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14. ABSTRACT Standard therapy for advanced Prostate Cancer (PCa) consists of anti-androgens, which provide respite from disease progression, but ultimately fail resulting in incurable mCRPC. Drugs aimed at the DDR were limited by toxicity and need better molecular targets. We show that androgen deprivation of LNCaP increases expression of TLK1B, a key kinase upstream of NEK1 and ATR and mediating the DDR that results in a cell cycle arrest of androgen responsive PCa cells. Following DNA damage, addition of the TLK inhibitor, thioridazine (THD), impairs ATR and Chk1 activation, resulting in apoptosis. Treatment with THD suppressed the outgrowth of androgen-independent colonies of LNCaP. THD also inhibited growth of several PCa lines (including androgen independent). Administration of THD or bicalutamide was not effective in long-term inhibition of LNCaP xenografts, while combination remarkably inhibited tumor growth via bypass of the DDR. Xenografts of LNCaP cells overexpressing a NEK1-T141A mutant were durably suppressed with bicalutamide.

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DNA Damage Response (DDR); TLK1; Nek1; Androgen Deprivation Therapy (ADT); Cell Cycle Checkpoint

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TABLE OF CONTENT:

Cover page (form 298) – 1
Introduction - 2
Keywords - 2
Summary of Accomplishments - 2-3
Impact - 3 and 5
Accomplishments vs. SOW -3-4
Challenges 5
Products 5-6
Participants 6
Appendix 6-30

INTRODUCTION

Prostate Cancer (PCa) is one of the most common urological malignancies in men in the United States. Tousled Like kinases (TLKs) are involved in numerous cellular functions, including the DNA Damage Response (DDR). During the tenure of this grant, through a novel proteomic approach, we have identified that NIMA kinase NEK1 strongly interacts and co-localizes with TLKs and has a role in the DDR, upstream of ATR and Chk1, and regulates the checkpoint in response to oxidative stress or replication arrest. We have then tested Thioridazine (THD), an anti-psychotic drug and specific inhibitor of TLKs in inhibiting the activity of NEK1 in prostate cancer cell lines and LNCaP cell derived xenografts in NOD/SCID mice and the subsequent role of ATR-Chk1 axis following DNA damage. Treatment with THD suppresses the outgrowth of androgen-independent (AI) colonies of LNCaP cells cultured with bicalutamide. Administration of THD or Bicalutamide long-term was not very effective in inhibiting tumor growth. In contrast, combination therapy of THD and Bicalutamide remarkably inhibited the tumor growth in LNCaP xenografts model. Further p-ATR and p-Chk1 axis was inhibited by treatment with THD and in combination with bicalutamide as evident by immunoblotting of residual tumors. In cells, following DNA damage, addition of the TLK inhibitor THD impaired ATR and Chk1 activation, indicating the existence of a TLK1>NEK1>ATR>Chk1 pathway. In order to publish these results, we wre required to confirm them in at least another cell line that recapitulates AS to AI conversion (we chose TRAMP-C2), which was not part of our original proposal. Following that publication (IJC 2019 –Appendix 1), we continued with the goals of the SOW, and showed that TRAMP mice treated with THD, following castration did not recover cancerous growth of their prostates, had no sign of metastases, and failed to convert to the classic NE phenotype at 28 weeks. In addition, as part of the original proposal, we had suggested that we may generate a pT141-Nek1 specific antiserum (although this was not part of the SOW), which we proposed would become handy in as a biomarker for PCa biopsies. We reported the first study of a PCa TMA of p-Nek1-T141 and correlation to the Gleason score. Finally, we recapitulated the process of translational increase in TLK1B expression in a naïve PDX model that was established from an AR+ adenocarcinoma. Hence, in future work (not part of this grant) we can rely on such PDX model that more closely resembles the human disease that comining ADT with a TLK inhibitor (like THD) can suppress the outgrowth of AI tumors. This work was published in Cancer Letter 2019 (Appendix 2).

Keywords

TLK1; Nek1; ATR; Chk1; DNA Damage Response (DDR); Cell Cycle Checkpoint; Androgen (In)Sensitive PCa; Xenograft models

Summary of key accomplishments for current year of work

First paper: Targeting the TLK1/NEK1 DDR axis with Thioridazine suppresses outgrowth of Androgen Independent Prostate tumors.

Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Hari K. Koul, Xiuping Yu, Arrigo De Benedetti.

Abstract: Standard therapy for advanced Prostate Cancer (PCa) consists of anti-androgens, which provide respite from disease progression, but ultimately fail resulting in the incurable phase of the disease: mCRPC. Targeting PCa cells before their progression to mCRPC would greatly improve the outcome. Combination therapy targeting the DNA Damage Response (DDR) has been limited by general toxicity, and a goal of clinical trials is how to target the DDR more specifically. We now show that androgen deprivation therapy (ADT) of LNCaP cells results in increased expression of TLK1B, a key kinase upstream of

NEK1 and ATR and mediating the DDR that typically results in a temporary cell cycle arrest of androgen responsive PCa cells. Following DNA damage, addition of the TLK specific inhibitor, thioridazine (THD), impairs ATR and Chk1 activation, establishing the existence of a ADT>TLK1>NEK1>ATR>Chk1, DDR pathway, while its abrogation leads to apoptosis. Treatment with THD suppressed the outgrowth of androgen-independent (AI) colonies of LNCaP and TRAMP-C2 cells cultured with bicalutamide. Moreover, THD significantly inhibited the growth of several PCa cells *in vitro* (including AI lines). Administration of THD or bicalutamide was not effective at inhibiting long-term tumor growth of LNCaP xenografts. In contrast, combination therapy remarkably inhibited tumor growth via bypass of the DDR. Moreover, xenografts of LNCaP cells overexpressing a NEK1-T141A mutant were durably suppressed with bicalutamide. Collectively, these results suggest that targeting the TLK1/NEK1 axis might be a novel therapy for PCa in combination with standard of care (ADT).

Second paper: The TLK1-Nek1 axis promotes Prostate Cancer progression

Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Hari K. Koul, Xiuping Yu, Arrigo De Benedetti.

Abstract: We recently uncovered the critical TLK1>NEK1>ATR>Chk1 axis in mediating the DDR and cell cycle checkpoint while transiting from Androgen Sensitive to Insensitive growth for LNCaP and TRAMP-C2 cells. However, we did not know the generality of this pathway in PCa progression since there are few cell lines where the transition has been studied. Furthermore, the identification of Nek1, and more importantly the TLK-mediated phosphorylation of T141, has never been studied in PCa biopsies. We now report the first study of a PCa TMA of p-Nek1-T141 and correlation to the Gleason score. In addition we found that TRAMP mice treated with the TLK inhibitor, thioridazine (THD), following castration did not recover cancerous growth of their prostates. Moreover, we recapitulated the process of translational increase in TLK1B expression in a naïve PDX model that was established from an AR+ adenocarcinoma. Therefore, we believe that this TLK1-Nek1 mediated DDR axis is likely to be a common adaptive response during the transition of PCa cells toward androgen-insensitive growth, and hence CRPC progression, which has the potential to be targeted with THD and other TLK or Nek1 inhibitors.

OVERALL IMPACT: Our data indicated that THD repressed growth of PCa cells *in vitro and in vivo*, and for Androgen-dependent (LNCaP) cells, in combination with bicalutamide it was very effective at preventing outgrowth of AI cells and hence as a targeted ADT adjuvant therapy. Moreover, THD impaired DDR. Collectively, these results strongly suggest that THD might be a novel anti-tumor agent for use in prostate cancer.

What were the major goals of the project? We have targeted a specific liability that incurs in Androgen Responsive PCa cells when shifted to ADT, by adding an inhibitor (THD) of the TLK1>Nek1>ATR>Chk1 DDR axis in order to abrogate the checkpoint and promote apoptosis. This axis was initially hypothesized at the onset of submitting this grant.

Outline of accomplishments in line with SOW

Specific Aim 1 (Probing the functional interaction between TLK1B and NEK1**)**	**Timeline**
Major Task 1 Test the hypothesis that TLK1 regulates NEK1 activity	Months
Subtask 1.1 Establishing the TLK1 phosphorylation site(s)	1-6
Subtask 1.2 Establish if phosphorylation of NEK1 alter its kinase activity	5-9
Subtask 1.3 TLK1/1B affect the activity of NEK1 after ADT	6-12
Subtask 1.4 TLK1B acts as a chaperone or a kinase for NEK1	7-15
Subtask 1.5 Phosphorylation of NEK1 by TLK1/1B is important for the DDR	8-18

We have completed and published all the subtasks of SA1, with the exception of subtask 1.4, since we have established that TLK1 is critical for the activation of Nek1 as a kinase (by phosphorylating T141). We have recently established that TLK1 mediates the interaction of Nek1 and ATR even in absence of phosphorylation. Hence we have not established that TLK1 acts also as a chaperone, for example in the localization of Nek1 to sites of DNA damage. In fact, we have established that TLK1 and Nek1 both relocalize to nuclear foci after induction of DBSs with doxorubicin, but we have not yet done experiments to determine if TLK1 critically mediates the relocalization of Nek1. One difficulty in addressing this problem is the fact that we have evidence that TLK2 can substitute for TLK1 in phosphorylating Nek1 and likely in mediating its relocalization even in

the absence of TLK1. Knocking down both TLK1 and TLK2 is extremely deleterious to cells and generally resulting in loss of cell viability. We have also obtained a very specific phospho-Ab to Nek1(pT141) that has proven to be very useful as a reporter of the level of Nek1 activity, and that we have begun to use in cell cycle studies and also in LNCaP cells before and after androgen deprivation (ADT). In fact, in the first paper in the Appendix (IJC 2019), we were able to show that the Nek1 phosphorylation of T141 correlated perfectly with the activation of ATR and Chk1 (Subtask 1.5) in the LNCaP model (both in vitro and in xerografts). In addition, we have demonstrated biochemically that the phosphorylation of T141 is critical for maximal activity of Nek1. In addition, in the second paper published in Cancer Letters 2019, we have shown in PCa TMA that the expression of TLK1 and the phosphorylation of Nek1-T141 correlate with each other and with the Gleason Score.

Specific Aim 2 (Attacking the TLK1-NEK1 axis in mouse models)	
Major Task 2 Generating GEM models for Attacking the TLK1/NEK1 axis in vivo	
Subtask 2.1 LNCaP model in SCID mice	6-30
Subtask 2.2 Breeding of TRAMP, NEK1^{kat} and TRAMP/NEK1^{kat} mice	8-24
Subtask 2.3 Testing THD/castration in the TRAMP model	10-30
Subtask 2.4 Test for progression to CRPC in TRAMP/NEK1^{kat} mice	20-36
Subtask 2.5 Generate NeoTag-$^{NEK-/-}$ and NeoTag-$^{TLK1-/-}$cells by CRSPR/Cas9	12-18
Subtask 2.6 Tissue recombination in SCID with the NeoTag deleted cells	18-36

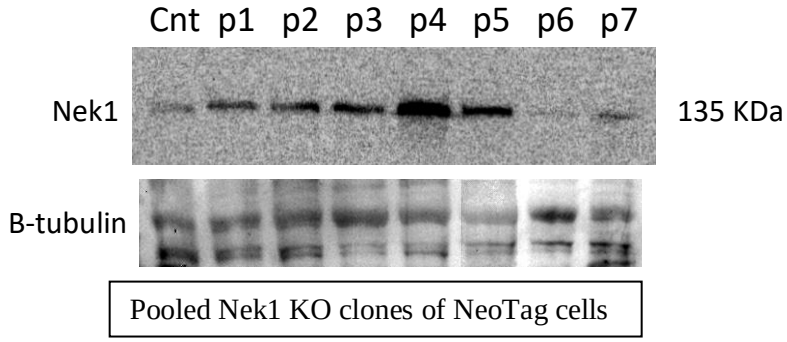
Subtask 2.1. We have tested extensively the role of Nek1-T141 phosphorylation by TLK1/1B in the LNCaP xerograft model. We have found that administration of THD or bicalutamide was not effective in inhibiting long-term tumor growth of LNCaP xenografts. In contrast, combination therapy remarkably inhibited tumor growth via bypass of the DDR. Moreover, xenografts of LNCaP cells overexpressing a NEK1-T141A mutant were durably suppressed with bicalutamide. We have confirmed the in vitro results with the TRAMP-C2 model, strongly suggesting that this DDR pathway is also evolutionarily conserved. Collectively, these results strongly suggest that targeting the TLK1/NEK1 axis (with THD) might be a novel therapy for PCa in combination with standard of care (ADT) at least in the well-established LNCaP model. However, we also found that THD inhibited the growth of also a panel of human PCa lines in vitro but not the non-tumorigenic line RWPE1. As previously reported, THD at a concentration equivalent to that used in schizophrenic individuals was not toxic to the mice and they displayed no behavioral anomalies.

Subtask 2.2, 2.3. We have completed the proposed TRAMP mice studies. The results were published in Cancer Letters 2019 (Appendix 2). about 6 months ago, and we now begin have about 40 males that we are ageing to start the experiments with castration and THD treatment.

Subtask 2.4 We have established a colony of Nek^{kat} mice (KO), and we will soon begin to cross them to TRAMP females. In the proposal we hypothesized that castration in the Nek^{kat} /TRAMP mice will be sufficient to regress the prostate cancer, without latter progression to CRPC.

Subtask 2.5. We have generated some pooled clones of the NeoTag cells with Nek1 the gene disruption. An example from 7 primary clones is shown below, but we are not sure if p6 is clonal, so we started subcloning it for complete characterization by RT-PCR and gene sequencing.

Subtask 2.6 will begin after we have characterized the KO cells more completely in vitro.



Pooled Nek1 KO clones of NeoTag cells

How were the results disseminated to communities of interest? We published two papers and presented two poster at AACR 2019, in addition to various seminars presented at our Institution.

Impact

What was the impact on the development of the principal discipline(s) of the project?

Standard therapy for advanced Prostate Cancer (PCa) consists of anti-androgens, which only provide temporary respite from disease progression to metastatic castrate-resistant prostate cancer (mCRPC). Here, the authors show in the LNCaP cell model that the increased expression with ADT of TLK1B, a prosurvival checkpoint pathway that is enacted before conversion to androgen-independent growth, offers a unique target for attacking more specifically PCa cells before their conversion to CRPC. Moreover, we suggest to re-purpose thioridazine or other phenothiazine antipsychotic drugs as inhibitors of the TLK1 > Nek1 > ATR > Chk1 DNA Damage Response (DDR) axis for the early treatment of advanced PCa still responsive to ADT

What was the impact on other disciplines? Nothing to report

What was the impact on technology transfer?

Inventions, patent applications, and/or licenses. A Provisional Patent was filed for the technology "Targeting the TLK1/NEK1 axis in Prostate Cancer" on March 29, 2019, and is expires one year from that date. We plan to file a full patent.

What was the impact on society beyond science and technology? Nothing to report

CHANGES/PROBLEMS:

We had some difficulties in publishing our first paper in a high IF Journal (such as IJC) because we originally had only one cell line model – LNCaP. This was because there really aren't other human PCa lines that rapidly convert form AS to AI growth. VCaP do convert so, but only in xenografts and not in vitro. We thus resorted to TRAMP-C2 to provide and additional model. Not yet satisfied with this, we have also implemented a PDX model, which we have begun characterizing, but which we cannot yet use for tumor regression studies because this has not been approved in this current grant and by the ACURO.

