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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.

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

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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). This year of funding, 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. Moreover, THD significantly inhibited the colony formation efficiency of several PCa cells *in vitro* (even of androgen independent lines). Intra-Peritoneal (I.P.) 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.

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: The Tousled Like kinases (TLKs) are involved in numerous cellular functions, including the DNA Damage Response (DDR), but only a handful of substrates have been identified thus far. Through a novel proteomic screen, we have now identified 165 human proteins interacting with TLK1, and we have focused this work on NEK1 because of its known role in the DDR, upstream of ATR and Chk1. TLK1 and NEK1 were found to interact by coIP, and their binding is strengthened following exposure of cells to H2O2. Following incubation with doxorubicin, TLK1 and NEK1 relocalize with nuclear repair foci along with γH2AX. TLK1 phosphorylated NEK1 at T141, which lies in the kinase domain, and caused an increase in its activity. Following DNA damage, addition of the TLK1 inhibitor, THD, or overexpression of NEK1-T141A mutant impaired ATR and Chk1 activation, indicating the existence of a TLK1>NEK1>ATR>Chk1 pathway. Indeed, overexpression of the NEK1-T141A mutant resulted in an altered cell cycle response after exposure of cells to oxidative stress, including bypass of G1 arrest and implementation of an intra S-phase checkpoint.

Second paper: 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.

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.

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) but we have not yet establish if 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 (our extensive observations and see also [1]). So, we are not sure that we will be able to obtain a definitive result, but we will try to determine if the reported interaction of Nek1 with ATR/ATRIP depends on presence of TLK1 We have also obtained a 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 second paper in the Appendix, 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, in the paper published in Cell Cycle, we have demonstrated biochemically that the phosphorylation of T141 is critical for maximal activity of Nek1.

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. 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 started a colony of TRAMP mice 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. We have not yet started the time-consuming breeding of the Nek^{kat} mice for their crossing to TRAMP because we reasoned that successful completion of subtasks 2.1 and 2.3 took precedence and form the essential logical basis for subsequently generating the more complex transgenic model.

Subtask 2.5. We have purchased the CRSPR/Cas9-sgRNA construct from Origene, and have begun growing the NeoTag cells for the gene disruption. **Subtask 2.6** will begin after we have characterized the KO cells more completely in vitro.

Participants

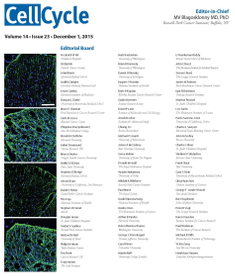
The following people (all in the Department of Biochemistry and Molecular Biology and the Feist Weiller Cancer Center at LSUHSC) have participated in this research: Vibha Singh, Praveen Kumar Jaiswal, Ishita Ghosh, Hari K. Koul, Xiuping Yu, and Arrigo De Benedetti

1. Segura-Bayona S, Knobel PA, Gonzalez-Buron H, Youssef SA, Pena-Blanco A, Coyaud E, Lopez-Rovira T, Rein K, Palenzuela L, Colombelli J *et al*: **Differential requirements for Tousled-like kinases 1 and 2 in mammalian development**. *Cell Death Differ* 2017, **24**(11):1872-1885. doi: 1810.1038/cdd.2017.1108. Epub 2017 Jul 1814.

Appendix (2 papers: One published, one under review at IJC):

1. Singh, V, Connelly, Z, Shen, X, and De Benedetti, A (2017) Identification of the proteome complement of humanTLK1 reveals it binds and phosphorylates NEK1 regulating its activity. Cell Cycle 16:915-926.

2. Singh, V., Jaiswal, P., Ghosh, I., Koul, H., Yu, X., De Benedetti, A. Targeting the TLK1/NEK1 DDR axis with THD suppresses outgrowth of Androgen Independent PCa cells



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EXTRA VIEW

Identification of the proteome complement of humanTLK1 reveals it binds and phosphorylates NEK1 regulating its activity

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ABSTRACT

The Tousled Like kinases (TLKs) are involved in numerous cellular functions, including the DNA Damage Response (DDR), but only a handful of substrates have been identified thus far. Through a novel proteomic screen, we have now identified 165 human proteins interacting with TLK1, and we have focused this work on NEK1 because of its known role in the DDR, upstream of ATR and Chk1. TLK1 and NEK1 were found to interact by coIP, and their binding is strengthened following exposure of cells to H2O2. Following incubation with doxorubicin, TLK1 and NEK1 relocalize with nuclear repair foci along with γH2AX. TLK1 phosphorylated NEK1 at T141, which lies in the kinase domain, and caused an increase in its activity. Following DNA damage, addition of the TLK1 inhibitor, THD, or overexpression of NEK1-T141A mutant impaired ATR and Chk1 activation, indicating the existence of a TLK1>NEK1>ATR>Chk1 pathway. Indeed, overexpression of the NEK1-T141A mutant resulted in an altered cell cycle response after exposure of cells to oxidative stress, including bypass of G1 arrest and implementation of an intra S-phase checkpoint.

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ATR; cell cycle checkpoint activation; Nek1; regulation of DNA damage response; replication stress; TLK1

Introduction

The human Tousled kinases (TLKs) are involved in DNA repair and are frequently overexpressed in several cancers (rev in[1]). From a proteomic screen to identify the targets of TLK1B, we have identified NEK1, an important kinase that mediates the DNA damage response (DDR), thereby opening up a new avenue for a possible role of TLKs in dealing with the damage.

