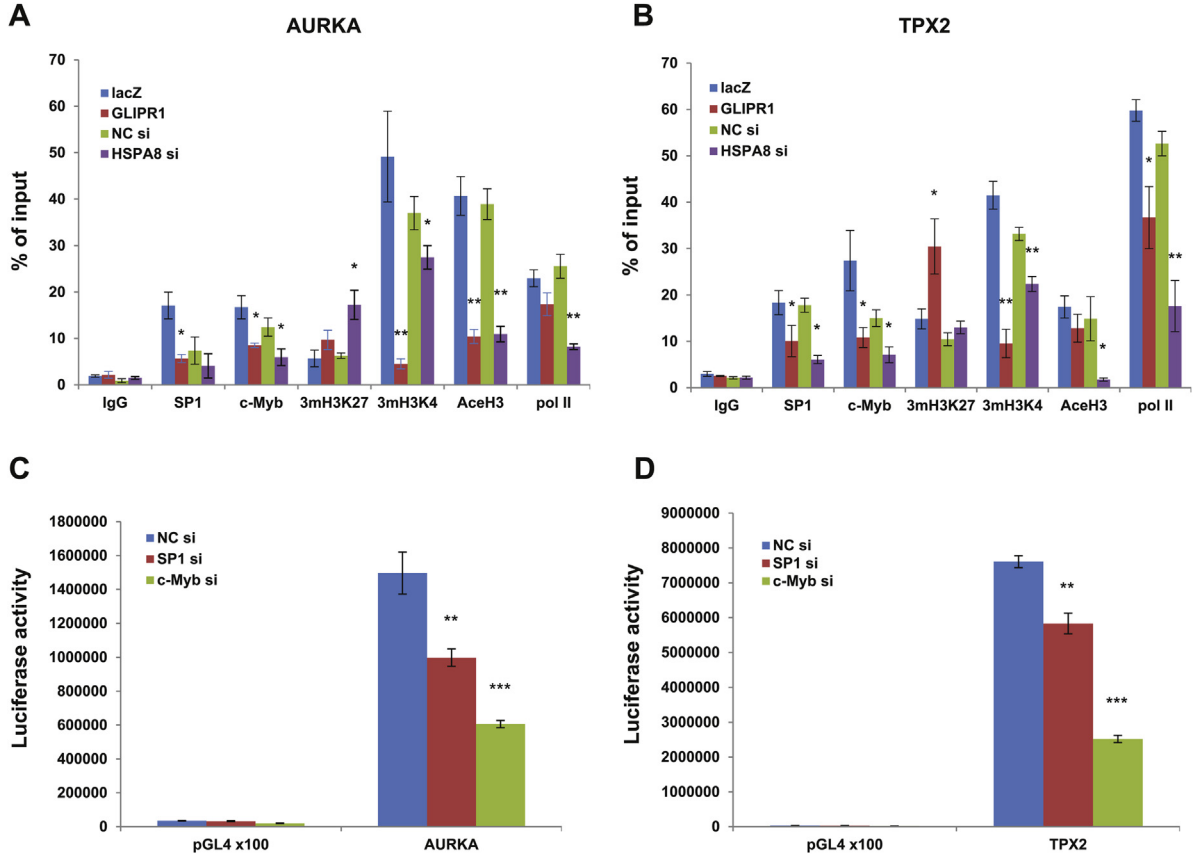

Figure 5 – Downregulation of SP1 and c-Myb is responsible for the GLIPR1 expression- or Hsc70 inhibition-induced suppression of AURKA and TPX2 promoter activities. (A–B) Results of ChIP assays show activities of SP1, c-Myb, and selected chromatin modifiers on AURKA (A) and TPX2 (B) promoters in GLIPR1-enforced and in HSPA8 siRNA-transfected PC-3M cells. Error bars indicate SE. (C–D) Luciferase reporter assays for AURKA (C) and TPX2 (D) promoters in SP1- and c-Myb siRNA-transfected PC-3M cells. Error bars indicate SD. *$p < 0.05$; **$p < 0.001$; ***$p < 0.0001$ in all panels when compared with the corresponding control.