What do you plan to do during the next reporting period to accomplish the goals? The final part of the grant and in line with the SOW was the generation of a Nek1-KO mouse in TRAMP background, so that we can determine genetically that these mice, following castration do not progress to CRPC. This will provide direct genetic evidence for the critical importance of the TLK1>Nek1>DDR axis in progression to CRPC. Should the bredding prove to be too difficult to obtain sufficient males for this work, our alternative is the kidney capsule tissue recombination experiment with Nek1-KO NeoTag cells. This will complete all the specific aims

PRODUCTS: (2 publications, 2 Abstracts):

1. Targeting the **TLK1**/NEK1 DDR axis with Thioridazine suppresses outgrowth of androgen independent prostate tumors Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Hari K. Koul, Xiuping Yu, Arrigo De Benedetti. Int J Cancer. 2019. 145(4): 1055–1067. Published online 2019 Feb 25. doi: 10.1002/ijc.32200.
2. The TLK1-Nek1 axis promotes Prostate Cancer progression. Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Hari K. Koul, Xiuping Yu, Arrigo De Benedetti. Cancer Letters 2019. 453:131-141. https://doi.org/10.1016/j.canlet.2019.03.041

3. **Meetings Attended and Papers Presented.**

   AACR March 29-April 3, 2019. Design, synthesis and biological evaluation of new phenothiazine derivatives as potential Tousled-like Kinase 1 inhibitors in Prostate Cancer Treatment

   Siddhant Bhoir, Javeena Hussain, Vibha Singh, Rupesh Chikhale, Richard Bryce, Sivapriya Kirubakaran, and Arrigo De Benedetti

AACR March 29-April 3, 2019. Over expression of Protein kinase Nek1 and its modulation by Thioridazine in Prostate cancer
Vibha Singh, Praveen Kumar Jaiswal, Hari K Koul, Xiuping Yu, and Arrigo De Benedetti

Participants

The following people (all in the Department of Biochemistry and Molecular Biology and the Feist Weiller Cancer Center at LSUHSC) have participated in this research: Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Hari K. Koul, Xiuping Yu, and Arrigo De Benedetti

What opportunities for training and professional development has the project provided?

Vibha Singh is a postdoc in my lab, and she had limited training in animal work. During this period she has become proficient in xenografts implants and with surgical castration.

Ishita Ghosh is a graduate student in my lab, and has learned numerous techniques in vitro and with animal work, including castration.

APPENDIX (2 PAPERS)

IJC
International Journal of Cancer

Targeting the TLK1/NEK1 DDR axis with Thioridazine suppresses outgrowth of androgen independent prostate tumors

Vibha Singh[1], Praveen Kumar Jaiswal[1], Ishita Ghosh[1], Hari K. Koul[1,2,3], Xiuping Yu[1] and Arrigo De Benedetti[1]

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[3]Overton Brooks VA Medical center, Shreveport, LA

Standard therapy for advanced Prostate Cancer (PCa) consists of antiandrogens, which provide respite from disease progression, but ultimately fail resulting in the incurable phase of the disease: mCRPC. Targeting PCa cells before their progression to mCRPC would greatly improve the outcome. Combination therapy targeting the DNA Damage Response (DDR) has been limited by general toxicity, and a goal of clinical trials is how to target the DDR more specifically. We now show that androgen deprivation therapy (ADT) of LNCaP cells results in increased expression of TLK1B, a key kinase upstream of NEK1 and ATR and mediating the DDR that typically results in a temporary cell cycle arrest of androgen responsive PCa cells. Following DNA damage, addition of the TLK specific inhibitor, thioridazine (THD), impairs ATR and Chk1 activation, establishing the existence of a ADT › TLK1 › NEK1 › ATR › Chk1, DDR pathway, while its abrogation leads to apoptosis. Treatment with THD suppressed the outgrowth of androgen-independent (AI) colonies of LNCaP and TRAMP-C2 cells cultured with bicalutamide. Moreover, THD significantly inhibited the growth of several PCa cells *in vitro* (including AI lines). Administration of THD or bicalutamide was not effective at inhibiting long-term tumor growth of LNCaP xenografts. In contrast, combination therapy remarkably inhibited tumor growth *via* bypass of the DDR. Moreover, xenografts of LNCaP cells overexpressing a NEK1-T141A mutant were durably suppressed with bicalutamide. Collectively, these results suggest that targeting the TLK1/NEK1 axis might be a novel therapy for PCa in combination with standard of care (ADT).

Introduction

Prostate cancer (PCa) is diagnosed in over 200,000 men in the US each year and results in approximately 25,000 deaths. The mainstay therapy for patients with locally advanced prostate cancer, metastatic prostate cancer and biochemically recurrent disease after failure of localized treatments, is androgen-deprivation therapy (ADT) with gonadropin-releasing hormone analogs and antiandrogens. ADT is known to provide remission of the disease, best evidenced by a decline of prostate-specific antigen (PSA) in about 90% of patients. After a mean time of 2–3 years, however, the disease progresses despite continuous hormonal manipulation. This type of cancer is known as metastatic castrate-resistant prostate cancer (mCRPC). mCRPC is associated with a poor prognosis and mean survival time of only 16–18 months.[1] Thus, the best window of opportunity is before development of mCRPC, and this requires a clear understanding of the process of PCa cells' mechanisms of adaptation to ADT. The best characterized model so far for studying this is the LNCaP. Androgen deprivation of LNCaP cells results in loss of AR function with a compensatory prosurvival activation of mTOR[2] and concomitant implementation of a cell division arrest by activation of the DNA Damage Response (DDR) mediated by ATR-Chk1[3] or ATM-Chk2.[4] However, it is not well understood what signals the activation of the DDR and ATR (rev. in Ref. [5]). Then, after a quiescent period of ADT adaptation of 2–3 weeks, androgen independent (AI) colonies begin to form.[6]

Additional Supporting Information may be found in the online version of this article.

V.S. and P.K.J. contributed equally to this work.

Conflict of interest: No potential conflicts of interest were disclosed.
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What's new?

Standard therapy for advanced Prostate Cancer (PCa) consists of anti-androgens, which only provide temporary respite from disease progression to metastatic castrate-resistant prostate cancer (mCRPC). Here, the authors show in the LNCaP cell model that the increased expression with ADT of TLK1B, a prosurvival checkpoint pathway that is enacted before conversion to androgen-independent growth, offers a unique target for attacking more specifically PCa cells before their conversion to CRPC. Moreover, they suggest to re-purpose thioridazine or other phenothiazine antipsychotic drugs as inhibitors of the TLK1 › Nek1 › ATR › Chk1 DNA Damage Response (DDR) axis for the early treatment of advanced PCa still responsive to ADT.

An attractive strategy to prevent this process would be to bypass the cell cycle arrest *via* inhibition of ATM or ATR, causing the cells to undertake replication with damaged DNA that would cause mitotic catastrophe, a strategy that was in fact implemented in LNCaP treated concomitantly with bicalutamide and ATM inhibition.[4] But a limitation of this approach is how to make the inhibition of ATM or ATR specific to PCa cells.

The Tousled Like kinases (TLK1 and TLK2) function in several processes including chromatin assembly, replication, transcription, DNA repair, and chromosome segregation (rev. in[7]). We recently uncovered the proteome target of TLK1 and identified the NIMA-related kinase, NEK1, as a major substrate,[8] a key kinase which in turn activates ATR and the DDR.[9,10] Inhibition of the TLK1/NEK1 axis with thioridazine (THD), a rather specific inhibitor of TLKs,[11] suppresses the DDR. THD was previously shown to improve therapeutic response in combination with DNA damaging agents.[11–15] Furthermore, THD was shown to cause growth inhibition of ovarian cancer cells *via* suppression of AKT activity[16] by an unidentified mechanism downstream of PI3K and mTOR, but which we now propose could be the result of inhibition of TLK1 and another of its targets, AKTIP, which is a direct activator of AKT. What is noteworthy is that the concomitant inhibition of TLK1 along with ADT (the current standard of care for advanced PCa) can result in a specific killing of Androgen Responsive PCa cells, instead of the more generalized, and thus more prone to side effects, inhibition of ATR or ATM. A similar strategy targeting a specific AR/CDC6-ATR-Chk1 signaling pathway has been used in a recent preclinical model,[17] but in that case, androgen deprivation resulted in reduction of CDC6 expression (a AR target gene), whereas in our work we show that ADT results in increased expression of TLK1B, a positive prosurvival mechanism, leading to activation of NEK1 and the DDR, that can be optimally targeted in PCa. Indeed, treatment with THD bypasses cell cycle arrest elicited by ADT and causes unencumbered replication-induced DNA damage, leading to apoptosis *in vitro* and in mouse xenografts models.

Materials and Methods

Cell lines

All PCa cell lines were obtained from the ATCC, recently authenticated, and cultured according to their guidelines. LNCaP cells stably overexpressing Nek1 (wt and mut) were generated as previously described.[18]

Animal studies

All animals used in this study received humane care based on guidelines set by the American Veterinary, and approved by the Institutional Animal Care and Use Committee of LSU Health Sciences Center at Shreveport. Male NOD-SCID (Taconic) mice (5 per group) were used. For xenografts, 1×10^6 LNCaP cells were resuspended in 100 μL of matrigel solution (Corning Matrigel Basement Membrane Matrix High Concentration, Catalog Number 354248) and injected in 1-month-old mice subcutaneously into the both flanks. After appearance of palpable tumors (~2 weeks) the mice were divided into the 4 study groups making sure that the tumors match in size at the start of the study. Tumor size and body weight were measured twice weekly and tumor volume were calculated by the formula V = (length × Width^2)/2. Tumor volumes and weights were statistically analyzed with ANOVA. Mice were treated with THD (dissolved in sterile water—2.5 mg/mL) at dosages similar to those reported previously in noncancer mouse studies every other day by ip injection or oral gavage—0.1 mL). Bicalutamide was dissolved in DMSO and diluted in corn oil and administered at a dose of 100 mg kg^{-1} by intraperitoneal injection twice a week.

Immunohistochemistry (IHC) of tumors

Sectioning and processing of the tissues were carried out in the FWCC Histology Service, using automated processes and equipment to provide uniform and standardized results. Indirect labeling was with ABC Elite: RTU Vectastain Elite Reagent, Vector #PK-7100; DAB: ImmPact DAB, Vector #SK-4105. Light counterstaining was with hematoxylin. Representative IHC images were taken at 40× magnification with the help of Olympus BX51 microscope. Positive staining were quantified by NIH ImageJ software with an average of three fields. Two-way ANOVA analysis was carried to compare the intensity of staining,

Results

Androgen deprivation results in translational increase in TLK1B expression

Our lab identified a splice variant of TLK1 that encodes a 65 kDa protein (named TLK1B) that becomes translationally upregulated in response to genotoxic stress *via* the mTOR-4EBP1 pathway.[19] TLK1B is similar to TLK1 but lacks the first 169 amino acids. TLK1 and TLK1B share the same catalytic domain and we sometime refer them to as TLK1/1B. TLK1B

overexpression protects cells from the genotoxic effects of IR.[20] We now show that androgen deprivation in LNCaP cells results in a rapid mTOR-dependent, rapamycin suppressible, strong increase in translation of TLK1B, whereas its mRNA does not change (Figs. 1*a* and 1*b* and Supporting Information Fig. S1A). Consistent with this mechanism, the phosphorylation of 4EBP1 increases in cells incubated in charcoal-stripped-serum (CSS) and this is suppressible with rapamycin (Supporting Information Fig. S1*b*). Notably, the TLK1 gene was identified by co-expression analysis using WGCNA as a key driver of PCa, highly enriched among candidate genes collected from expression Quantitative Trait Loci (eQTL), somatic copy number alterations (SCNA), and prognostic analyses.[21] But since expression of TLK1B is largely controlled at the post-transcriptional level it may have been largely underestimated in transcriptomics analyses of PCa. Recently we have identified the proteome complement of TLK1/1B, and have identified the protein kinase Nek1 as one of its principal targets.[18] TLK1/1B activates Nek1, by phosphorylating T141, and we established that this mechanism is an early DDR mediator upstream of ATR and Chk1 upon oxidative damage or replication arrest,[18] extending earlier studies on Nek1.[10] It seemed possible that a similar pathway becomes activated upon AR inhibition by ADT, leading to a prosurvival cell cycle arrest.

Nek1 expression in PCa and its TLK1-dependent phosphorylation/activation

The involvement of Nek1 in PCa development or progression is unknown, but interestingly C-Bioportal analysis of Nek1 revealed upregulation in 33% of patients and gene amplification in 12% of patients with CRPC-Neuroendocrine Prostate Cancer (NEPC, a type of AR negative CRPC). Moreover, Nek1 mRNA is upregulated in 21% of patients with metastatic PCa. Finally, Nek1 is upregulated in PCa patients in PAN-CANCER analysis of whole genome Dataset (Figs. 1*c*–1*e*). It is tempting to speculate that Nek1 may be involved is some mechanism(s) leading to survival of PCa cells particularly at the stage of mCRPC and importantly in AR-negative NEPC. We thus analyzed the Nek1 protein expression and its phosphorylation status (pT141- the site that is phosphorylated by TLK1) with a custom-made antibody (Pierce) in a panel of human PCa lines (Fig. 1*f*). This analysis revealed that Nek1 is ubiquitously expressed and is phosphorylated at T141 in all cells, including PC3 that do not express TLK1[22] although PC3 express TLK2, therefore suggesting that TLK2 can replace TLK1 in phosphorylating Nek1-T141. The specificity of the phospho-antibody (pAb) is shown in Figure 1*g*, in which we have analyzed two LNCaP derivatives overexpressing wt-Nek1 or a T141A mutant. While overexpression of wt-Nek1 resulted in a strong signal with the pAb, overexpression of the T141A gave a weak signal, likely attributable to the endogenous Nek1. Parallel probing for the AR and Ponseau S staining of the blots confirmed similar protein loading. Our main hypothesis is that ADT of LNCaP cells (with CSS or bicalutamide) should result in increased expression of TLK1B, and in turn in increased level of phosphorylated Nek1. Moreover, the specific phosphorylation of T141 by TLK1B should be suppressible with THD, a specific inhibitor of TLKs. These expectation were confirmed in Figure 2*a*. Moreover, treatment with THD of LNCaP overexpressing wt-Nek1 resulted in greatly reduced signal of pNek1 compared to bicalutamide-treated cells (Fig. 2*b*), whereas in cells overexpressing the T141A mutant, only the overexpressed unphosphorylated form of Nek1 was detected (note that this blot was probed with crude antiserum that reacts also with unphosphorylated Nek1, or the T141A mutant).

Treatment of LNCaP cells with ADT + THD prevents formation of AI colonies

Our hypothesis is that the activation of the DDR and cell cycle checkpoint *via* the TLK1/Nek1 axis,[18] in this case in response to ADT in the Androgen-sensitive (AS) line LNCaP, is a protective effect resulting in a quiescent state, before these cells convert to a Androgen-Independent (AI) growth. We postulate that this DDR axis is critical for survival of the cancer cells. We have tested this by monitoring the inhibition of emergence of AI colonies following treatment of LNCaP cells with bicalutamide (antiandrogen) and increasing concentrations of THD. AI colonies were scored after 3 weeks. At a concentration of 3–9 μM THD, which inhibits growth by only 50% in androgen containing medium (FCS), few or no AI colonies emerged (Figs. 2*c* and 2*d*). In addition, after 4 weeks with bicalutamide+3 μM THD the remaining cells became apoptotic and lifted from the plate—see example in 2D right panel where we compare a colony without (top) and with THD treatment (bottom). On the other hand, THD has been reported to have anticancer activity, although the precise mechanisms for this have not been elucidated. We thus also tested a panel of AI cell lines, in addition to LNCaP, in androgen containing medium—FCS. This resulted in a dose-dependent inhibition of colonies formation with maximal efficacy around 10 μM (Fig. 2*e*). Note however, that the 'normal' prostate cell line RWPE1 was largely insensitive to THD, except at the highest concentration. This suggests that THD may indeed be a more targeted anticancer agent, or it is possible that the TLK1/Nek1 axis remains critical even for CRPC cells. However, we previously reported that THD delays, but does not eventually prevent, the growth of PC3 subcutaneous tumors.[11] Note also that LNCaP cells grown in FCS-medium were not much affected by THD, again suggesting that a large part of inhibition of the AI growth of these cells in CSS could be mediated *via* the TLK1/Nek1 DDR axis. We have also tested the AI growth of TRAMP-C2 cells (see below) in Figure 2*f*.