The ability of cells to sense and signal DNA damage is critical for genomic stability, and defects in this process are intimately linked to genomic instability and cancer etiology. In human cells, 2 checkpoint kinases, ATM and ATR are central effectors of the DNA damage relay.[2] Whereas ATM primarily responds to double-stranded breaks (DSB), ATR activity is elicited by a variety of DNA damaging agents and replication stress,[3] typically resulting in regions of ssDNA. ATR functions in a complex with its activating partner ATRIP.[4] RPA-coated ssDNA, a common intermediate of DNA replication and repair, plays a key role in recruiting and activating ATR-ATRIP, as well as recruiting the Rad9-Rad1-Hus1 (9–1–1) complex,[5,6] another effector of the DDR that we identified as being regulated by TLK1.[7,8] Once activated ATR phosphorylates its downstream effector kinase, Chk1, and together orchestrate the repair pathway and the cell cycle checkpoint. While the ATR-Chk1 kinases constitute a backbone of the DDR, other protein kinases modulate the strength of the response, including ATM, CDKs, PLK1, AKT, and important to this proposal, NEK1 (Never in Mitosis A-related kinase 1)[9-11] and TLK1.[7,12,13]

Tousled Like kinases (TLK1 and TLK2) are serine/threonine kinases, which localize to the cytoplasm and nucleus and are known to autophosphorylate and dimerize.[14] Our laboratory identified a splice variant of TLK1 that encodes a 65 kDa protein (named TLK1B) that becomes translationally upregulated in response to genotoxic stress, including IR.[13] TLK1B is similar to TLK1 but lacks the first 169 aa. TLK1 and TLK1B share the same catalytic domain and we often refer them to as TLK1/1B. TLK1B overexpression protects cells from the genotoxic effects of IR.[12] Until now only a few direct interacting substrates of TLK1/1B have been identified, primarily histone H3, the histone chaperone Asf1, and Rad9.[7,15,16] As expected from the interacting substrates various studies have shown that TLKs play an important role in chromatin assembly, DNA repair, transcription, and chromosome segregation.[17-19] Knockout of the single TLK in *Drosophila* and *C. elegans* leads to an arrest in embryonic development,[17,18] and expression of a dominant negative mutant in mouse cells causes missegregation of chromosomes resulting in aneuploidy.[20] TLK1/1B overexpression is observed in breast cancer, prostate cancer and cholangiocarcinoma, which often corresponds to reduced sensitivity toward chemotherapy due to efficient repair in those tumors (rev in[1]). TLKs are regulated in a cell-cycle dependent manner and are maximally active during the S phase [21] and M.[22] Recently, we

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Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/kccy.

[†]Equal co-contributors

Supplemental data for this article can be accessed on the publisher's website.

and others have found that TLK1 and TLK2 play critical roles in DDR recovery,[8,23] and as guardians of the genome.[1] Hence, it was critical to identify additional substrates/interacting partners of TLK1/1B to elucidate the function of these proteins in the DDR.

The founding member of the NIMA family of protein kinases was originally identified in *Aspergillus nidulans* as a protein kinase essential for mitosis.[24] NIMA related kinases have adapted to a variety of cellular functions in addition to mitosis.[11] In human cells, 11 NEKs were identified that are involved in several functions. For example NEK2 is critical for centrosome duplication,[11] whereas NEK6, 7, 9 are regulators of the mitotic spindle and cytokinesis.[25] NEK1,[9,10,26] and NEK4[27] have been linked to the DDR and DNA repair pathways. NEK1 mediates Chk1 activation via an unclear mechanism since 2 papers reported opposite results using siRNA depletion experiments. One paper reported that NEK1 functions independently of ATR and does not affect ATR activity.[10] The other reported that NEK1 associates with ATR-ATRIP and primes ATR for the DDR.[28] NEK1 activity and relocalization to nuclei was reported to increase upon a variety of genotoxic stresses.[9,10] A defect in DNA repair in NEK1-deficient cells is suggested by persistence of DSBs after low dose IR. 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.[26] NEK1-deficient cells suffer major errors in mitotic chromosome segregation and cytokinesis, and become aneuploid.[29] Genomic instability is also manifested in NEK1+/− mice, which late in life develop lymphomas with a higher incidence than wild type littermates.[29] However, it is not known what regulates NEK1 activity and its nuclear and cytoplasmic functions. Recently NEK1 was found to phosphorylate Rad54 at S572 regulating its activity in HR that in turn promotes the dissociation of Rad51 from DNA filaments during HR in G2 or during S at stalled forks.[30] We propose that TLK1, which we herein identify as interacting strongly with NEK1, is a key regulator of its activity, particularly in the DDR.

Results

Identification of TLK1B interacting proteome

To identify additional TLK1B interacting proteins, we obtained from Life Technologies a proteomic interaction study using biotinylated TLK1B to interrogate their ProtoArray system (9000 full length and native human proteins arrayed in duplicates on glass slides). The result included 165 proteins identified with high confidence. In Table S1 the hits are shown with signal intensity, negative signal (streptavidin only), concentration of TLK1B used in the assay, Z-score, variance between the 2 spots, and CI P-value (Chebyshev's Inequality P-value). The proteins were grouped by function with Reactome pathway database (http://www.reactome.org/) (Table 1), and the majority of hits were proteins involved in the cell cycle or DNA repair, although clearly TLK1B interacts with proteins involved in a variety of functions.

For DNA repair, the proteins are in order of signal intensity: NEK1; APEX2; UBR2; RAD54; Deoxynucleotidyltransferase; LATS1; AlkB; FANCM; UVSSA. From these, we decided to focus our immediate attention on NEK1. NEK1 was reported to be a key modulator and interacting partner of ATR/ATRIP [28], and we had already identified a pathway by which TLK1 could regulate ATR via modulation of Rad9 activity.[8]

Table 1. Functional pathway interactions hierarchal order (Reactome Pathway Analysis)

Cell Cycle
DNA Repair
Cell-Cell communication
Cellular responses to stress
Chromatin organization
Circardian Clock
Developmental Biology
DNA Replication
Extracellular matrix organization
Gene expression
Hemostasis
Immune system
Mitophagy
Metabolism
Metabolism of proteins
Cell motility
Neuronal system
Organelle maintenance
Programmed cell death
Signal transduction
Transmembrane transport of small molecules
Vesicle-mediated transport

NEK1 interacts with TLK1B

We have confirmed the interaction of TLK1B with NEK1, as GST-TLK1B pull-down (Fig. 1A) and by reciprocal immunoprecipitation of TLK1 and NEK1 (Fig. 1C, D). In addition, we found that the association of TLK1 with NEK1 is strengthened by DNA damage (H2O2 which induces SSBs and DSBs). The function of the interaction of TLK1/1B with NEK1 remains to be established. With 2 previously known substrates of TLK1/1B (Asf1 and Rad9) the TLK1 protein functioned both as a chaperone and as a kinase, with distinct activities depending on the reactions under study.[7,8,31] The NEK1 protein typically migrated as 2 well-separated bands, and the slower migrating form can be converted to the faster form by treatment with phosphatase CIP (Fig. 1B). It remains to be established if the phosphorylated form of NEK1 is the catalytically more active and if its phosphorylation is somehow regulated by TLK1/1B.