3.4.2. *GLIPR1 expression or Hsc70 inhibition decreases protein stability of SP1 and c-Myb*

To determine the effect of reduced Hsc70 binding to SP1 and c-Myb, we performed protein stability analyses for SP1 and c-Myb, using cell lysates from GLIPR1-transduced or HSPA8 siRNA-transfected PC-3M cells. Both GLIPR1 overexpression and Hsc70 inhibition markedly reduced SP1 and c-Myb protein half-lives (Figure 4E–H). Thus, our data demonstrated that interaction of GLIPR1 with Hsc70 disrupts Hsc70's chaperone protection of SP1 and c-Myb, leading to destabilization of SP1 and c-Myb proteins.

3.4.3. *Downregulation of SP1 and c-Myb is responsible for the GLIPR1 expression- or Hsc70 inhibition-induced suppression of AURKA and TPX2 promoter activities*

To examine the role of SP1 and c-Myb in the transcription of AURKA and TPX2, we performed chromatin IP (ChIP) and luciferase reporter assays for AURKA and TPX2 promoters. The ChIP assays indicated that both *GLIPR1* overexpression and *HSPA8* inhibition led to significantly reduced SP1 and c-Myb binding, increased levels of the trimethyl-histone H3 (Lys27) (3mH3K27) inactivation mark; decreased levels of trimethyl-histone H3 (Lys4) (3mH3K4) and acetyl-histone H3 (Lys27) (AceH3) activation marks; and RNA polymerase II (pol II) on AURKA and TPX2 (Figures 5A and B) promoters. The luciferase reporter assays further demonstrated that SP1 and c-Myb positively regulate *AURKA* and *TPX2* transcription, since suppression of *SP1* or *c-MYB* by siRNA led to significantly reduced *AURKA* and *TPX2* promoter activities (Figures 5C and D).

3.5. *GLIPR1 is secreted, internalized by PCa cells, downregulates Aurora A and TPX2, induces apoptosis and MC in PCa cells, and suppresses tumor growth in vivo*

3.5.1. *GLIPR1 secretion, internalization and induction of apoptosis and MC in PCa cells*

GLIPR1 is a member of the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (CAP)