Treatment of LNCaP cells with ADT + THD results in apoptosis

We propose that bypass of the DDR induced by ADT by inhibition of TLK1/Nek1 with THD results in abrogation of the checkpoint and in accumulation of replication-dependent DNA damage and induction of apoptosis. To establish if this is the case,

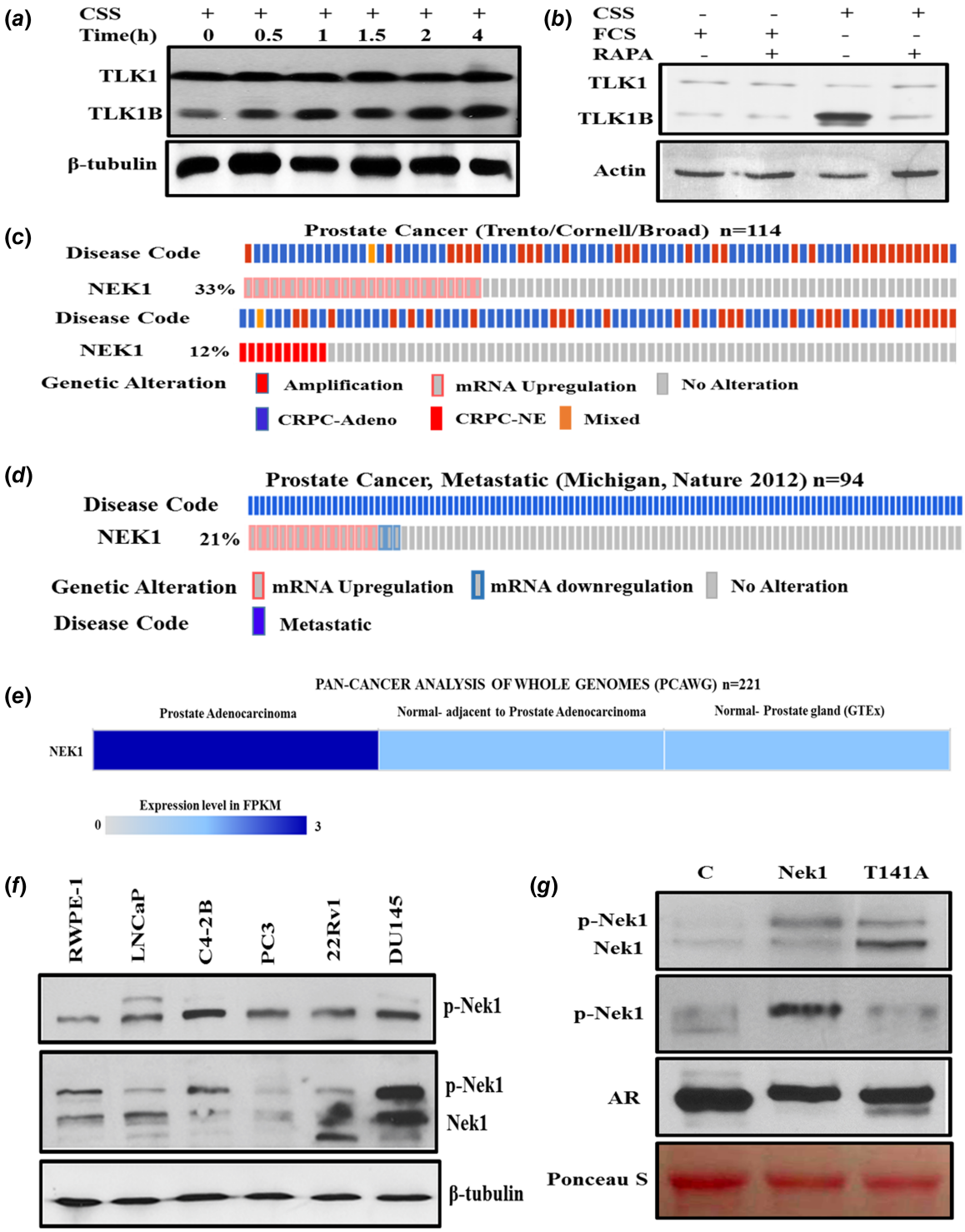

Figure 1. (*a*) Androgen Deprivation results in time-dependent increase in TLK1B expression correlated with 4EBP1 phosphorylation. LNCaP Cells were grown in medium containing FCS (t = 0) then transferred to CSS during a 4 h time-course. (*b*) The increased expression of TLK1B after ADT is mTOR dependent and suppressible with rapamycin. LNCaP cells were shifted to CSS for 17 h with and without rapamycin (20 nM) to inhibit mTOR, and cell extracts were prepared and probed with TLK1 antiserum. (*c*)–(*e*) Nek1 expression and Gene Amplification in PCa samples analyzed as described in Ref. 38. (*c*) Nek1 mRNA is upregulated and amplified in patients in CRPC-Neuroendocrine Prostate Cancer dataset. (*d*) Nek1 mRNA is upregulated in patients with Metastatic Prostate Cancer. (*e*) Nek1 is upregulated in PCa patients in PAN-CANCER ANALYSIS OF WHOLE GENOME Dataset. (*f*)–(*g*) Expression and phosphorylation of Nek1 in a panel of PCa lines and in LNCaP cells overexpressing Nek1 (wt) and T141A mutant. (*f*) Top panel probed with anti-pNek1 (T141) antiserum. Middle panel was probed with pan-Nek1 antiserum. (*g*) WB of Nek1. Top panel probed with pan-Nek1 antiserum. Second panel was probed with anti-pNek1 (T141) antiserum. Third panel was probed for the Androgen receptor (AR). [Color figure can be viewed at wileyonlinelibrary.com]

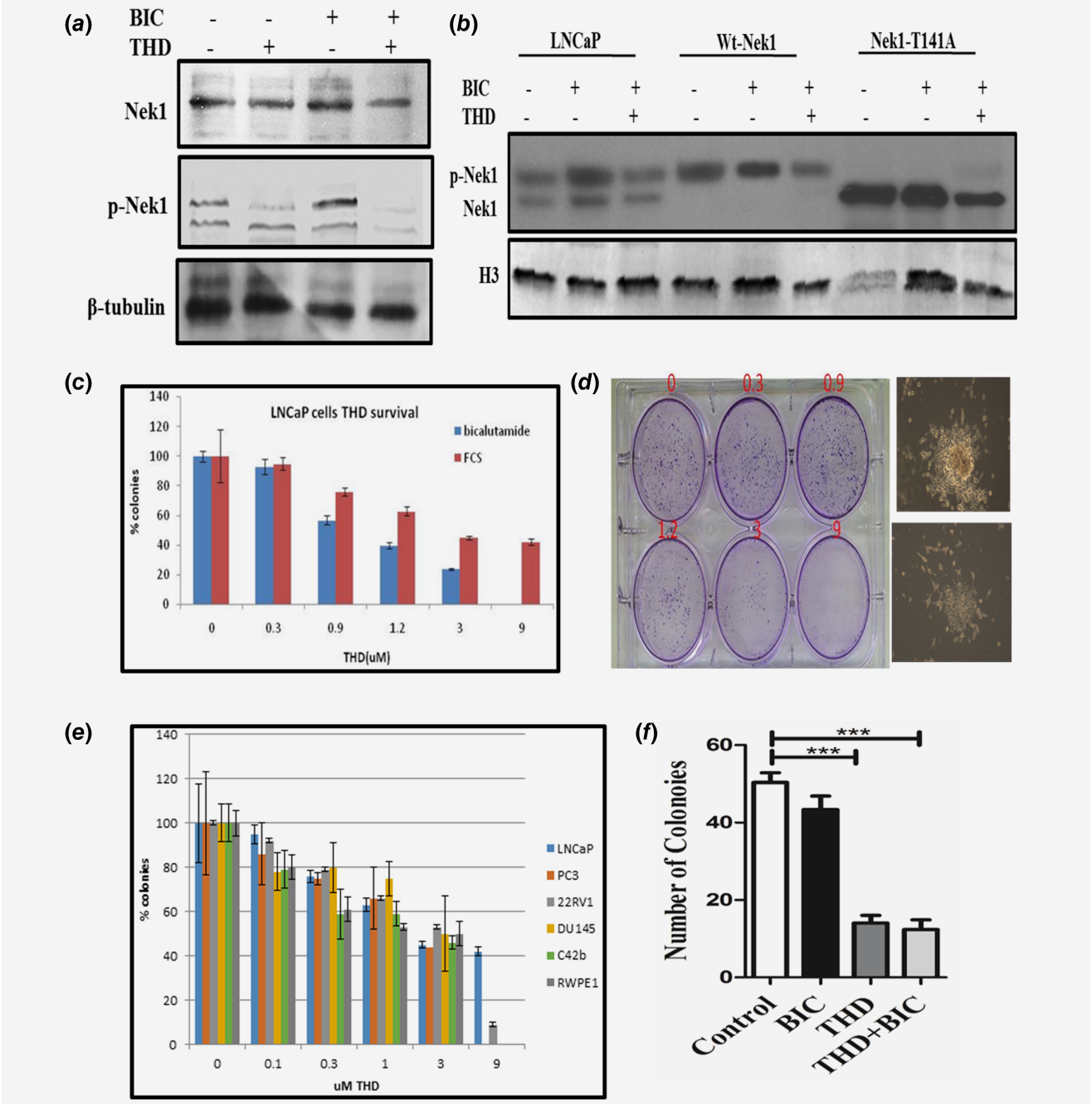

Figure 2. (*a*)–(*b*) Phosphorylation of Nek1 in parental LNCaP cells and overexpressing Nek1 (wt) and T141A mutant after incubation with bicalutamide +/− THD. The WB in panel *b* was probed with the pNek1 antiserum before affinity purification, so that it also reacts with the unphosphorylated Nek1, although more weakly. (*c*)–(*d*) AI colonies do not form with THD (concentration dependent). LNCap cells were plated in duplicates in 6-well plates at 4000 cells with bicalutamide (10 nM) to score developing AI colonies, or 400 cells in control medium (FCS) to monitor for general clonogenic inhibition by THD. (*d*) An example of crystal violet-stained plate from which the quantitation in panel C was derived. Here the AI colonies incubated with increasing concentrations of THD were scored after 3 weeks. After 1 month, a typical AI colony is shown in *d* (right top) without THD, or with addition of 3 μM THD (right bottom). (*e*) Percent of colonies treated with different conc. of THD in different PCa cell lines with increasing concentrations of THD. F. TRAMP-C2 cells do not form AI colonies when cultured with THD. Clonogenic assays of TRAMP-C2 cells untreated (control) or treated with bicalutamide (10 μM), THD (5 μM), or combination are displayed. [Color figure can be viewed at wileyonlinelibrary.com]

we analyzed the cell cycle distribution of LNCaP cells treated with bicalutamide, THD, or combination for 24 h to determine if there is cell cycle arrest or induction of apoptotic—sub-G1 cells (Fig. 3*a*). As expected, treatment with bicalutamide alone resulted in accumulation of cells in G1, 77% *vs* 61 (untreated). In contrast, THD (10 μM) did not induce accumulation of cells in G1, but

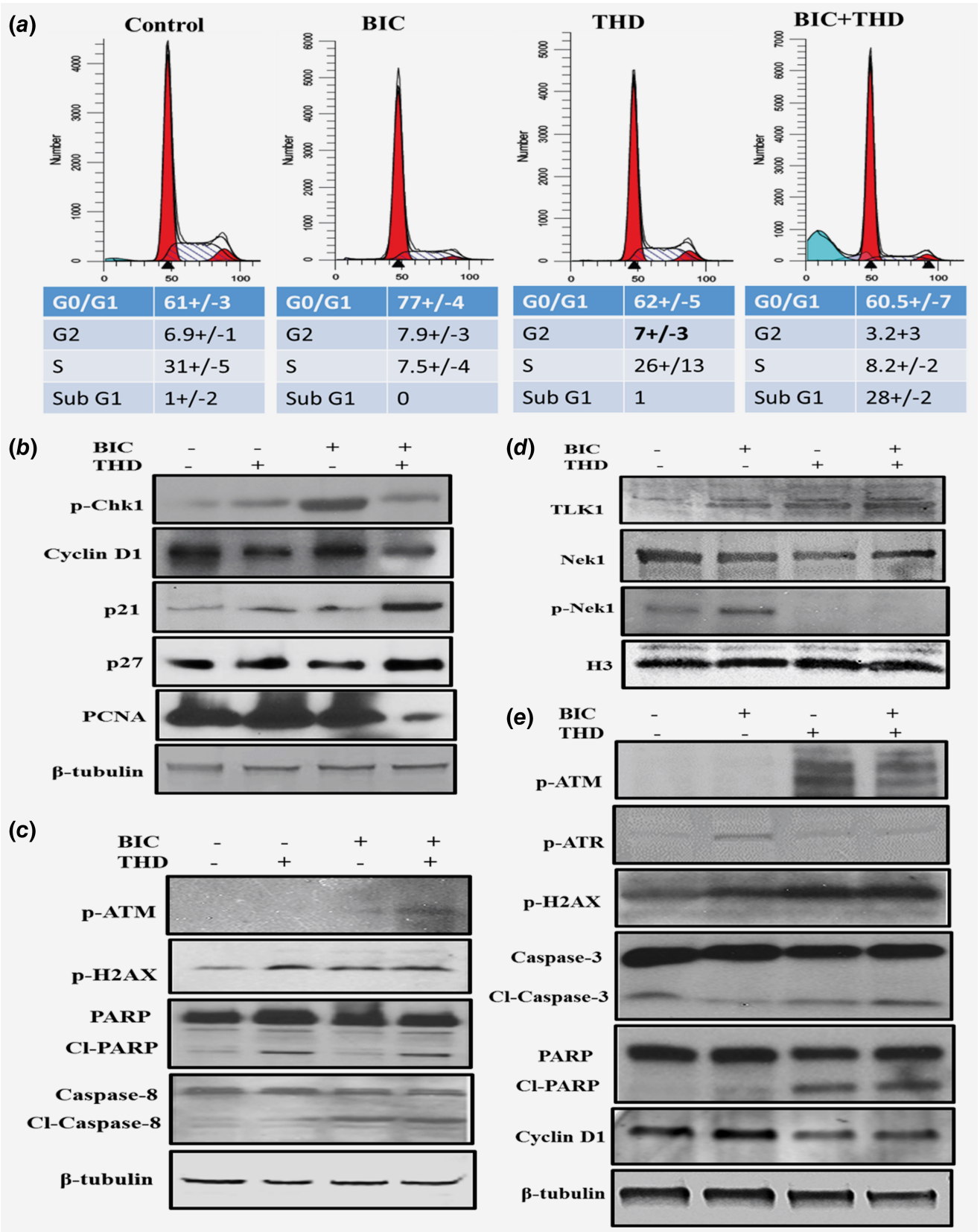

Figure 3. (*a*) Treatment of LNCaP cells with ADT + THD results in apoptosis. LNCaP cells were incubated bicalutamide (10 μM), THD (10 μM), or bicalutamide + THD for 24 h for cell cycle analysis of PI-stained cells. (*b*)–(*c*) LNCaP-Western Blots of cell cycle and apoptotic indicators. Cells from the four treatment groups as in A were analyzed by WB for several indicators of DNA damage/apoptosis and cell cycle arrest/proliferation. (*d*)–(*e*) TRAMP-C2-Western blots of several proteins involved in cell cycle and apoptosis. [Color figure can be viewed at wileyonlinelibrary.com]

when combined with bicalutamide it resulted in a drastic ~30% fraction of apoptotic cells. This result can well explain the fact that AI cells are unlikely to form in these conditions (see Figs. 2*c* and *d*).