TLK1 and NEK1 relocalize with γH2AX nuclear foci after induction of DSBs

We determined by IF the localization of TLK1, NEK1, and pH2AX (γH2AX) in untreated cells or cells treated for 4h with doxorubicin, which results in production of DSBs that rapidly become decorated with pH2AX. After DNA damage, NEK1, which is mostly cytoplasmic in untreated cells (Fig. 2), relocalizes to the nuclei at sites of damage[10] and colocalizes with pH2AX staining. The NEK1 signal also increases as it was reported that its expression rapidly increases in presence of DSBs.[32] In undamaged cells, the distribution of TLK1 is equally localized in both cytoplasm and nuclei (Fig. 2) as previously reported.[16] Upon induction of DSBs with doxorubicin, the expression of the TLK1B splice variants is rapidly induced,[13]

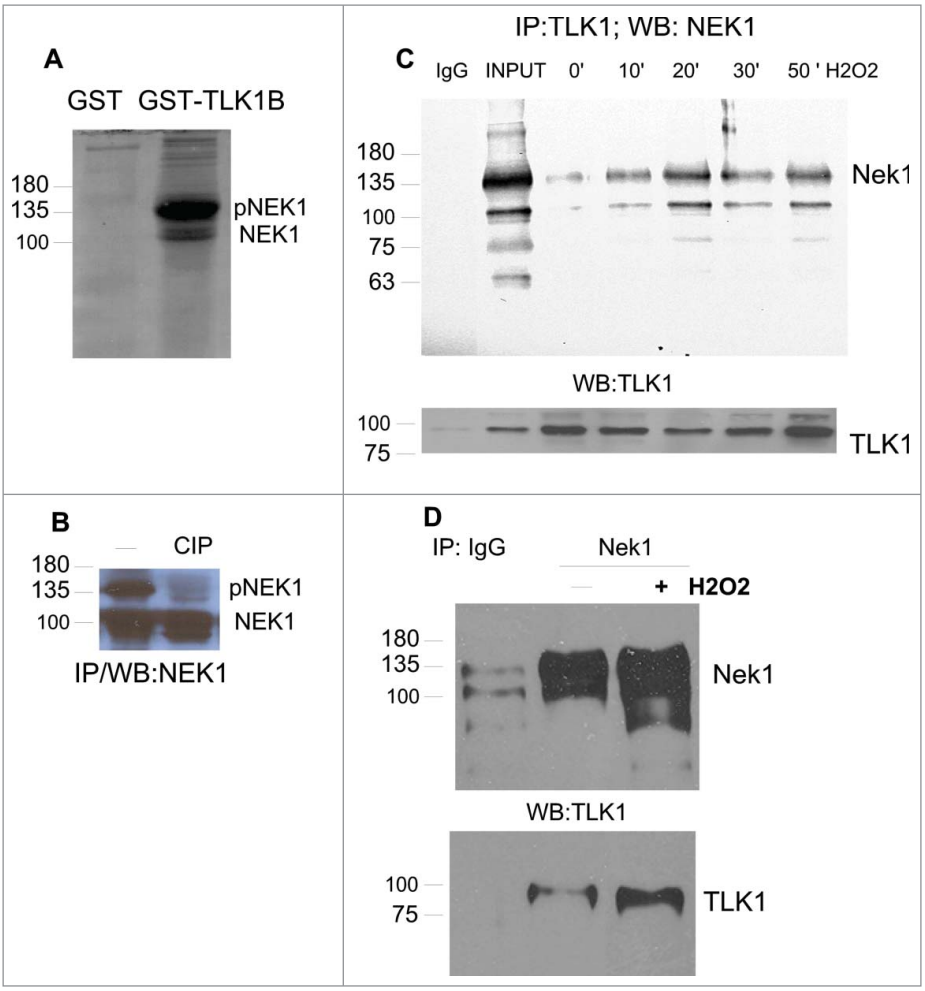


Figure 1 . TLK1B binds NEK1. (A) NEK1 was pulled-out with GST-TLK1B, but not GST, from 0.3 mg of Hek293 cell extract. (B) Immunoprecipitated NEK1 was treated with CIP to establish the mobility of the phosphorylated form. (C) The interaction between TLK1 and NEK1 is strengthened upon DNA damage. Hek293 cells were exposed to 0.2 mM H2O2 for the indicated time. Extracts were prepared and immunoprecipitated with TLK1 antiserum and probed for coIP with NEK1 serum, or for TLK1 (bottom). EXT (cell extract).

resulting in the increased IF signal in these cells. We previously reported that, in contrast to the TLK1 isoform, TLK1B preferentially localizes to nuclei.[16] Therefore, treatment with doxorubicin results in both overall IF signal increase and in particular nuclear, although simple relocalization of TLK1/1B may also occur and explain the observation. Using a different NEK1 antibody (a mouse monoclonal) we were also able to show that a large fraction of TLK1 colocalizes in the nuclei with NEK1 after treatment with doxorubicin (Fig. 2), further demonstrating that the 2 proteins interact more avidly in presence of DSBs. In conclusion, both NEK1 and TLK1 colocalize preferentially to nuclei and with pH2AX upon induction of DSBs. Since we have shown that the TLK1-NEK1 interaction is strengthened upon DNA damage, it is likely that they both form a complex at sites of damage, decorated with pH2AX.