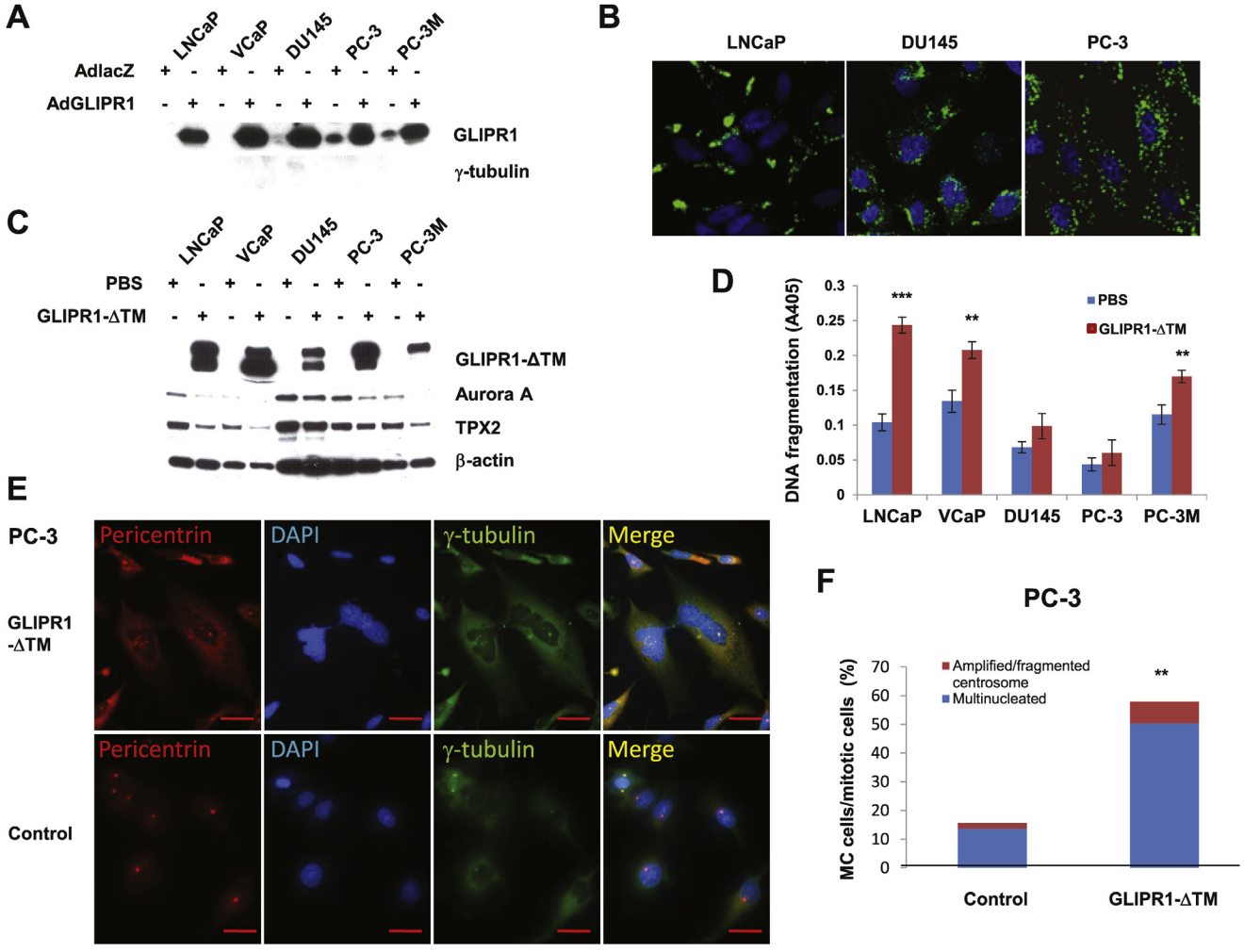

Figure 6 – Induction of apoptosis and MC in PCa cells with purified GLIPR1-ΔTM protein. (A) GLIPR1 secretion from cultured PCa cells. Proteins in the conditioned medium were precipitated by trichloroacetic acid and separated on SDS-PAGE, and GLIPR1 was detected by Western blotting. (B) Images show internalization of Alexa Fluor 488-conjugated GLIPR1-ΔTM (green). Scale bars = 15 μm. (C) Western blotting analysis of cell lysates from PCa cells that were incubated with GLIPR1-ΔTM in growth medium containing 0.5% fetal bovine serum for 48 h (D) DNA fragmentation assay. Error bars indicate SD. **$p < 0.001$; ***p 0.0001. (E) GLIPR1-ΔTM treatment induced MC in PC-3 cells, characterized by multinucleated cells with multiple/fragmented centrosomal and/or spindle poles (arrows), as shown on double immunofluorescence of pericentrin and γ-tubulin. Scale bars = 20 μm. (F) Quantitative comparison of MC frequency in GLIPR1-ΔTM- and control PBS-treated PC-3 cells.

superfamily. Most CAP proteins are secreted and have extracellular endocrine or paracrine functions (Gibbs et al., 2008). The N-terminal signal peptide and two extracellular protein signature motifs on the GLIPR1 protein (Ren et al., 2006) suggest that GLIPR1 is potentially secreted or shed from the membrane. Indeed, both endogenous and enforced GLIPR1 were detected in the culture medium (Figure 6A). Purified recombinant membrane domain-truncated GLIPR1 (GLIPR1-ΔTM) was effectively internalized by PCa cells in vitro (Figures 6B and C) through a clathrin-dependent endocytosis pathway (data not shown). Of importance, exogenous GLIPR1-ΔTM protein functioned like transduced GLIPR1 with regard to downregulation of Aurora A and TPX2 expression (Figure 6C), induction of apoptosis indicated by increased DNA fragmentation (Figure 6D) and induction of MC (Figures 6E and F) in PCa cells in vitro.