To solidify these results, we collected the cells from the four treatment groups and determined by WB several indicators of DNA damage/apoptosis and cell cycle arrest/proliferation (Figs. 3*b* and 3*c*). Treatment with bicalutamide resulted in the anticipated increase in pChk1 (Fig. 3*b*), an indicator of its activation, and consistent with the G1-arrest shown above. In contrast, the simultaneous addition of THD suppressed Chk1 phosphorylation by abrogating the TLK1 > NEK1 > ATR > Chk1 pathway as we previously showed.[18] Bicalutamide also caused an increase in pH2AX (Fig. 3*c*), an indicator of DNA damage, all consistent with previous reports of activation of the DDR and ATR or ATM upon ADT of LNCaP cells.[3] In addition, combining THD with bicalutamide lead to activation of ATM (pS1981, Fig. 3*c*), suggesting that bypass of the Nek1 > ATR > Chk1 checkpoint may result in replication forks collapse and leading to ATM activation. THD also caused an increase in the appearance of a fraction of cleaved PARP, which is consistent with the fact that it induced an increase in apoptotic cells. However, the combination treatment did not result in further increase in cleaved PARP, although there was a significant increase in cleaved Caspase 8 in comparison to THD alone. PCNA, a marker of proliferating cells, was not strongly reduced in cells treated with bicalutamide, unlike what we had anticipated judging from the increase in G1-arrested cells. However, it was drastically reduced in the combination treatment group. Cyclin D1 was not changed with bicalutamide treatment (Fig. 3*b*), but was reduced in the THD and in the combination group. Finally, it seemed possible that bypass of the DDR elicited by bicalutamide *via* THD-mediated inhibition of the TLK1/Nek1 axis[18] would cause the emergency activation of the P53 > p21 pathway, as shown for treatment of LNCaP-C42B cells with a Chk1 inhibitor.[17] Indeed, there was a strong increase in p21 expression in the combination treatment group (Fig. 3*b*). P27 was also slightly increased in the THD treated groups.

The ADT › TLK1 › NEK1 › ATR, DDR axis is conserved in mouse TRAMP-C2 cells and is likely fundamental in PCa progression

It was our desire to be able to extend our observations to additional PCa models. Unfortunately, other than LNCaP there are not other human cell lines that can recapitulate the AS to AI conversion in culture in a short time (most are AI for growth as they were derived from metastatic lesions of ADT-patients). However, the TRAMP-C2 cells convert in culture from AS to AI growth, displaying a NE phenotype, and they are wt PTEN unlike LNCaP—we were concerned that the bicalutamide mediated activation of the AKT > mTOR>TLK1 axis would be a peculiar response to loss of PTEN. We thus tested if the DDR axis we uncovered in LNCaP cells following ADT was conserved in TRAMP-C2 cells. Treatment of TRAMP-C2 cells with bicalutamide for 16 h resulted in a strong increase in TLK1 expression (Fig. 3*d*—note that the equivalent of the TLK1B splice variant is not reported in mouse EST data). This result indicates that the compensatory prosurvival pathway of mTOR activation following ADT is conserved in the TRAMP mouse-derived model. Next, we tested if the remainder of the DDR pathway is also conserved. First we checked pNek1, whose kinase domain, including the T141 residue is completely conserved between mouse and human (94% identity). As for LNCaP, treatment of TRAMP-C2 cells with bicalutamide resulted in increased phosphorylation of Nek1-T141 without change in total Nek1 (Fig. 3*d*). In contrast, addition of THD completely abolished pNek1, even when combined with bicalutamide. ATR followed the same pattern of activation (Fig. 3*e*—pATR) consistent with its dependence on Nek1. However, THD treatment in TRAMP-C2 cells resulted in significant ATM activation (Fig. 3*e*—ATM-pS1981) even in the absence of bicalutamide, suggesting that loss of the TLK1 > Nek1 > ATR axis in these cells may result in replication forks collapse and induction of some DSBs, as also demonstrated by the presence of pH2AX. In fact, THD and more so in combination with bicalutamide resulted Caspase3 and PARP cleavage (Fig. 3*e*), suggesting that the inhibition of this DDR axis eventually results in apoptosis. Cyclin D1 was reduced in the THD and combination group, just as in LNCaP cells, an indication that proliferation was also impaired. In fact, clonogenic assays of C2 cells treated with bicalatumide, THD, or combination showed that whereas AI colonies (bicalutamide resistant) formed readily within 2 weeks, THD and THD + bicalutamide strongly impaired their growth or was toxic (Fig. 2*f*). It is important to point out that TRAMP-C2 cells, unlike LNCaP, carry a truncation mutant of TP53,[23] and therefore our observations seem to apply to at least two cell models regardless of TP53 status.

Combination bicalutamide and THD suppresses growth of LNCaP xenografts via suppression of the pNek1-pATR-pChk1 DDR pathway

We wanted to establish if the results obtained with LNCaP in culture could be translated *in vivo* as a method to suppress emergence of CRPC in a model of LNCaP cells xenograft. Following formation of sizeable tumors (~200 mm^3), castration or antiandrogens halt the progression of LNCaP xenografts for some time (2–3 weeks), while typically the subsequent regrowth is refractory to ADT (AI). Therefore, we injected LNCaP cells in matrigel subcutaneously to both of the flank in NOD-SCID mice, and then randomly assigned them to four treatment groups, as shown in Figures 4*a*–4*c*. As expected, bicalutamide arrested the growth of tumors for 10 days, but later the tumors began growing again, although slowly. Importantly, treatment with bicalutamide resulted in an increase in TLK1B expression in the tumors of 3/3 mice tested compared to controls (Supporting Information Fig. S2D), consistent with the results previously shown *in vitro*. Interestingly, treatment

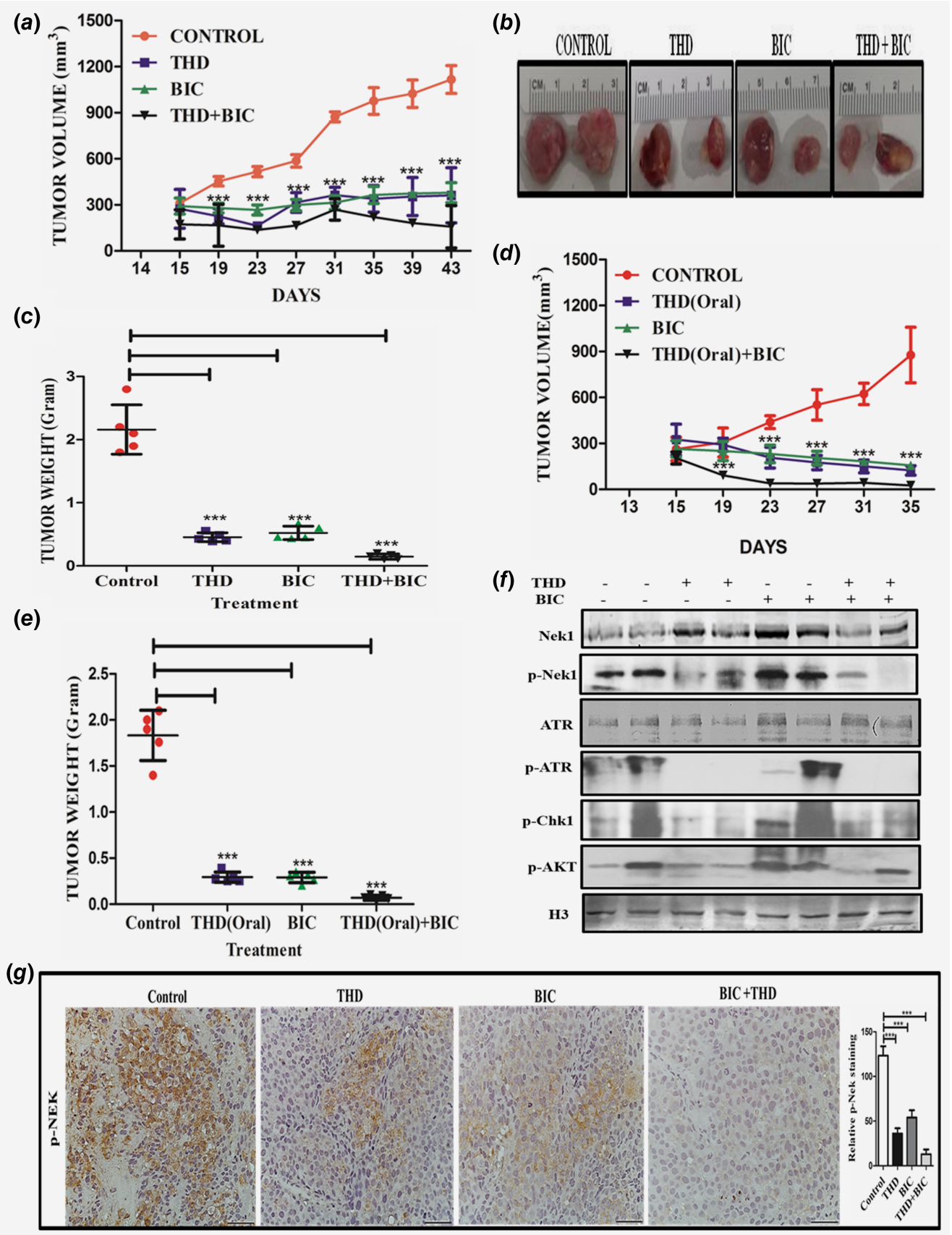

Figure 4. Legend on next page.

with THD alone resulted is a similar temporary suppression of tumor growth (***$p < 0.005$), showing that THD can have antitumor activity by itself. Importantly, the combination treatment with THD and bicalutamide resulted in complete suppression of tumor growth (***$p < 0.005$) and actually upon removal of the tumors, both volume and weight showed some regression compared to the beginning of the treatment Figures 4*b* and 4*c*.

As THD is normally given orally for schizophrenic individuals, we also tested the efficacy of THD through oral administration in the LNCaP xenografts model. We found that oral route of THD administration alone or in combination with bicalutamide (IP) was also very effective (***$p < 0.005$) in reducing the tumor burden (volume and weight) as shown in Figures 4*d* and 4*e*. Oral administration would be a much better method of treatment for PCa patients in conjunction with ADT. Further, we checked the status of the pNek1, pATR and pChk1 axis in protein extracted from residual tumors after 3 weeks of treatment, and found a significant decrease in this DDR pathway in THD alone or in combination with bicalutamide. In particular, ATR was highly phosphorylated in tumors from control mice, possibly the result of replication stress induced DDR. THD resulted in complete loss of pATR and reduced level of pNek1 and pChk1 *in vivo* (Fig. 4*f*), suggesting that abrogation of the DDR in the growing tumors may have forced their demise by inducing apoptosis, as we have shown in tissue culture. In support of this, immunohistochemistry analysis of tissue sections from LNCaP xenografts revealed that THD treatment alone or in combination with bicalutamide inhibits pNek1 (Fig. 4*g*) and decreases Ki67 (Fig. 5*a*) and Proliferating Cell Nuclear Antigen (PCNA) (Supporting Information Fig. 2C), which are markers of proliferation. The combination treatment also induces apoptosis (as shown by cleaved PARP [Fig. 5*b*] and cleaved Caspase 3 [Fig. 5*c*]) and results in increased DNA damage, as indicated by γH2AX staining (Fig. 5*d*), which can be attributed to a failure to properly activate the DDR. Representative tumor sections H&E stained are shown in Supporting Information Fig. S2B. In addition, and consistent with what was reported for treatment of ovarian cancer cells with THD,[16] tumors isolated from mice treated with THD and in combination showed a decrease in pAKT (Fig. 4*f*), an event that was linked to decreased viability of ovarian cancer cells.[16] It is noteworthy that AKT-interacting-protein (AKTIP) is a target of TLK1/1B[18] and we are investigating if THD may inhibit AKT phosphorylation *via* a TLK1 > AKTIP pathway. We also determined that bicalutamide and THD treatments did not cause apparent behavioral changes or feeding habits nor lead to significant changes in body weights (Supporting Information Fig. S2A), although we have not checked for specific organ toxicity since both bicalutamide and THD have been already extensively tested in mice and humans.

Overexpression of wt-Nek1, but not the T141A mutant, counters the growth arrest from bicalutamide and the apoptosis induced by combination with THD.

Treatment of LNCaP cells with bicalutamide results in a temporary growth arrest and morphological changes already noticeable the next day (Fig. 6, LNCaP parental). Eventually, the cells become AI independent for growth after about 2 weeks and form colonies (Fig. 2), although in combination with THD the cells instead undergo apoptosis. This can be seen in Fig. 6 (BIC + THD), which shows few residual cells with evidence of blebbing and nuclear condensation. In stark contrast, LNCaP cells overexpressing (OE) Nek1 do not appear to growth arrest when incubated with bicalutamide or even in combination with THD, suggesting that Nek1 overexpression (or increased activity) could be a key factor in the conversion to AI growth of at least LNCaP cells. Consistent with the fact that it is not just the overexpression of Nek1, but that also the kinase activity that is critical for the AI growth, we show that overexpression of the T141A mutant, previously shown to be significantly less catalytically active,[18] was unable to confer AI growth in presence of bicalutamide and resistance to apoptosis when combined with THD.

LNCaP overexpressing Nek1-T141A mutant form slow-growing tumor xenografts which remain sensitive to ADT

Overexpression of Nek1-T141A in LNCaP cells did not alter their doubling time (~32 h) or their cell cycle profile (not shown, but also see)[18] in FCS-containing medium (i.e., with androgen). In contrast, these cells had very different properties when grown as tumor xenografts (Fig. 6). First of all, in untreated animals the tumors grew much more slowly than those from parental LNCaP cells (Fig. 6*b*). In fact, at the end of the experiment (51 days from time of implantation) the tumors had just reached the size of the start point for LNCaP tumors (200 mm^3—compare with Fig. 4, day 16). Moreover, in mice treated bicalutamide starting at day 17, the tumors actually regressed. This was clearly visible at the end of the

FIGURE 4 (*a*)–(*e*) Combination bicalutamide and THD suppresses growth of LNCaP xenografts. A. Time course of tumor growth of LNCaP cells xenografts in 4 treatment groups. Treatment started after 2 weeks of implantation when the tumors measure ~200 mm^3. Bicalutamide was injected IP twice/week and THD IP an alternate days. (*b*) Examples of tumor size at the end of the experiment. (*c*) Tumor weights at the end of the experiment. (*d*) Same as in (*a*) but the mice were treated with THD given orally an alternate days. Same as in C but the mice were treated with THD given orally. (*e*) Tumor weights. (*f*) Proteins extracted from the tumors of two representative mice in each group were analyzed by western blot for the indicated key proteins of cell cycle checkpoint and also for pAKT and histone H3. (*g*) Representative sections from tumors resected from mice in the 4 treatment groups analyzed by IHC for pNek1. Note the absence of pNek1 stain in the bicalutamide+THD tumor, consistent with the results shown in F. [Color figure can be viewed at wileyonlinelibrary.com]