TLK1B phosphorylates NEK1 in the NT-kinase domain

NEK1 was identified as a dual specificity kinase during a screen to identify proteins phosphorylated on Tyr.[33] NEK1 contains an N-terminal kinase domain of approximately 260 aa, while a prominent portion of the NEK1 middle and C-tail is composed of several predicted coiled coil domains that were reported to promote dimerization and autophosphorylation[33] as well as interaction with other proteins.[34]

To determine if TLK1B can phosphorylate NEK1 directly, we produced all recombinant proteins: GST-TLK1B; His-NEK1-FL (full length); His-NT-NEK1 amino-terminus kinase domain (first 480 aa); and His-CT-NEK1 (carboxy terminus 777 aa containing the coiled-coil repeats). NEK1 isolated from bacteria was already autophosphorylated (see below) and had very weak additional autophosphorylation with γ-ATP[32] (see Fig. 4B). Thus, we were able to carry out phosphorylation studies with TLK1B. In Fig. 3, we show that TLK1B phosphorylated very efficiently the NEK1-NT but weakly the NEK1-CT. A parallel western blot shows the NEK1-CT and NEK1-NT purified proteins.

TLK1B phosphorylates NEK1-NT at T141

We performed phosphorylation reactions with FL-NEK1 or NT-NEK1 and GST-TLK1B, to determine the phosphorylation sites by LC-MS of tryptic digests (see Methods). This analysis revealed that the purified NEK1 protein in itself was autophosphorylated at several previously identified sites (PhosphoSitePlus), namely, T156, S417; and was additionally phosphorylated at S14 and

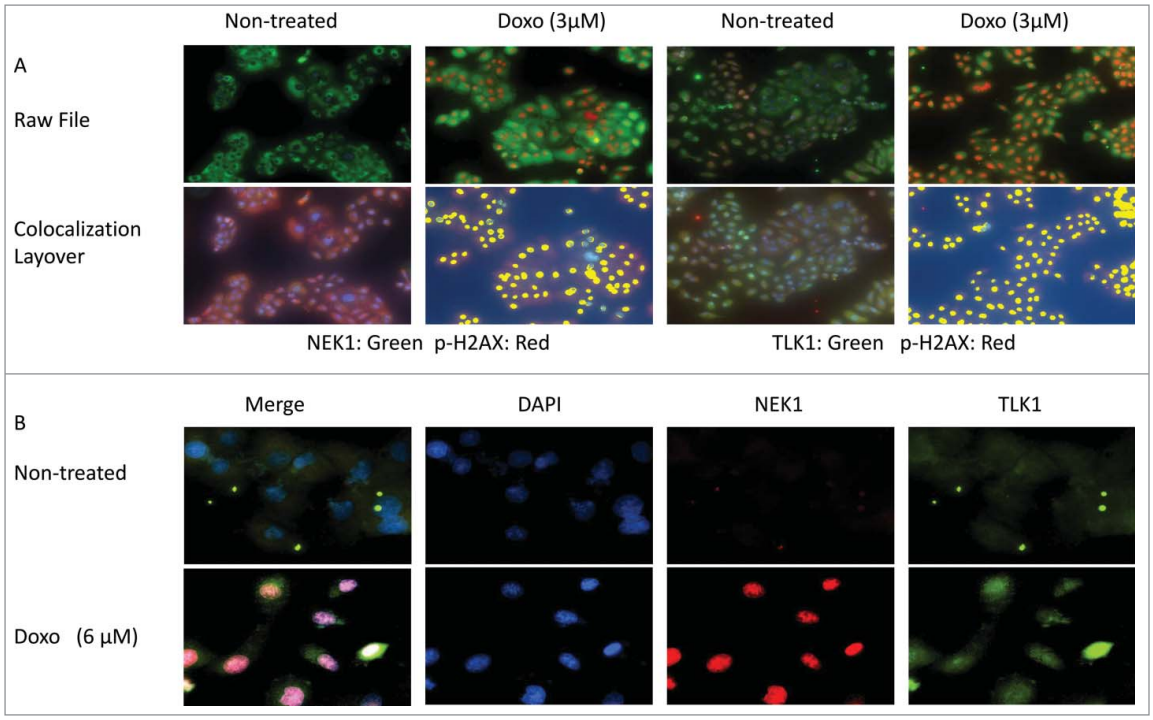


Figure 2. Colocalization of TLK1, NEK1, and γH2AX at sites of DNA damage following incubation with doxorubicin. A. Cells treated with or without doxorubicin (3uM) and then processed for IF as described in.[8] Cells were imaged on the Perkin elmer Mantra scope (20x). Raw files were obtained using the InForm. Colocalization layover was applied using InForm software which generates a pseudo pixel (yellow) where the 2 fluors colocalize. Antibodies used were: Rabbit anti-NEK1, Rabbit anti-TLK1, mouse anit γH2AX. B. Microscopy was performed following treatment with doxorubicin. Imaged were obtained at 60X and analyzed with Nikon software. Colocalization of NEK1 and TLK1 is shown in pink.

S299, that are sites not reported previously (see Sup. Table 2 + Mass Spec Sup data). In the phosphorylation reaction with TLK1B, 2 additional phosphorylation sites were identified in both FL-NEK1 and NT-NEK1: T141 and Y315. Y315 was previously held as a putative autophosphorylation site of NEK1, whereas T141 is a novel site, which we attribute directly to the activity of TLK1B. In addition, since Y315 was not detected in the reaction with NEK1 alone, the simplest explanation is that the phosphorylation of T141 by TLK1B resulted in activation of the NEK1 kinase and autophosphorylation at Y315. Indeed it is noteworthy that TLK1B, which we previously showed acted primarily on the NT-NEK1, phosphorylated specifically T141 of NEK1 that lies within the kinase domain and may activate it.

We should stress that our analysis was somewhat limited since the peptide coverage was only 26% for the NT-kinase domain fragment (and 11% for the full length protein) and it is therefore possible that we have not identified all the sites phosphorylated by TLK1. However, additional site-directed mutagenesis work indicated that T141 is the main phosphorylation target of TLK1B (see below).