3.5.2. *GLIPR1-ΔTM treatment inhibited growth of VCaP-Luc and PC-3-Luc orthotropic prostate tumors*

Administration of GLIPR1-ΔTM to mouse prostate tumors that had been induced by prostatic orthotopic inoculation of PC-3 or VCaP cells harboring a lentivirus expressing luciferase significantly suppressed tumor growth, as indicated by the reduction of luminescence photon signal (Figure 7A) and tumor wet weight (Figure 7B). GLIPR1-ΔTM protein also induced tumor cell apoptosis, demonstrated by an increased level of cleaved (activated) caspase 3 (Figure 7C) and induction of MC, as shown by increased frequencies of multipolar mitotic cells (Figure 7D). There

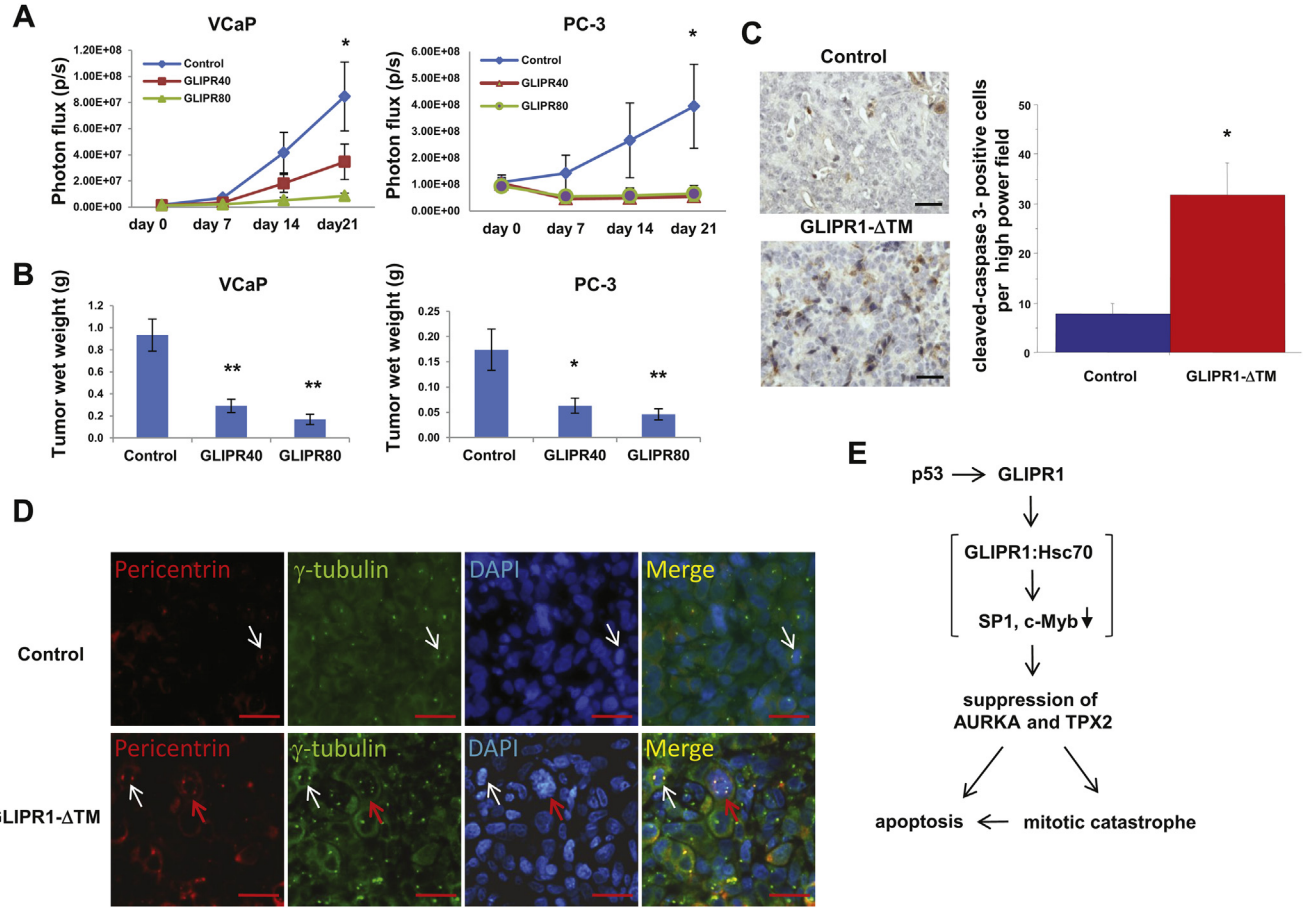

Figure 7 – GLIPR1-ΔTM treatment inhibited growth of VCaP-Luc and PC-3-Luc orthotropic prostate tumors. Prostate tumors were induced by orthotropic inoculation of VCaP-Luc and PC-3-Luc and the tumors were allowed to grow for 7 days for PC-3–Luc and 14 days for VCaP–Luc before treatment. The control group was treated with PBS, and the experimental groups with GLIPR-ΔTM protein (40 or 80 μg/dose) intravenously (IV) through the tail vein for VCaP model and intraperitoneally (IP) for PC-3 model three times per week for 2 weeks. Each group contained 9-14 mice. Tumor growth was monitored in vivo by bioluminescence measurement (A) and tumor wet weight (B). Significant tumor growth inhibition by GLIPR1-ΔTM protein was observed in both VCaP-Luc (IV route) and PC-3-Luc (IP route) tumors. Error bars indicate SE. *$p < 0.05$ and **$p < 0.01$. (C) Quantitative analysis of activated caspase 3 immunostaining in the PC-3 tumors collected 21 days after initial GLIPR1-ΔTM (80 μg) and control (PBS) injections. *P* value was derived with Mann–Whitney testing. Scale bars = 80 μm. Error bars indicate SE. *$p < 0.05$. (D) Dual immunofluorescence labeling of pericentrin (red) and γ-tubulin (green) and DAPI nuclear staining (blue) show