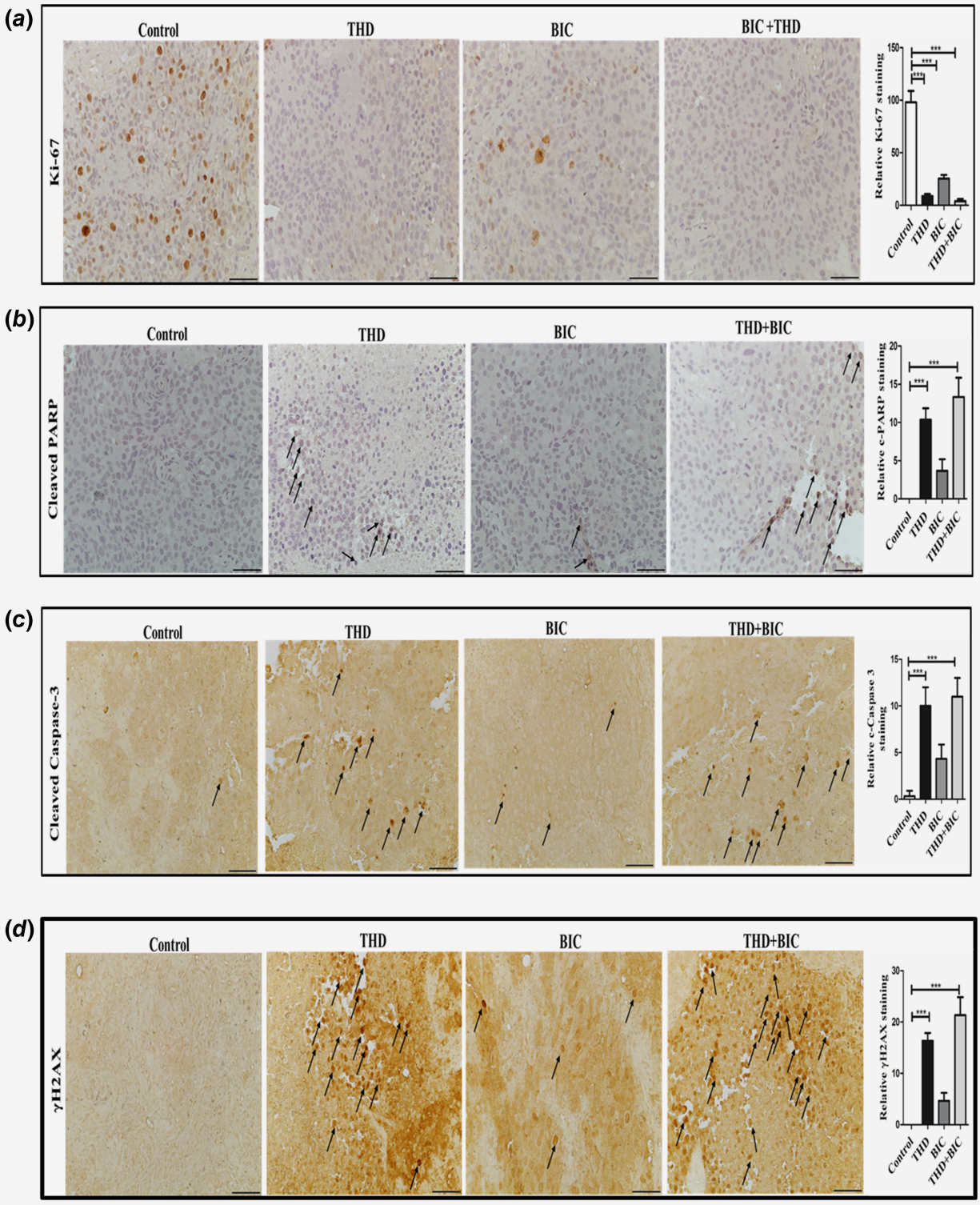

Figure 5. (*a*)–(*d*) IHC analysis of markers of proliferation (Ki67—(*a*)), apoptosis (Cleaved PARP and Caspase 3—(*b*), (*c*)), and presence of γH2AX (an indicator of DSBs—d*a*)). [Color figure can be viewed at wileyonlinelibrary.com]

experiments, where all the LNCaP-T141A tumors isolated from mice treated with bicalutamide were clearly smaller than those in the untreated group (Figs. 6*b*–6*d*). Note that the average body weights were a little less in the mice treated with bicalutamide, but all still in the normal range for their age (Fig. 6*e*).

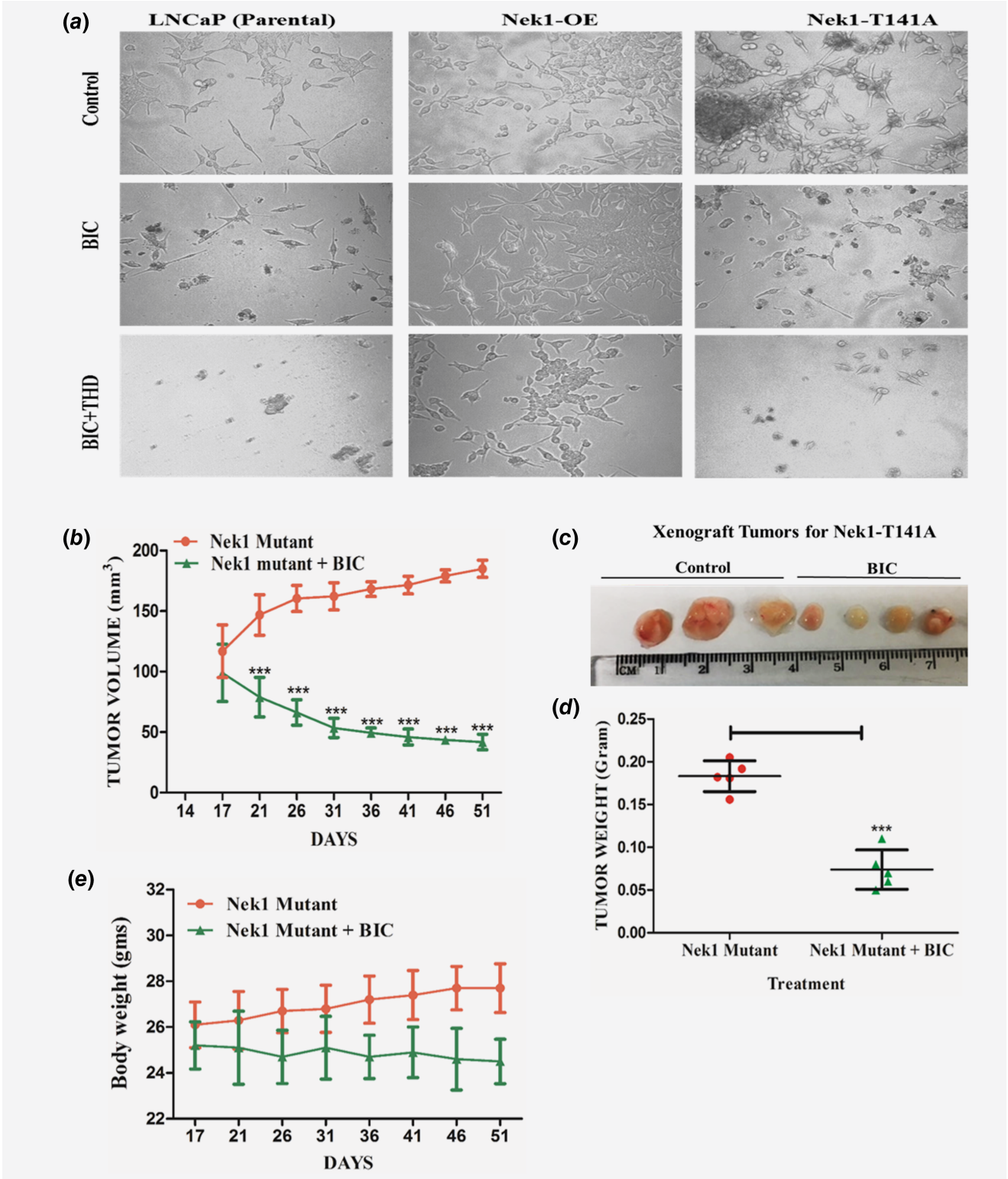

Figure 6. (*a*) Overexpression of wt-Nek1, but not the T141A mutant, counters the growth arrest from bicalutamide and the apoptosis induced by combination with THD. LNCaP parental cells and overexpressing Nek1 (wt or T141A mutant) were plated at low density and incubated for 1 week without or with bicalutamide and +/− THD. (*b*) LNCaP overexpressing Nek1-T141A form slow-growing tumor xenografts which remain sensitive to ADT. Time course of tumor growth LNCaP cells xenografts in mice treated or not with bicalutamide. Treatment started after 2 weeks of implantation when the tumors measure ~100 mm^3 (note that at this time, tumors from parental LNCaP had already reached 200 mm^3—Fig. 4). (*c*) Examples of tumor size at the end of the experiment. (*d*) Tumor weights at the end of the experiment. (*e*) Body weights of mice treated or not with bicalutamide. [Color figure can be viewed at wileyonlinelibrary.com]

Discussion

Use of phenothiazines TLK inhibitors as adjuvant to standard PCa treatment

TLK1/1B and Nek1 are functionally linked kinases[18] involved in DNA repair and are frequently overexpressed in PCa cell lines and patients' samples. The DDR is activated during ADT and is a mechanism to ensure survival from replication forks arrest induced damage. During a high throughput screen to identify inhibitors of TLK1 we have found several in the class of phenothiazine (PTH) antipsychotics, most notably THD,[11] which we propose to repurpose for the treatment of PCa to improve response to ADT. PTH antipsychotics have been used extensively for the treatment of severe psychiatric disorders for over 30 years, for prolonged periods with relatively low side effects. These include a small increased risk of cardiac arrhythmia with long term use,[24] and extrapyramidal toxicity that can be potentially permanent. Notably, several PTH antipsychotics were reported to have significant antitumoral effects with several cancer cell lines[25] and their repurposing has been proposed[26] even though their cellular targets have not been identified, and they were generally assumed to work largely through inhibition of dopamine receptors.[27] Interestingly, an analysis from five independent studies of PCa incidence in individuals with schizophrenia revealed a significant decrease in Standardized Incidence Ratio ranging from 0.49 to 0.76 (95% CI).[28] Proposed explanations for this were: specific genetic factors; antipsychotic drug effects, either by being cancer protective or decreasing testosterone; and lifestyle differences. The most compelling study specifically pointed to the use of PTH as the possible cause.[29] We propose that this is in fact the result of inhibition of TLK1/1B by PTH antipsychotics. PCa cells are subject to elevated DNA damage from replication stress, which we propose require the activity of the TLK1/NEK1 axis to repair the damage and mediate the checkpoint/DDR. Hence, inhibition of the axis will result in lethal levels of damage, causing suppression of PCa emergence in schizophrenic patients. Given these considerations, we believe that the relatively small increased risk for side effects from the use of PTH inhibitors of the TLK1/NEK1 axis can be outweighed by a more complete response to ADT, perhaps preventing progression to mCRPC altogether.

Combination ADT and THD suppresses growth of androgen responsive PCa cells via suppression of the pNek1-pATR-pChk1 DDR pathway

We previously showed that treatments that result in activation of the DDR, like oxidative stress and replication arrest, are mediated *via* the TLK1 > Nek1 > ATR > Chk1 pathway,[18] and that its suppression with THD results in checkpoint bypass and then induction of apoptosis. Likewise, treatment of androgen-responsive LNCaP and TRAMP-C2 cells with ADT results in activation of the DDR,[3] as shown here. We have shown that combination treatment of ADT and THD results in increased apoptosis and in complete arrest of progression to AI growth *in vitro* (formation of colonies). Moreover, we have shown that in xenografts, treatment of LNCaP tumors with THD resulted in bypass of ATR and Chk1 activation, resulting in increased apoptosis and suppression of tumor growth. In addition there was an increase in markers of apoptosis, like cleaved Caspase 3 and PARP, and presence of numerous γH2AX-expressing cells, a marker of DNA damage and especially of presence of DSBs. This is in support of our model that bypass of the DDR with THD can result in progression into mitosis with damaged DNA, resulting in accumulation of unrepaired DSBs and then cell death (see model in Supporting Information Fig. S3). However, we should stress that the mechanism(s) we are proposing is not quite proven, and that THD could have other effects. Nonetheless, this proposed mechanism appears to be evolutionarily conserved, as both LNCaP and TRAMP-C2 gave similar results. Moreover, we have shown in LNCaP cells that overexpression of wt-Nek1 counteracts the growth inhibition of bicalutamide and loss of viability in presence of THD, whereas the T141A mutant (a single amino acid change in Nek1) does not. Since this is a complete bypass of the TLK1/1B activity on Nek1 phosphorylation/activation, it seems like a direct effect, although we cannot rule out some other activity of Nek1 in addition to its regulation of the DDR.

NEK1 and TLK1/1B in DNA repair and DDR

The founding member of the NIMA family of protein kinases was originally identified in *Aspergillus nidulans* as a protein kinase essential for mitosis.[30] NIMA related kinases (NEKs) have adapted to a variety of cellular functions in addition to mitosis.[31] In human cells, 11 NEKs were identified that are involved in several functions. For example Nek2 is critical for centrosome duplication,[31] whereas Nek6, 7, 9 are regulators of the mitotic spindle and cytokinesis.[32] Nek1[9,33,34] and Nek4[35] have been linked to the DDR and DNA repair pathways. Nek1 promotes Chk1 activation *via* its association with ATR-ATRIP and primes ATR for the DDR.[10] Nek1-deficient cells fail to activate the checkpoint kinases Chk1 and Chk2, and fail to arrest properly at G1/S or G2/M-phase checkpoints after DNA damage. Nek1-deficient cells suffer major errors in mitotic chromosome segregation and cytokinesis.[36] However, it was not known what regulates Nek1 activity and its nuclear and cytoplasmic functions. We have recently shown that TLK1 is a key regulator of Nek1 activity, particularly in the DDR.[18] While specific inhibitors of Nek1 have not been identified so far,[37] we have now shown that reduction of Nek1 activity *via* TLK1/1B inhibition (or the T141A mutant) can sensitize PCa cells and tumors to ADT. While similar strategies have been proposed recently for treatment with ADT in combination with ATM or Chk1 inhibitors,[3,17] one problem that was apparent with those studies is how to limit the effects of general inhibitors of the DDR pathway to make them more PCa cells-specific. We are now showing that the increased expression of TLKB with ADT in LNCaP cells, a prosurvival checkpoint pathway that is enacted before conversion to AI growth, offers a unique target for attacking more specifically

PCa cells before their conversion to CRPC. Moreover, we suggest the use of THD or similar PTH derivatives, which have already a long history of relatively safe use with patients suffering from severe psychotic conditions, for the early treatment of advanced PCa still responsive to ADT.

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Authors' Contributions

V.S., P.J., I.G., H.K., X.Y. and A.D.B.: Conception and design

V.S., P.J., I.G., H.K., X.Y. and A.D.B.: Development of methodology

V.S., P.J., I.G. and A.D.B.: Acquisition of data

V.S. and P.J.: Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis)

V.S., P.J., I.G., H.K., X.Y. and A.D.B.: Writing, review, and/or revision of the manuscript

V.S., P.J., I.G., H.K., X.Y. and A.D.B.: Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases)

H.K., X.Y. and A.D.B.: Study supervision

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Original Articles

The TLK1-Nek1 axis promotes prostate cancer progression

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ABSTRACT

We recently uncovered the critical TLK1 > NEK1 > ATR > Chk1 axis in mediating the DDR and cell cycle checkpoint while transiting from Androgen Sensitive to Insensitive growth for LNCaP and TRAMP-C2 cells. However, we did not know the generality of this pathway in PCa progression since there are few cell lines where the transition has been studied. Furthermore, the identification of Nek1, and more importantly the TLK-mediated phosphorylation of T141, has never been studied in PCa biopsies. We now report the first study of a PCa TMA of p-Nek1-T141 and correlation to the Gleason score. In addition we found that TRAMP mice treated with the TLK inhibitor, thioridazine (THD), following castration did not recover cancerous growth of their prostates. Moreover, we recapitulated the process of translational increase in TLK1B expression in a naïve PDX model that was established from an AR + adenocarcinoma. Therefore, we believe that this TLK1-Nek1 mediated DDR axis is likely to be a common adaptive response during the transition of PCa cells toward androgen-insensitive growth, and hence CRPC progression, which has the potential to be targeted with THD and other TLK or Nek1 inhibitors.

1. Introduction

Standard therapy for Prostate Cancer (PCa) that has progressed beyond localized treatment control consists of androgen-deprivation therapy (ADT) which provide respite from disease progression, but ultimately fail resulting in the incurable phase of the disease: mCRPC. Targeting PCa cells before their progression to mCRPC would greatly improve the outcome. An important area of clinical work is how to target the DNA Damage Response (DDR) to kill the cancer cells, but this has been limited by general toxicity of checkpoint inhibitors, and much effort is being devoted to finding specific cancer cells' molecular targets. We have recently uncovered in two Androgen Sensitive (AS) lines – LNCaP and TRAMP-C2 – that androgen deprivation results in a temporary cell cycle arrest that protects the cancer cells from dividing with damaged DNA, and have uncovered the likely signal transduction pathway that in brief follows this pattern: ADT > mTOR > TLK1 > Nek1 > ATR > Chk1 [1]. In the rapid first step following ADT, TLK1 is translationally upregulated via the compensatory mTOR > 4EBP1 pathway [2], thereby initiating the DDR cascade that results in the cell cycle arrest. Abrogation of the DDR via adding the specific TLK inhibitor, thioridazine (THD), results in accumulation of DNA damage and DSBs that eventually lead to apoptosis, thereby preventing the progression of AS cells toward the Androgen Independent (AI) growth, both in vitro and in xenografts of LNCaP cells. In fact, tumor growth was suppressed by the combination treatment of bicalutamide plus THD. However, this work was largely mechanistic and insufficiently generalized, as only the LNCaP model, and partly the TRAMP-C2 models were used in that study. We now set up to confirm this in two more models, including a PDX and the TRAMP mice, as well as in a tissue microarray (TMA) study the generality of the TLK1-Nek1 axis as one of the earliest events during the process of adaptation of PCa cells to ADT. We further show that this is a step that can be optimally targeted in the clinics by adding specific inhibitors of TLKs, like THD, to the standard of care for advanced PCa (ADT).