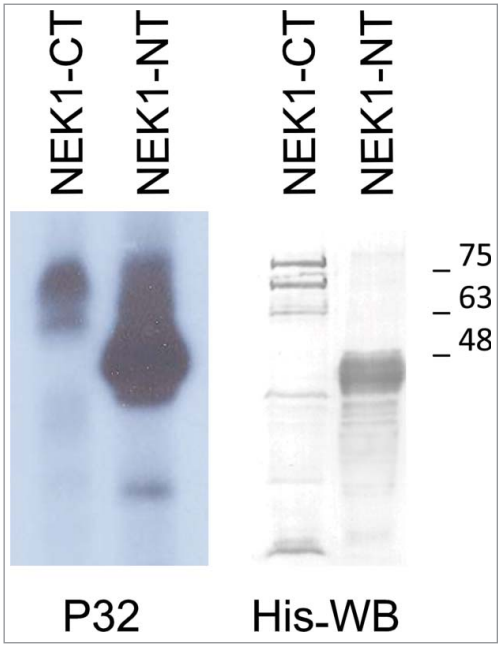


Figure 3. TLK1 phosphorylates in vitro preferentially NEK1N-terminal fragment (NT). 3 μg of Ni-Sepharose purified recombinant NEK1-CT and NEK1-NT proteins expressed in bacteria following induction with IPTG were phosphorylated in a kinase reaction using 50 ng recombinant TLK1B and γP32-ATP. The samples were separated by SDS/PAGE and either exposed to film (autoradiography) or immunoblotted with anti-His antibody.

Kinase activity with recombinant NEK1 protein and its modulation by TLK1

We first asked with recombinant proteins if the activity of NEK1 can be regulated by TLK1B. The activity of NEK1 is typically determined using β-casein as the substrate, whereas TLK1 does not phosphorylate casein efficiently.[21] In fact, recombinant TLK1 even at high concentration (100 ng/μl) phosphorylated very weakly casein Fig. 4A. NEK1 at that concentration phosphorylated casein efficiently, maximally between 10 and 15 minutes, but its activity was greatly stimulated when co-incubated with a small amount of TLK1B (5 ng/μl). Even after only 5 minutes of incubation, the signal was stronger than 15

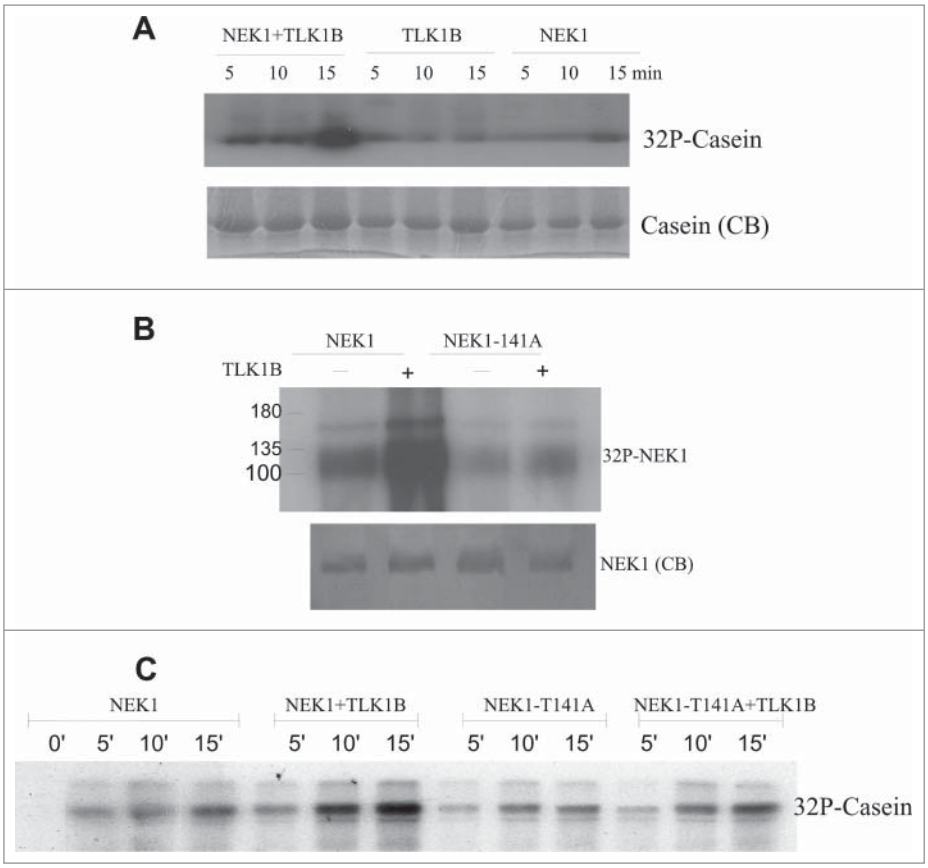


Figure 4. TLK1B addition stimulates the NEK1 kinase activity in vitro. (A) Kinase reactions (see methods) contained 100 ng/μl of the indicated recombinant proteins and 250 ng/μl β-casein as the substrate. The supplemented reaction with NEK1 contained 5 ng/μl TLK1B. After separation on a 10% PAGE, the gel was stained with coomassie blue (CB), dried and exposed to XR film for 4 h. (B) Phosphorylation of NEK1 by TLK1B occurs on T141. In kinase reactions, 1 μg of recombinant NEK1 or the NEK1-T141A mutant were incubated in absence or presence of 50 ng TLK1B. After separation on an 8% PAGE, the gel was stained with coomassie blue (CB), dried and exposed to XR film for 4 h. Note that the autophosphorylation of NEK1 was similar and weak for both wt and T141A NEK1 proteins. (C) The NEK1-T141A mutant kinase is not activated by incubation with TLK1B. Reactions were like in (A) but in addition we tested the activity of NEK1-T141A kinase.

minutes w/o TLK1B. This also suggested that TLK1B rapidly activates NEK1.