the presence of MC cells which demonstrate multiple centrosomal/spindle poles (red arrows) in the GLIPR1-ΔTM-treated tumors. In the untreated tumors, only bipolar mitotic cells were found (white arrows). Scale bars = 30 μm. (E) Diagram illustrates molecular signaling of GLIPR1-induced apoptosis and MC in PCa cells.

were no obvious signs of toxicity, and analysis of multiple nontumor tissues did not reveal cellular damage (data not shown).

4. Discussion

This study showed that GLIPR1-induced apoptosis and/or MC in PCa cells is associated with activation of cell cycle checkpoints and proapototic genes and with downregulation of a substantial subset of spindle- or centrosome assembly-related genes, including *AURKA* and *TPX2*. *Glipr1*$^{-/-}$ MEFs showed higher AURKA and TPX2 expression, enhanced cell proliferation, and significantly greater colony formation than *Glipr1*$^{+/+}$ MEFs did. These *Glipr1*$^{-/-}$MEF features, together with the high frequency of spontaneous tumors in *Glipr1*$^{-/-}$mice found in our previous study (Li et al., 2008), suggest that endogenous, physiologic levels of Glipr1 are sufficient to influence the progression of PCa.

The results of our mechanistic studies show that GLIPR1's binding to Hsc70 chaperone protein is associated with disruption of an oncogenic signaling pathway that involves Hsc70's interaction with SP1 and c-Myb and activation of AURKA and TPX2 transcription (Figure 7E).

Furthermore, GLIPR1 was shown to be secreted and internalized by PCa cells, suggesting that it has autocrine and possibly paracrine functions. Treatment of cultured PCa cells with recombinant GLIPR1-ΔTM protein effectively inhibited Aurora A and TPX2 expression and induced apoptosis and MC. Finally, systemic administration of GLIPR1-ΔTM protein to mice bearing PCa xenograft tumors induced apoptosis and MC in the tumor cells and suppressed tumor growth without any signs of toxicity.