2. Materials and methods

Analysis of the PCa-TMA. IRB approval was obtained for the use of the TMA, and the TMA was obtained with Informed Consent and following The Code of Ethics of the World Medical Association. TMA block

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of prostate cancer FFPE tissues was built using the Galileo CK4500 semiautomatic Tissue Microarrayer (ISENET-USA LLC, Philadelphia, USA) per manufacturer's protocol. 34 cancer and 1 benign prostate blocks were cored with a 1.0 mm core needle in triplicate. 2 Additional cores that contained only normal prostate tissue that was adjacent to PCa areas were also included. The layout was set to 12 columns by 9 rows with the first set of triplicate cores left intentionally blank for orientation purposes. Sectioning and processing of the tissues were carried out in the FWCC Histology Service, using automated processes and equipment to provide uniform and standardized results. IHC staining for this TMA and the TRAMP prostate tissues was as described in Ref. [3]. Representative IHC images were taken at ×40 magnification with an Olympus B×51 microscope. Positive staining were quantified by NIH ImageJ software with an average of three fields. 2-way ANOVA analysis was carried to compare the intensity of staining.

Animal use. All animals used in this study received humane care based on guidelines set by the American Veterinary, and approved by the Institutional Animal Care and Use Committee of LSU Health Sciences Center at Shreveport. All animal experiments were also approved by the DoD-ACURO and fully complied with the ARRIVE guidelines. 12-week-old TRAMP mice were used to observe PIN regions. We used ~16 weeks old TRAMP mice and treated with 9 dose of THD (10 mg/kg/body weight). In another set of experiment, ~20 weeks old TRAMP mice were castrated and treated with 9 dose of THD (10 mg/kg/body weight). Whole GU was collected, weighted and fixed in 4% formalin for further processing of tissues. The NSG-TM00298 PDX model was purchased from the Jackson laboratory, and propagated in 6 SCID mice until the tumors grew to ~1000 mm^3. Two mice were then treated with Bic on alternate days for one week, after which all the mice were sacrificed and the tumor isolated for protein and RNA analysis.

2.1. Cell lines

The TRAMP-C2 cell line was recently purchased from the ATCC and cultured according to their guidelines. Analysis of cell cycle by PI staining was carried out as described in Ref. [4].

2.2. List of antibodies

TLK1 antibody [N2C2], Internal-Genetex- Cat. #GTX102891.
pNek1 antibody - Purified custom - Thermofisher Scientific/Pierce.
Anti-Phospho-Histone H2A.X (Ser139) – Millipore–Cat. #05-636.
Cleaved Caspase-3 (Asp175) (D3E9) - CST-Cat. #9579.
Cleaved PARP (Asp214) (D64E10) Rabbit mAb CST-Cat. #5625.
Chr-A Antibody (C-12) Santa Cruz Cat. #393941.
Ki-67 (D3B5) Rabbit mAb – Cell Signaling - Cat #12202.
Alpha –rabbit IgG-HRP – CST-Cat.# 7074S.
Alpha -mouse IgG-HRP CST-Cat.# 7076.
Anti-beta Tubulin antibody - Loading Control (HRP)-Abcam- Cat.# (ab21058).
Tubulin Antibody (2-28-33) -Loading Control HRP Santa Cruz Cat.# −23949 HRP.

2.3. Cell cycle analysis

Tramp C2 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and treated. The next day cells were treated for one day with Biacalutamide and THD at 10 μM. Cells were washed with 1X PBS and trypsinized, washed twice in ice-cold 1X-PBS and fixed in 70% ethanol overnight. Samples were then stained with Propidium Iodine 50 μg/ml (Sigma Aldrich P4170, 3.8 mM Sodium Citrate and 0.5 μg/ml RNase A. Samples were then imperiled to FACS Calibur flow cytometer (Becton–Dickinson). Quantitative amounts of cell cycle phases (G1, S, G2, M) were determined by FACSDiva and Modfit modeling software.

2.4. Western blot analysis

Cells were lysed in 1X SDS sample buffer. Lysates were sonicated for 15 s and heated at 100 °C for 5 min. Proteins were separated on 6–12% SDS-PAGE gels and transferred to PVDF membranes (Millipore). Membranes were incubated with PBS containing 0.05% Tween 20 and 5% non-fat dry milk to block non-specific binding and were incubated with primary antibodies; membranes were then incubated with appropriate secondary antibodies conjugated to horseradish peroxidase. Immunoreactive bands were visualized using chemiluminescence reagent.

2.5. Quantification of the TLK1B mRNA expression in PDX

Total RNA was extracted from ~10 mg of tumor tissue using the QIAGEN RNeasy MiniKit. The cDNA synthesis and analysis was carried out as described in Ref. [1].

3. Results

Nek1-T141 phosphorylation increases with prostate cancer stage in primary biopsies of untreated patients. We recently reported a C-Bioportal analysis of Nek1 that revealed upregulation in 33% of patients and gene amplification in 12% of patients with CRPC-Neuroendocrine Prostate Cancer (NEPC, a type of AR negative CRPC). Moreover, Nek1 mRNA is upregulated in 21% of patients with metastatic PCa. This may implicate a function of Nek1 in PCa progression. However, this must be only part of the story because Nek1 is conformationally constrained in inactive state by an activation loop [3]. And we previously reported that TLK1 phosphorylates T141 that lies adjacent the activation loop, leading to its activation [4]. So that the phosphorylation status of Nek1-T141 is really the more critical information that we set to study in order to uncover the real significance of Nek1 in PCa progression. For this, we analyzed a TMA generated by the department of Pathology and the FWCC at LSUHSC that contained 35 PCa biopsies, one benign sample (BPH), and two cancer-free tissues in two patients that also had a cancer-positive biopsy, arranged by tumor stage and Gleason Score (GS). We previously showed that the p-Nek1affinity-purified antiserum we have generated is highly specific (does not react with Nek1-T141A mutant) and is suitable for IHC [1]. Our TMA analysis by IHC revealed that p-Nek1-T141 was elevated in tumor biopsies compared to the normal specimens (***$p < 0.001$), and that the intensity of staining correlated with the GS and tumor stage (GS7 ***$p < 0.001$, GS8 **$p < 0.01$), and where staining was weaker in the stroma and most elevated in the luminal cancer cells niches (Fig. 1A–C). Note that a pan-Nek1 antiserum we have purchased from Bethyl labs is not suitable for IHC analysis of FFPE samples, so that we cannot rule out that there was an increase in total Nek1.

The p-Nek1 staining correlates with the expression of TLK1B. We previously showed that the TLKs are likely the only kinases that phosphorylate Nek1-T141, since overexpression of several dominant negative mutants of TLK1B suppressed Nek1 phosphorylation (judged by mobility on SDS/PAGE [4]), while subsequently we showed that the addition of the specific TLK inhibitor, THD, specifically suppressed the phosphorylation of T141 [1]. Notably, the TLK1 gene was identified by co-expression analysis using WGCNA as a key driver of PCa [5], so it seemed quite possible that the increased p-Nek1 could be in part a consequence of the GS-stage related increase in TLK1B expression, and therefore that there should be a correlation between TLK1B expression and p-Nek1 This expectation was partially met since in GS8 samples the intensity of TLK1B and p-Nek1 was maximally elevated and well correlated. The difference in staining between the normal sample and the cancer samples is significant (**$p < 0.01$), and correlated with GS (GS7 **$p < 0.01$, GS8 *$p < 0.05$). However, we should note that there was not much difference between GS6 and GS7 for TLK1B, whereas p-Nek1 was increased in those samples (Fig. 1D–F).

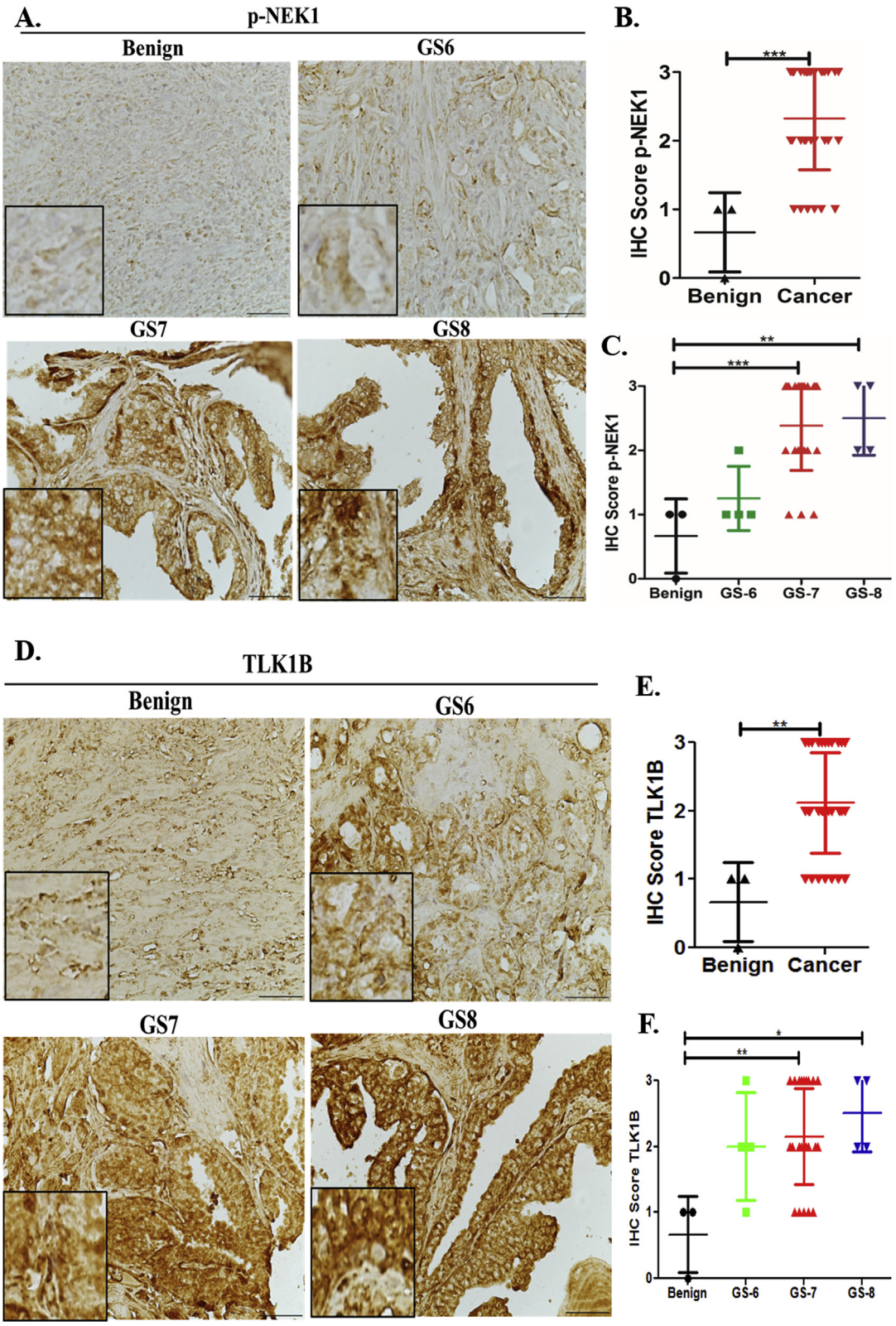

Fig. 1. A) TMA IHC analysis of phopho-Nek1-T141 (p-Nek1) is elevated in PCa in comparison to a BPH lesion and normal prostate tissue, and the staining correlates with the Gleason Score (GS). B) The expression of TLK1B increase in PCa compared to BPH and correlates with GS.

In TRAMP mice the progression to PCa correlates with increased p-Nek1 and TLK1 expression. In TRAMP mice, expression of the PB-Tag is restricted to lobes of the prostate and the temporal pattern of transgene expression correlates with sexual maturity. TRAMP mice display high grade PIN and/or well-differentiated prostate cancer by 10–12 weeks of age. Ultimately, TRAMP mice spontaneously develop invasive primary adenocarcinomas that involves most of the prostate and greatly increases its weight. Later on the cancer cells routinely metastasize to the lymph nodes and lungs [5]. Early castration (12 weeks) results in a decrease in tumor volume burden, but ultimately has

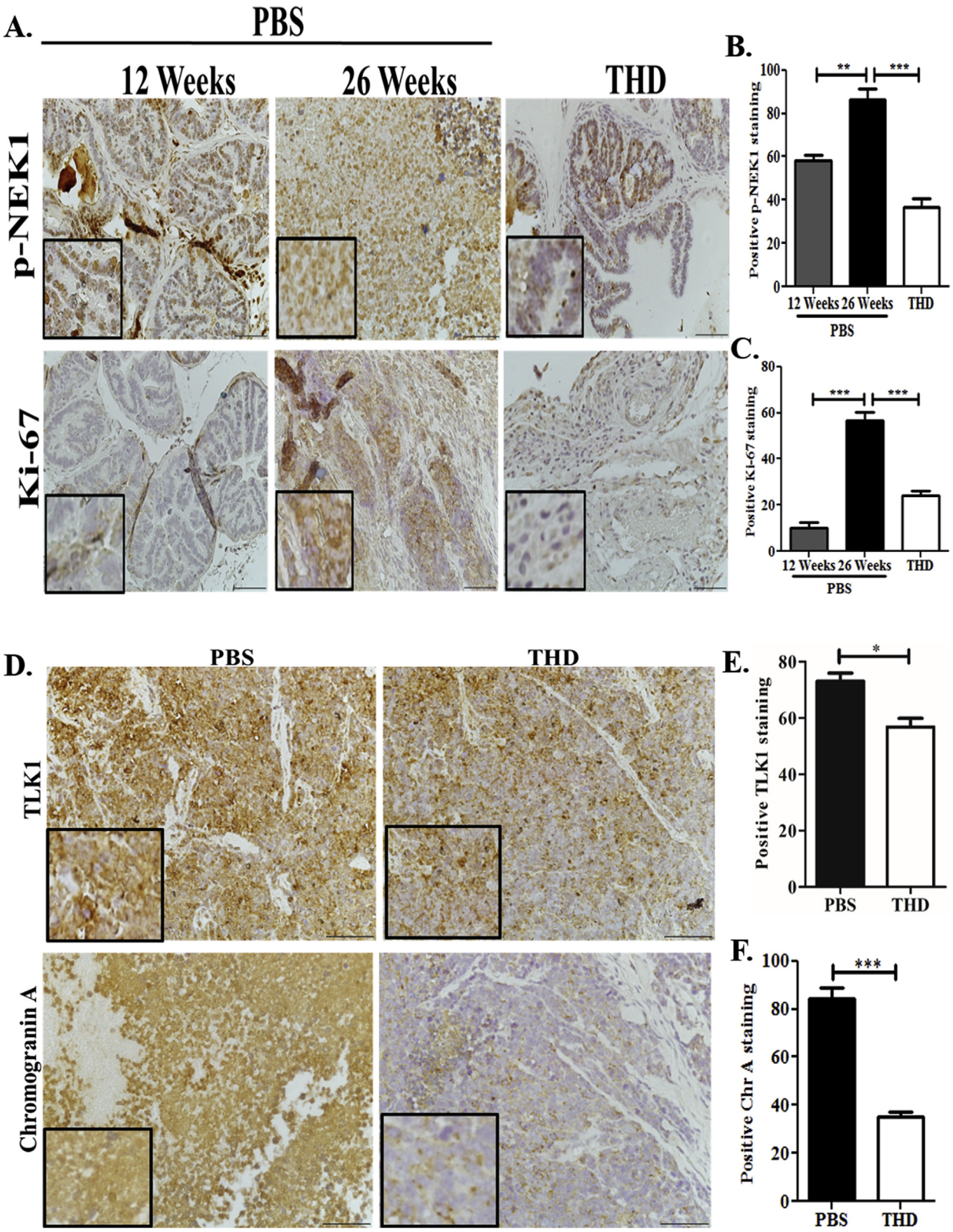

Fig. 2. IHC analysis of PCa progression in the TRAMP model. A-C) The phosphorylation of Nek1-T141 is evident by 12 weeks of age when PIN lesions begin to develop, and can be suppressed by systemic treatment with THD. In addition, the proliferative index, indicated by the % of Ki67-positive cells, can be reduced by THD treatment in 26 weeks-old mice. D-E) The expression of TLK1 is highly elevated in prostate cancer tissue from 30 weeks-old mice, but THD treatment decreases progression to NE lesions, as indicated by suppression of chromogranin staining.