To confirm that TLK1B phosphorylates NEK1primarily at T141, we prepared recombinant NEK1-T141A to compare the extent of phosphorylation (radioactivity incorporation) by recombinant TLK1B respect to wt NEK1. Addition of a small amount of TLK1B (5 ng/μl) resulted in a strong increase in labeling in wt NEK1, suggesting either that NEK1 is a direct substrate of TLK1B eliciting P32 incorporation (at T141) or that TLK1B promotes the autophosphorylation of NEK1. However, the phosphorylation of NEK1-T141A was not increased following incubation with TLK1B (Fig. 4B), therefore indicating that T141 is the primary phosphorylation target of TLK1B.

The increased activity of NEK1 in presence of TLK1B could be due to either an activating phosphorylation event (pT141), or simply via an interaction of the 2 proteins leading to a conformational change in NEK1 and increased activity. To establish which mechanism is more likely, we repeated the experiment in panel A, but using both wt NEK1 or NEK1-T141A recombinant proteins. Phosphorylation of casein was again used as readout for NEK1 activity. In Fig. 4C, we show that the addition of a small amount of TLK1B resulted in a strong stimulation of wt NEK1 activity (just as in Fig. 4A). However, the addition of TLK1B to NEK1-T141A had a minimal effect on the activity of NEK1, indicating that the stimulation of NEK1 by TLK1B is most likely due its phosphorylation at T141.

The activity of NEK1 is regulated by the Cell Cycle and DNA damage (doxorubicin)

Although there is no direct evidence that human NEKs are essential for mitotic entry, unlike NIMA in the fungus *Aspergillus nidulans*, it is clear that several NEK family members have important roles in cell cycle control. To our knowledge, it has not been reported what is the pattern of activity of NEK1 during the cell cycle. In contrast, maximal activity of TLK1 was reported to occur during S-phase and at G2/M,[21,22] and we wondered if a similar pattern of activity could be found for NEK1. To test this, cells were arrested at the G1/S boundary with hydroxyurea (HU), and then released into S-phase after washing with fresh medium. Finally, the cells were arrested in M-phase with nocodazole (see details in Materials and methods). Cell extracts from the different cell cycle phases (Fig. 5B) were prepared and equal amounts of protein were used to immunoprecipitate (IP) NEK1. Equal aliquots of the IP were then used to carry out kinase reactions, with 10 μCi γ-ATP^{32} and β-casein as the substrate, during a 15 minutes time course removing equal amounts at 5 minute intervals. Figure 5A shows that the kinase activity of IP-NEK1 was weak in