Our microarray data clearly showed that overexpression of GLIPR1 not only reduces expression of AURKA and TPX2, which is the main focus of this paper, but also regulates the expression of an array of apoptosis- or cell cycle-related genes. It is conceivable GLIPR1 activities result in a series of proapoptotic and/or cell cycle inhibitory events prior to GLIPR1 action in the mitotic phase. As we previously reported, GLIPR1 can induce apoptosis through ROS-JNK signaling (Li et al., 2008) and can disrupt the cell cycle through targeted destruction of β-catenin and c-Myc in PCa cells (Li et al., 2011). The newly defined GLIPR1-Hsc70-c-Myb/SP1-AURKA, TPX2 signaling pathway can be seen as an additional important mechanism that contributes to GLIPR1-induced PCa cell death.

GLIPR1 expression led to downregulation of androgen receptor (AR) expression in all of the three androgen-sensitive PCa cell lines tested. Since elevated expression of AR drives castration-resistance and overall cancer cell survival (Vakifahmetoglu et al., 2008), future studies to determine the role of AR in GLIPR1-induced apoptosis and/or MC are warranted.

Owing to their oncogenic activities, specific inhibitors targeting AURKA, TPX2, or both have been developed rapidly [reviewed in (Andrews, 2005; Asteriti et al., 2010; Cheung et al., 2011; Dar et al., 2010; Kelly et al., 2011; Perez Fidalgo et al., 2009)]. This study has shown that as an endogenous inhibitor for both AURKA and TPX2, GLIPR1-based therapy has the advantage of suppressing both AURKA and TPX2. An exciting result of our study was that a truncated, recombinant form of GLIPR1 protein, GLIPR1-ΔTM, was internalized by PCa cells. Internalization of therapeutic proteins is a challenging aspect of systemic protein cancer therapeutics, and further studies are required to define the GLIPR1 uptake pathway(s) and mechanisms involved. However, the structure of GLIPR1 that defines it as a membrane-tethered and/or secreted protein, together with our results that show efficient GLIPR1 secretion from PCa cells following gene transduction, suggest an autocrine and possibly a paracrine role for GLIPR1 in prostate. As shown in our in vitro and in vivo studies, GLIPR1-ΔTM treatment suppresses AURKA and TPX2 protein levels and has potent antitumorigenic activities in both androgen-sensitive and -insensitive PCa cells. An important note is that these therapeutic effects were observed in the absence of any nonspecific toxicity. Future studies that further define and exploit the target-rich Hsc70-SP1 and c-Myb-AURKA and -TPX2 transcriptional activation and that develop the potential of GLIPR1-based protein therapeutics are clearly warranted.

4.1. *Conclusion*

Although many lines of evidence have suggested that GLIPR1 has p53-dependent or -independent tumor suppressor functions in PCa, the mechanisms underlying those functions have not been fully illustrated. The relationship of GLIPR1 with Hsc70, c-Myb, AURKA and TPX2 is unknown. In this study we report that expression of GLIPR1 regulated numerous apoptotic, cell cycle, and spindle/centrosome assembly-related genes, including *AURKA* and *TPX2*, and induced apoptosis and/or MC in PCa cells, including p53-mutated/deleted, androgen-insensitive metastatic PCa cells, a category of therapy-resistant PCa. Mechanistically, GLIPR1 interacts with heat shock cognate protein 70 (Hsc70); this interaction is associated with SP1 and c-Myb destabilization and suppression of SP1- and c-Myb-mediated AURKA and TPX2 transcription. Inhibition of AURKA and TPX2 using siRNA mimicked enforced GLIPR1 expression in the induction of apoptosis and MC. GLIPR1 is secreted and internalized by PCa cells. Recombinant GLIPR1-ΔTM protein inhibited AURKA and TPX2 expression, induced apoptosis and MC, and suppressed orthotopic xenograft tumor growth. Our results define a novel GLIPR1-regulated signaling pathway that controls apoptosis and/or mitotic catastrophe in PCa cells and establishes the potential of this pathway for targeted therapies.

Conflict of interest

The data included in this manuscript are relevant to intellectual property that has been licensed by Baylor College of Medicine to Progression Therapeutics, Inc., a private biotechnology start-up company. T.C. Thompson and C. Ren are inventors of record on patents that are included in this licensing agreement.

Supplementary Information accompanies the paper on the Elsevier website (http://www.sciencedirect.com).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2012.12.005.

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