no effect on time and progression to mCRPC [6], thus creating an opportunity to identify molecular targets and therapies relevant to delay or prevent progression to PCa development and later to CRPC. We thus set out to determine at 12 weeks vs. 26 weeks of age the status of p-Nek1. A group of mice were also treated with THD (inhibitor of TLKs and hence p-Nek1) to determine if this could delay cancer progression. Representative samples are shown in Fig. 2A and the quantitation in Fig. 2B (p-NEK1, PBS 12 week vs PBS 26 week, **p < 0.01). At 12 weeks, there was clear evidence of hyperproliferation of the gland and possibly evidence of PIN formation, but the cells remained nested in clearly defined acini with no evidence of stromal invasion. Ki67, a maker of proliferation, gave weak staining indicating the low level of hyperproliferation of these lesions (Fig. 2A lower panel and the quantitation Fig. 2C (Ki-67, PBS 12 week vs PBS 26 week, ***p < 0.001). In contrast, at 26 weeks, there was clear evidence of extensive cancer spread of the lobe and invasion. Correspondingly, p-Nek1 was significantly increased both in the number of cells involved and intensity. Ki67 was likewise greatly increased. In contrast, in the group of mice that were treated with THD starting at 16 weeks, two effects were obvious: 1) the phosphorylation of Nek1 was almost completely suppressed, as one would expect from the inhibition of TLK activity. 2) There was little progression from the confined PIN (or early adenocarcinomas) to the highly invasive profile seen at 26 weeks. Similarly, Ki67 staining was much weaker indicating the low proliferative characteristics of these lesions in THD-treated mice (Fig. 2B, D, 26 week PBS vs THD***p < 0.001 for P-NEK1 and Ki-67). However, in other mice treated with THD there was progression to invasive adenocarcinomas around 28 weeks of age, indicating that THD alone does not prevent eventual progression to PCa in the TRAMP model.

THD treatment of TRAMP mice decreases progression to NE lesions. The pathobiology of TRAMP mice is that poorly differentiated tumors arising in > 28-week-old mice, or at a higher frequency in castrates, express neuroendocrine markers such as chromogranin-A and synaptophysin [6]. Indeed, around 28 weeks in the TRAMP model the PCa cells convert from AR-dependent adenocarcinomas to a AR-negative neuroendocrine (NE) phenotype, which is considered a more aggressive, less treatable form of the disease in humans. At 30 weeks, there was clearly evidence of invasive cancer spread in the prostate (Fig. 2). Moreover, treatment of mice with THD did not alter much the expression of TLK1 (Fig. 2D and E, *p < 0.05), as would be expected since THD only affects the activity of TLK1 and generally not its level. However, Nek1 would not be phosphorylated in the THD treated mice, and we found that this greatly affected the NE differentiation. Whereas, the tumors from the untreated mice show strong chromogranin-A staining, the tumors from THD-treated mice showed very few cells that were stained (Fig. 2D and E, ***p < 0.001). Interestingly, as stated above, Nek1 expression was found to be elevated in a significant proportion of human NE-PCa, but the TRAMP model described here is the first evidence that p-Nek1 may be important for NE differentiation around 28 weeks of age.

The TLK1 (mouse or TLK1B in human) > Nek1 > ATR DDR axis is conserved in all ADT PCa models tested. We previously proposed that spontaneous damage from replication stress in growing PCa tumors results in frequent activation of the DDR via the ATR > Chk1 pathway, and in fact we have shown strong presence of pATR in subcutaneous LNCaP tumors of control mice, while mice treated with THD showed suppression of pATR via inhibition of the TLK1 > Nek1 > ATR DDR pathway [1]. In fact, the suppression of pATR correlated with suppression of p-Nek1 by THD. However, we needed to verify that a similar suppression of p-Nek1 (via inhibition of TLK activity) was seen in 20 weeks-old mice treated for the last four weeks with THD, similar to the experiment analyzed in Fig. 1 by IHC. To obtain a more quantitative result, the tumors-containing prostates of control mice vs. mice treated with THD were homogenized and analyzed by western blots (Fig. 3A). The prostates from all 3 control mice showed similar levels pNek1, whereas the mice treated with THD showed complete suppression of Nek1-T141 phosphorylation. Conversely, we have previously showed that ADT results in a rapid mTOR-dependent increase in TLK1 expression and a corresponding increase in Nek1 phosphorylation, which is one of its main substrates [1,4]. And in fact, castration of all 3 TRAMP mice tested resulted in increased p-Nek1 (Fig. 3B) in parallel with increased TLK1 expression that was seen in all castrated mice we have analyzed (by IHC - Fig. 5), and as previously shown also for the TRAMP-derived cell line, TRAMP-C2 [1]. We wanted to confirm the generality of this pathway also in an additional primary human PDX model from an AR-expressing adenocarcinoma: NSG-TM00298. SCID mice were implanted subcutaneously with the PDX, and after the tumors grew to ~1000 mm^3, two mice were left untreated while two received Bicalutamide (Bic) twice in one week prior to sacrifice and excision of the tumors. As anticipated, the expression of TLK1B was strongly increased in the Bic-treated mice (Fig. 3C), and correspondingly there was a strong increase in p-Nek1 (Fig. 3D). Analysis of the expression of the TLK1B splice variant by qRT-PCR revealed a ~50% reduction in TLK1B mRNA (P = 0.01) in PDX tumors isolated from mice treated with BiC compared to those untreated (Fig. 3E), supporting the idea that the mechanism of TLK1B upregulation is most likely at the translation level following activation of the mTOR > 4EBP1 axis [2].

THD treatment of TRAMP mice results in slower prostate cancer progression by increasing DNA damage and frequency of apoptosis. We previously reported that treatment of LNCaP cells with Bic or charcoal-stripped serum results in a rapid increase in TLK1B expression and the parallel activation of the DDR via the Nek1 > ATR > Chk1 pathway, and resulting in a well-known G1/S cell cycle arrest prior to their latter conversion to AI-growth [1]. Suppression of this protective DDR-induced arrest, with concomitant THD treatment, resulted in bypass of G1/S arrest and subsequent mitotically-generated DSBs and induction of apoptosis. We have now confirmed the same results with the TRAMP-derived C2 model, wherein the combined Bic + THD treatment resulted in a 30% fraction of apoptotic cells (Fig. 3F; sub-G0). To determine if a similar effect was seen during progression of TRAMP PCa tumors, mice were sacrificed at 20 weeks following four weeks of treatment with THD. We hypothesized that suppression of the DDR by inhibition of p-Nek1 with THD could result in increased unrepaired DNA damage and hence greater apoptotic response. Indeed, THD treatment resulted in increased staining for γ-H2AX (both intensity and proportion of cells), as well as staining for cleaved Caspase 3 and PARP, which are indicators of presence of DSBs and of apoptotic cells, respectively (Fig. 4A–C, PBS vs THD***p < 0.001).

Combination of castration and THD suppresses the resumption of PCa growth, CRPC progression, and results in elevated apoptotic markers in prostate tumors. When TRAMP mice are castrated at about 20 weeks of age there is a temporary suppression of PCa progression and in fact some regression of their prostates' volumes. But by 28 weeks their PCa tumors have all resumed growth and become terminal in size (humane end-point). At that time, most tumors have converted to NE type and there are frequent metastases. However, we reasoned that concomitant treatment with castration, resulting in increased TLK1 expression, and THD to suppress the TLK1 > Nek1 DDR axis, would result instead in increased apoptosis and hence restrain of PCa tumor growth/progression. Therefore, we castrated a group of 20–21 weeks old mice, and then further treated one-half with THD for four weeks. One week after the end of treatment the mice were sacrificed for analysis of their prostates. As shown in Fig. 5A–C, the prostates from many control and castrated mice had reached the terminal growth point of 4–6 gr and completely filled with cancer cells, although the prostates from 3 castrated mice still showed the initial decrease in size (regression) compared to the weight of the prostates of untreated mice. In contrast, most mice that received THD in addition to castration showed generally small prostates that on appearance had still overall normal morphology (Fig. 5A). The prostates of mice that were left untreated (PBS) compared to those treated with THD (but no

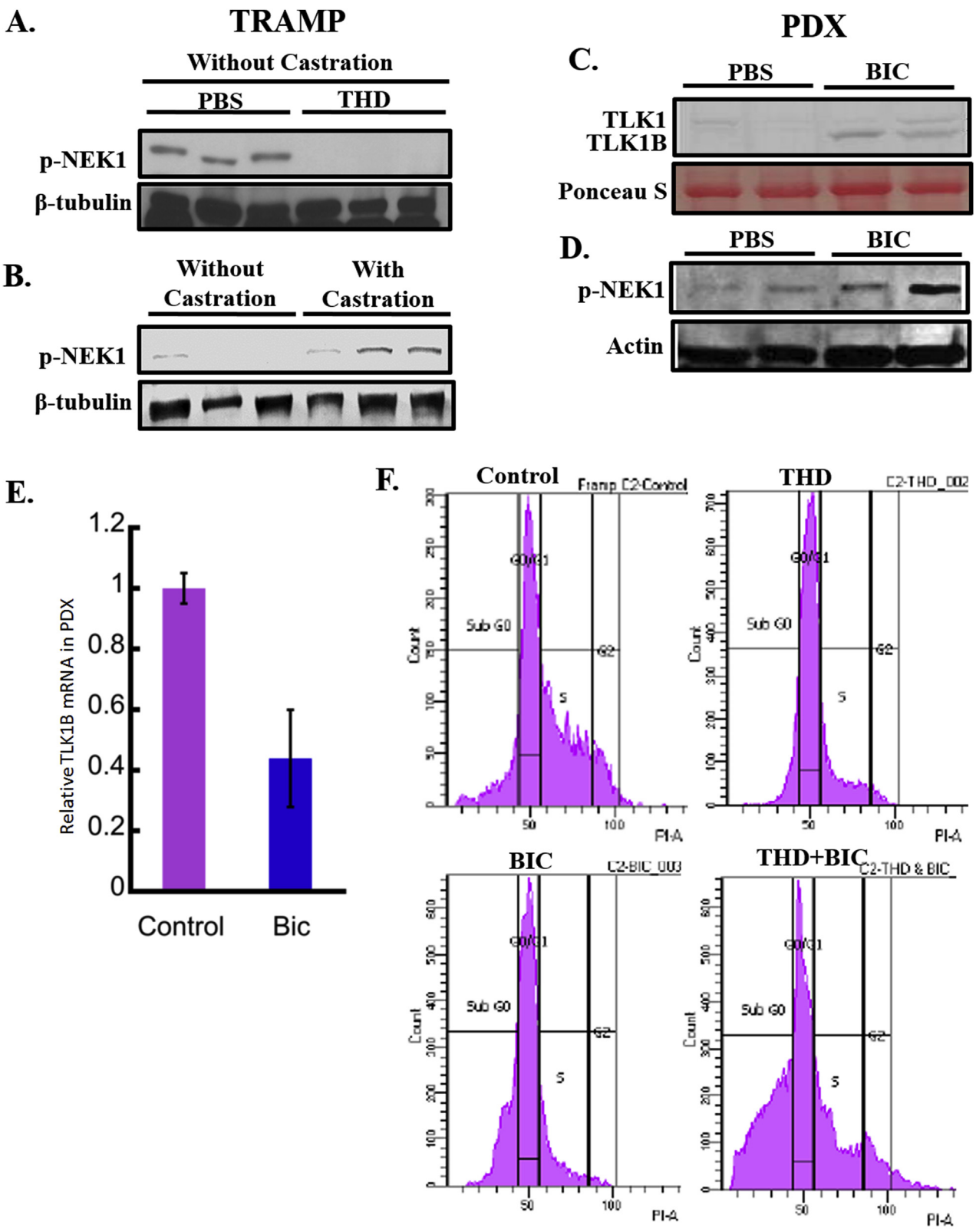

Fig. 3. A, B) WB analysis of p-Nek1 in a group of 20 weeks-old mice that have been treated with THD for 1 month (A) or one week following castration. C, D) WB analysis of TLK1B expression and P-Nek1 in the PDX model NSG-TM00298, without or with treatment with Bicalutamide (Bic), one bi-weekly dose. E) The expression of TLK1B mRNA, which was slightly reduced in the PDX mice treated with Bic (n = 4 tumors), does not correlate with the increased TLK1B protein expression. F) Treatment of TRAMP-derived C2 cells with ADT + THD results in apoptosis. TRAMP-C2 cells were incubated bicalutamide (10 μM), THD (10 μM), or bicalutamide + THD for 24 h before cell cycle analysis of PI-stained cells. Note the ~30% fraction of Sub-G0 cells in the combination treatment. Note also that whereas Bic induced a G1 cell cycle arrest with loss of the G2 population, concomitant treatment with THD prevented this, as shown by the presence of cells in S and G2.