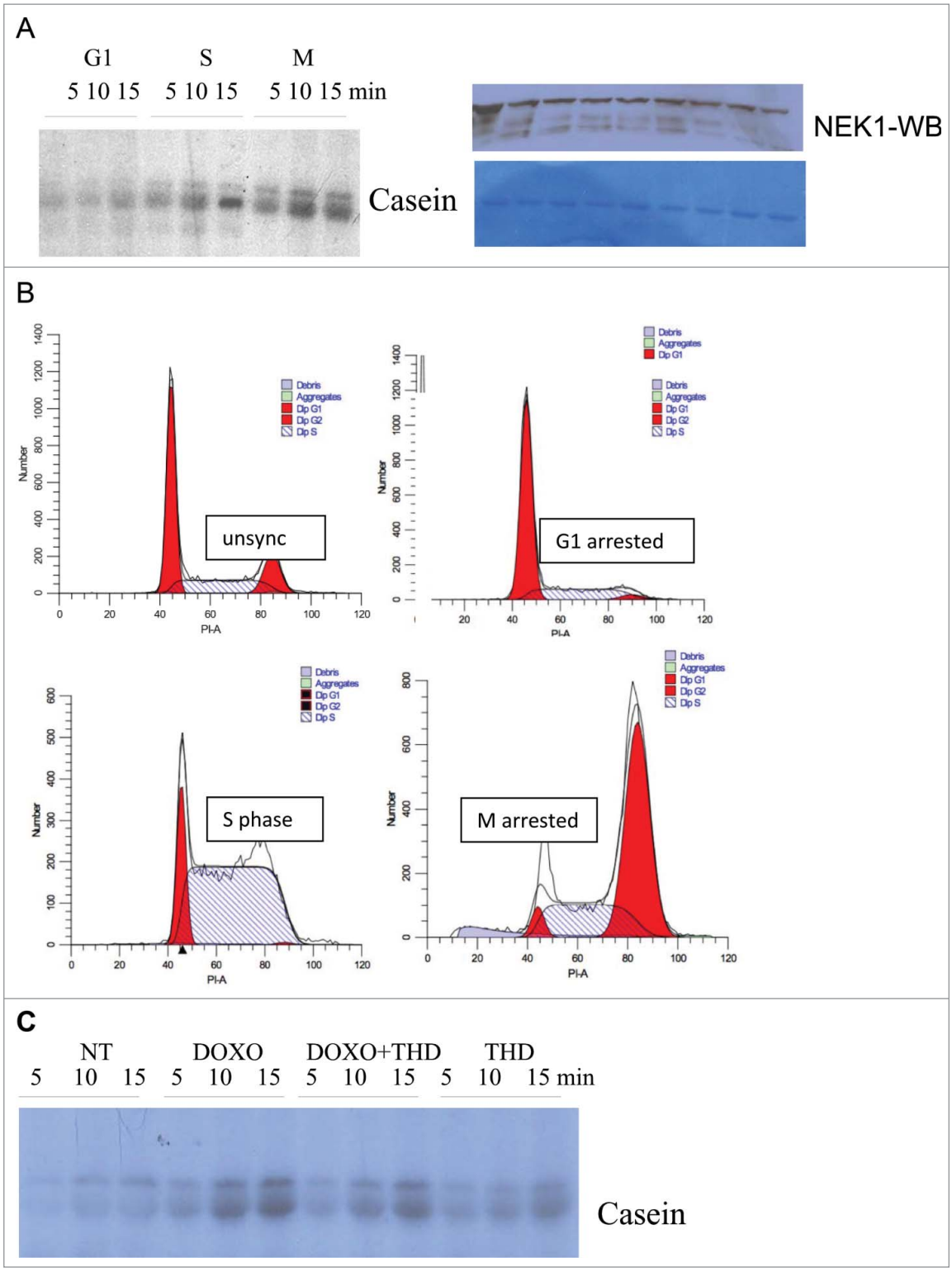


Figure 5. (A and B). The activity of NEK1 is cell cycle regulated. DU145 cells were arrested enriched in G1/S with HU, synchronously released in S after washing with fresh medium, and then arrested in M with nocodazole (see panel B). Cell extracts from equal cell numbers were prepared and NEK1 was isolated by IP (see methods) and tested for activity using β-casein as the substrate (panel A). C) The activity of NEK1 increases following addition of doxorubicin, but reduced by concomitant addition of THD. Cells were treated as described in Methods, and NEK1 was IP'd and tested for activity in vitro using β-casein.

G1-arrested cells, and increased in cells isolated from S and M phases, similar to what has been reported for TLK1 (the expression of NEK1 did not change during the cell cycle). It thus seems possible that both TLK1 and NEK1 may be required for specific functions during DNA replication and then mitosis, although it does not prove that the activity of TLK1 and NEK1 are linked. Unfortunately, it is difficult to test directly if TLK1 modulates the activity of NEK1 during the cell cycle since depletion of TLK1 with siRNA results in a S-phase arrest.[20]

The activity of NEK1 was reported to increase in response to various types of DNA damage.[10] This is confirmed in Figure 5C, where NEK1 was IP'd from cells treated with doxorubicin

(3 μM) and then tested *in vitro* for kinase activity. DNA damage with doxorubicin found NEK1 to be significantly more active than when isolated from untreated cells (NT). Whereas treatment of cells with THD, a specific inhibitor of TLK1 ,[35] had little effect on the activity of IP-NEK1, the addition of THD to cells treated with doxorubicin significantly reduced the activation of NEK1.

Overexpression of inactive TLK1B mutants found in cancers results in loss of phosphorylated NEK1

We wondered if dominant expression of kinase inactive mutants of TLK1B would have an effect on the pattern of phosphorylation of NEK1, as determined by mobility shift. We thus decided to focus on spontaneous TLK1 mutations found in cancers. There are, so far, 225 heterozygous mutations of TLK1 reported in COSMIC and we focused on 6 that were expected to affect the kinase activity (see Fig. 6). We generated HEK293 stable cell lines overexpressing mutants and wild-type (WT) TLK1B in the pIRES2 vector, after selection for high GFP positive cells. Hek293 cells express TLK1 and not TLK1B, but we are aware that this experiment can work only if the TLK1B mutants have a dominant negative effect over the endogenous TLK1. Two frame-shift truncations (G622* and A583*) that encompass the kinase domain, and the mutant G417C in the consensus ATP binding pocket, resulted in loss of the phosphorylated NEK1 band (Fig. 6). We do not know if this phosphorylated form is the result of autokinase activity that is altered or if it is directly due to TLK1B acting on NEK1 as a substrate.

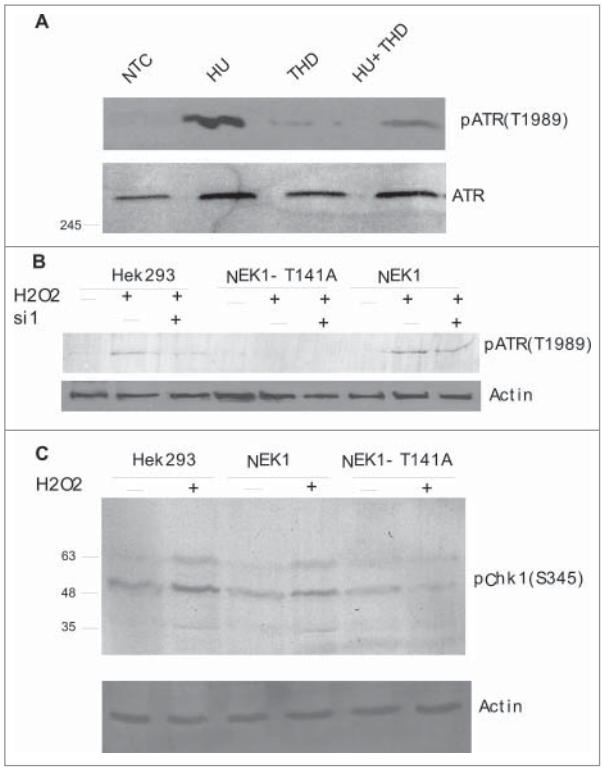
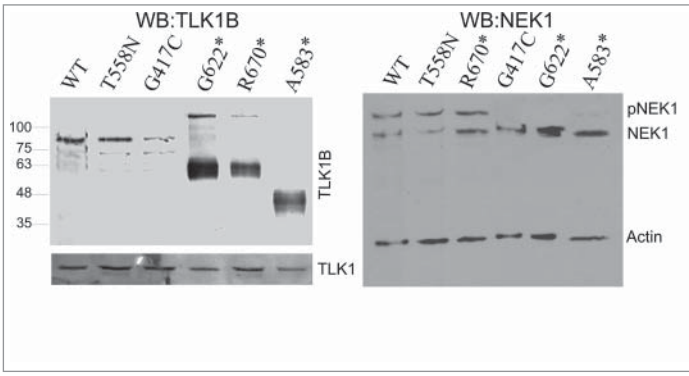


Figure 7. (A) The activation of ATR (autophosphorylation at T1989) after 6h incubation with hydroxyurea (HU) is strongly reduced by concomitant addition of the TLK1 inhibitor, thioridazine (THD, 10 μM). (B) Activation of ATR (pATR-T1989) is impaired following 1h incubation with 0.2 mM H2O2 in cells expressing NEK1-T141A in contrast to wt-NEK1 expressing cells. Where indicated, siRNA to nek1 (si1) was added 24h before H2O2. (C) Activation of Chk1 (pChk1-S345) is reduced in cells overexpressing NEK1-T141A.