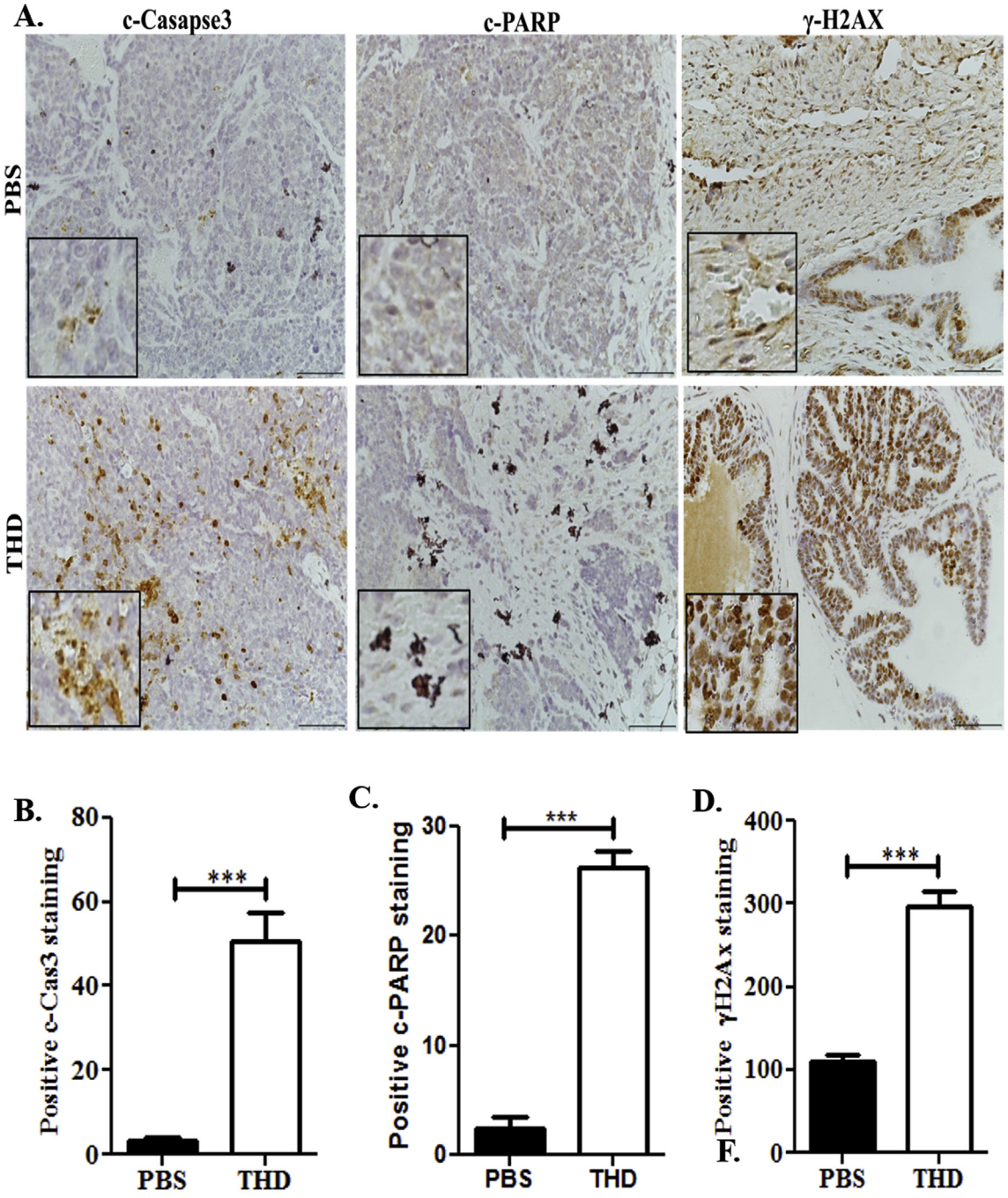

Fig. 4. THD treatment of TRAMP mice results in slower prostate cancer progression by increasing DNA damage and frequency of apoptosis. A) representative images of 26-old mice treated with THD or vehicle (PBS) showing that THD resulted in increased staining for γ-H2AX (both intensity and proportion of cells), as well as staining for cleaved Caspase 3 and PARP. B-D) Quantitations of the IHC analyses for cleaved Caspase 3 and PARP and for γ-H2AX.

castration) were similarly on average 2 gr in size, whereas those of mice that were treated with THD following castration were almost invariably regressed (~1 gr) and clearly different from most mice that were only castrated. IHC representative examples are shown in Fig. 5D and E for p-Nek1, TLK1 and Ki67. As expected, castration (ADT) resulted in increased expression of TLK1, while follow-up treatment with THD did not change this. In contrast, castration resulted in a strong increase in p-Nek1 (compare also with Fig. 1-PBS-26 weeks), whereas castration followed by THD strongly suppressed p-Nek1 (Fig. 5D and E; PBS vs THD $^{**}p < 0.01$). Cumulative evidence suggests that proliferating AS-cells are induced to undergo apoptosis by concomitant ADT + THD treatment, and consistent with this, there was a marked reduction in Ki67 (a marker of proliferating cells) staining in mice treated with THD following castration (Fig. 5D and G; PBS vs THD $^{***}p < 0.001$). To confirm that the combination of castration followed by THD results in induction of apoptosis during resumption of prostates' tumor growth at the onset of CRPC progression, we analyzed by IHC for the presence of cleaved Caspase 3 and PARP for apoptotic cells and of γ-H2AX for DSBs. Staining for all 3 markers was highly increased in castrated mice treated with THD compared to mice that were only castrated (PBS group), as shown in Fig. 6 (A-D; PBS vs THD $^{***}p < 0.001$). This is consistent with our model that treatment with THD in combination with ADT results in bypass of the DDR and hence induction of apoptosis.

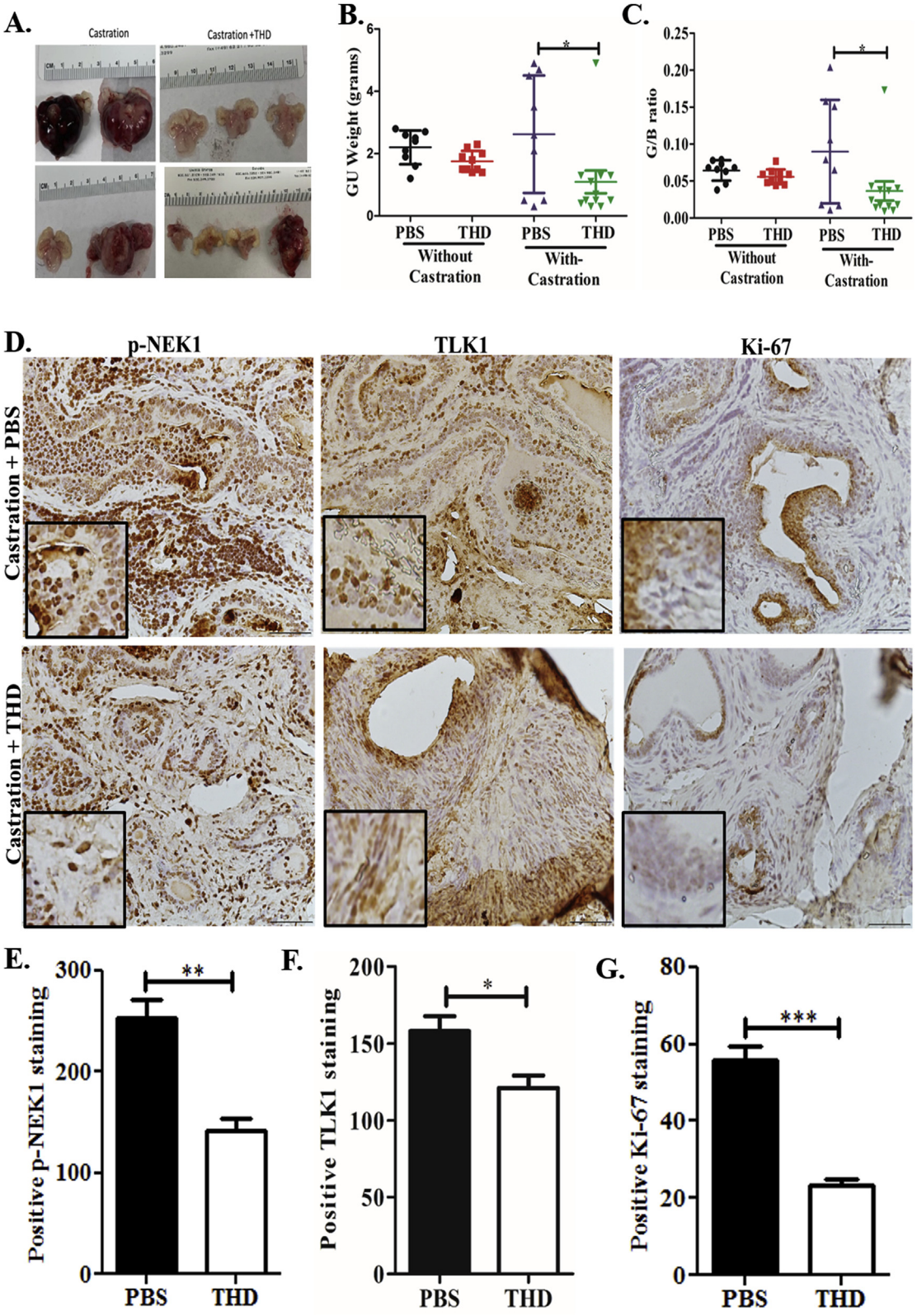

Fig. 5. A-C) Representative images of prostates and their weights in ~28-weeks old mice castrated at 20–21 weeks and treated or not with THD for 4 weeks, prior to sacrifice one week later. D-E) Representative IHCs stained for P-Nek1, TLK1 and Ki67 from the same animals. Note the reduction in P-Nek1 and Ki67 staining in the mice that were treated with THD.

4. Discussion

The Tousled Like kinases (TLK1 and TLK2) function in several processes including chromatin assembly, replication, transcription, DNA repair, and chromosome segregation (rev. in Refs. [7,8]). Expression of TLKs is frequently elevated in cancer and correlates with malignancy, prognosis and response to therapy [9,10], while their inhibition or suppression of expression sensitizes cancer cells to DNA

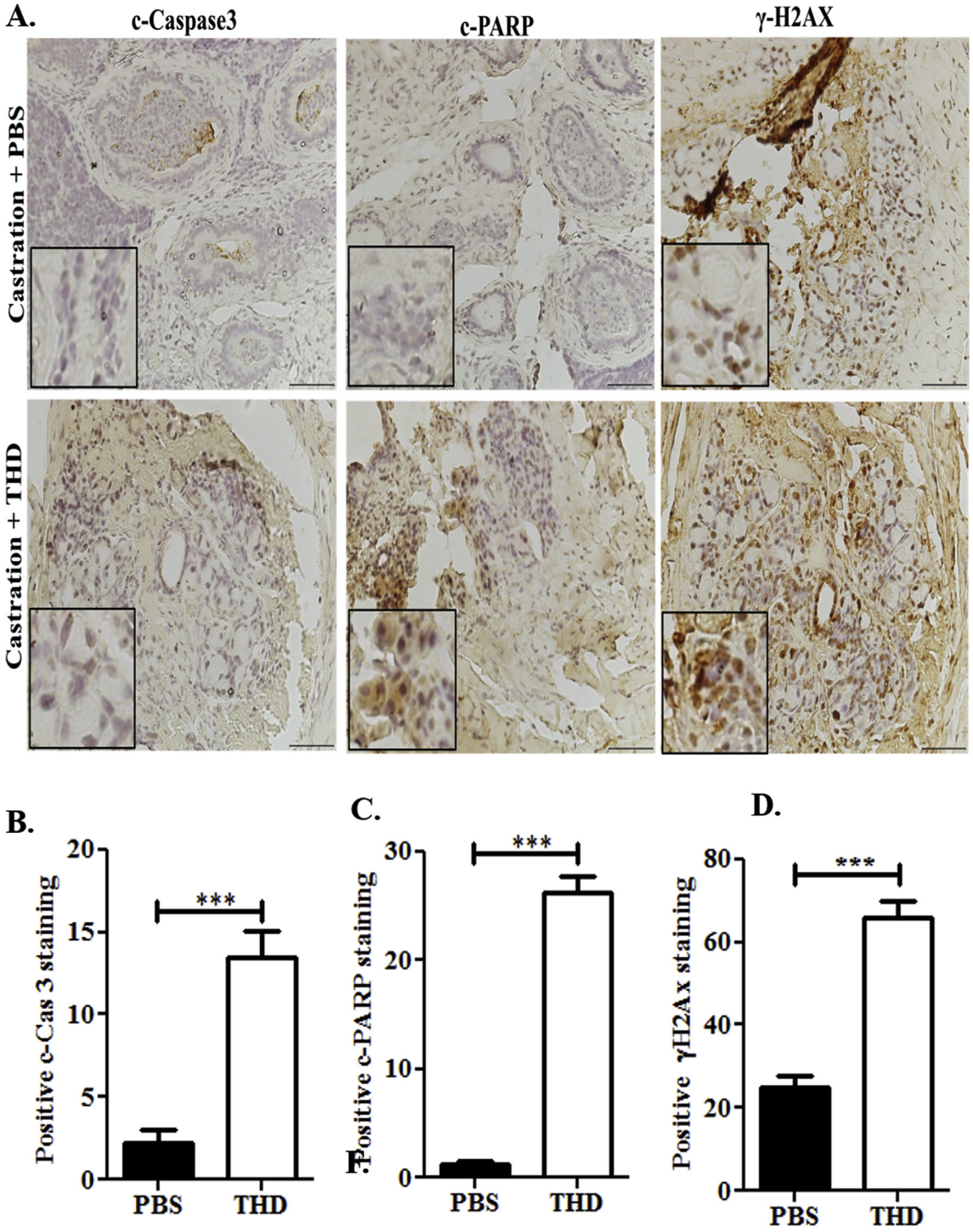

Fig. 6. A) Combination of castration and THD suppresses the resumption of PCa growth and results in elevated apoptotic markers in prostate tumors. Representative IHCs stained for cleaved Caspase 3 and PARP and for γ-H2AX. Note the significantly elevated staining for these markers in the mice treated with THD, which is quantitated in (B–D).

damaging agents [11] or PARP inhibitors that diminish repair [10]. Notably, the TLK1 gene was identified by co-expression analysis using WGCNA as a key driver of PCa, highly enriched among candidate genes collected from expression Quantitative Trait Loci (eQTL), somatic copy number alterations (SCNA), and prognostic analyses [12], and the expression of TLK1B was found elevated in several PCa cell lines in comparison to RWPE1 normal prostate line [13]. Expression of TLKs appears to be controlled at both transcription and translation levels [10,14,15] and the human splice variant TLK1B is particularly dependent on the mTOR-4EBP1-eIF4E pathway [14], a nexus which is frequently upregulated in cancer [16,17] and constitutes a key therapeutic target [18]. Recently we have identified the proteome complement of TLK1/1B, and have identified the protein kinase Nek1 as one of its principal targets [4]. Nek1 activation depends on its phosphorylation by TLK at T141, and this in an early DDR event upstream of ATR and Chk1 upon oxidative damage or replication arrest [4], extending earlier studies on Nek1 [19]. We have now shown that preventing Nek1 activation by inhibiting TLK functions with THD results in

significant delay in the progression of the TRAMP cancer model, both in carcinogenic progression of the prostate glands and even later during NE differentiation. In addition, the expression of TLK1 and p-Nek1 was correlated with each other and with the tumor grade (GS) in the (admitted limited) number of PCa biopsies that were available to us. This suggests that the TLK1 > Nek1 axis, in addition to being a critical mediator of the DDR [1,4] may have additional functions during progression of PCa, particularly during conversion to CRPC. In fact, expression analysis Nek1 revealed upregulation in 33% of patients and gene amplification in 12% of patients with NE-CRPC. Nek1 is the first identified member in human of the NIMA family of protein kinases that was originally identified in *Aspergillus nidulans* as a protein kinase essential for mitosis [20]. NIMA related kinases (NEKs) have adapted to a variety of cellular functions in addition to mitosis [21]. In humans, 11 NEKs were identified that are involved in several functions, whereby Nek1 [22–24] and Nek4 [25] have been mostly implicated in the DDR and DNA repair pathways [26]. Nek1 promotes Chk1 activation via its association with ATR-ATRIP and primes ATR for the DDR [19], and its suppression abolishes the checkpoint and results in genomic instability [22,27]. Not all the substrates of Nek1-11 have been fully elucidated, and the majority of NEKs are involved in more than one of the three NEKs core functions: (1) centrioles/mitosis; (2) primary ciliary function/ciliopathies; and (3) the DNA damage response (rev. in Ref. [21]). Deletion mutations in the Nek1 gene in mice cause polycystic kidney disease (PKD) and other pleiotropic effects, ranging from facial dysmorphism, dwarfing, male sterility, anemia and cystic choroid plexus. The pleiotropic nature of these phenotypes suggested a role of Nek1 early on in development [28]. But even later in life, it was recently discovered that certain mutations in Nek1 confer susceptibility to development of ALS [29], all of which suggest that Nek1 may be critically involved in controlling disease progression and development. We recently uncovered the TLK1 > Nek1 axis in regulating the DDR [4] and have recently reported that its inhibition with THD results in bypass of the checkpoint response and induction of apoptosis in vitro and in xenografts [1]. However, we have now shown that the TLK1 > Nek1 axis may affect additional aspects of PCa progression, and particularly regulate CRPC and NE progression, thus offering an even more important target for development of specific inhibitors of this pathway.

Authors' contribution

Conception and design: V. Singh, P. Jaiswal, I. Ghosh, H. Koul, X. Yu, A. De Benedetti.

Development of methodology: V. Singh, P. Jaiswal, I. Ghosh, H. Koul, X. Yu, A. De Benedetti.

Acquisition of data: V. Singh, P. Jaiswal, I. Ghosh, A. De Benedetti.

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Singh, P. Jaiswal.

Writing, review, and/or revision of the manuscript: V. Singh, P. Jaiswal, I. Ghosh, H. Koul, X. Yu, A. De Benedetti.

Administrative, technical, or material support**:** V. Singh, P. Jaiswal, I. Ghosh, H. Koul, X. Yu, A. De Benedetti.

Study supervision: A. De Benedetti, H. Koul, X. Yu.

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Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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