Phosphorylation of NEK1 by TLK1 is important for the DDR and cell cycle checkpoint

It was reported that NEK1 associates with ATR/ATRIP and primes it for activation in response to a variety of genotoxic agents, and that siRNA knockdown of NEK1 leads to a reduction in phospho-Chk1.[28] To determine the potential contribution of TLK1 to this pathway that converges on NEK1>ATR>Chk1, as a first experiment, we compared the activation of ATR following addition of hydroxyurea (HU) with that in cells simultaneously tread with thioridazine (THD, inhibitor of TLK1). We followed ATR autophosphorylation of T1989, which is a very early event in the activation of ATR. It was reported that cells lacking NEK1 fail to support ATR autophosphorylation at T1989.[28] In Figure 7A, we show that THD strongly inhibited the phosphorylation of ATR after treatment of cells with HU. We knew from a previous kinome study[35] that THD does not affect ATR activity directly, and we suggest that the mechanism is indirect and via reduced activation of NEK1 owing to inhibition of TLK1 by THD.

To establish more directly if the phosphorylation of NEK1-T141 has an effect on the DDR, we compared the activation of ATR and Chk1 in Hek293 cells overexpressing wt NEK1 or the



MUTANT	MUTATION TYPE	CANCER TISSUE
T558N	CATALYTIC DOMAIN	PROSTATE
R670*	TRUNCATION	LARGE INTESTINE
G417C	CONSESUS ATP POCKET	LARGE INTESTINE
G622*	INSERTION-fs-TRUNCATION	LIVER
A583*	INSERTION-fs-TRUNCATION	LUNG

Figure 6. Expression of some dominant K_D mutants of TLK1B result in loss of phosphorylated NEK1. (Left) Expression of TLK1B mutants in stably transfected 293T cells. (Right) WB of NEK1, where the main 2 mobility forms of the protein are indicated as NEK1 and pNEK1.

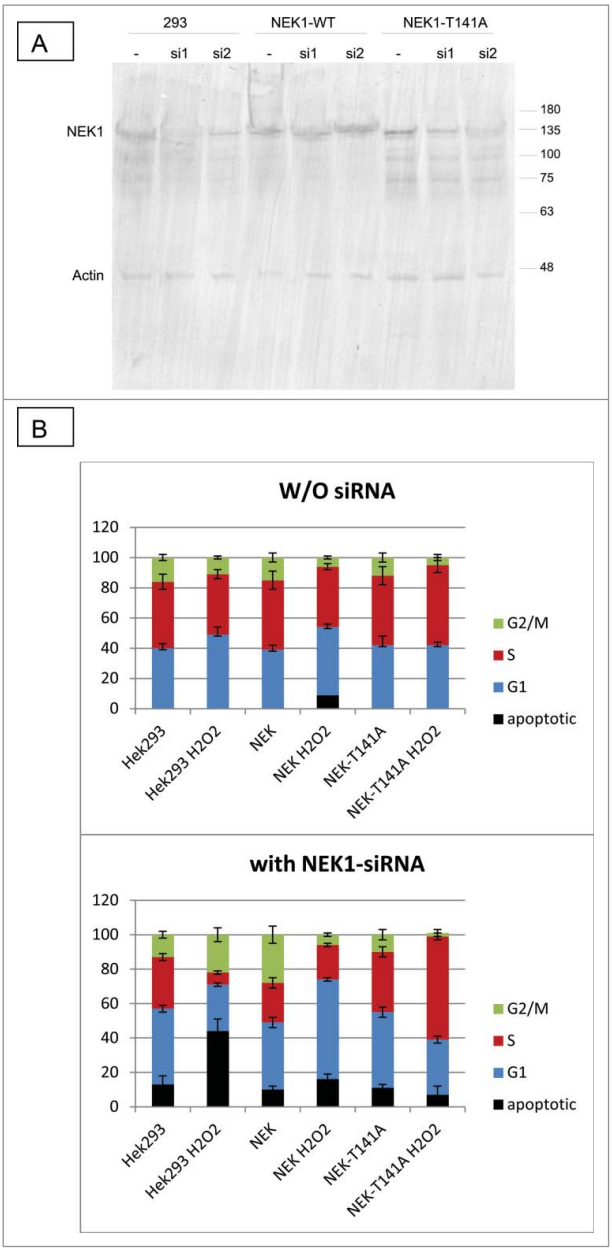


Figure 8. (A) Expression of endogenous NEK1 in control H3k293 (293) cells and in and stably transfected NEK1 overexpressing constructs. The control and NEK1 overexpressing cells (siRNA resistant constructs) were treated for 18h with 2 different siRNAs, and cell extracts were prepared and immunoblotted for NEK1 and actin. (B) Hek293 controls and cells overexpressing wt-NEK1 or the NEK1-T141A mutants were treated (or not) with si1 to knockdown endogenous NEK1. Cells were then treated with H2O2 for 4h, as described in text, and then processed for cell cycle analysis with PtdIns staining.

T141A mutant. To activate the DDR, the cells were treated for 30 min with H2O2 (0.2mM) to induce genomic damage, and where indicated cells were pretreated for 24h with siRNA to NEK1 (si1, see also Fig. 8). Figure 7B shows that H2O2 in Hek293 cells activated ATR (pATR-T1989) but the induction was reduced after treatment with si1. Cells expressing NEK1-T141A treated with H2O2 were unable to induce pATR. In contrast, cells expressing siRNA-resistant NEK1 induced pATR with H2O2. Figure 7C shows that cells overexpressing the T141A mutant are unable to activate Chk1, consistent with our proposed role for TLK1-mediated modulation of NEK1 activity via phosphorylation of T141.

To establish the consequences of the NEK1-T141A mutation on the cell cycle after genotoxic stress, we repeated the treatment with H2O2 in the absence of the endogenous NEK1 after depletion with siRNA. Two different siRNAs to the 3'UTR of NEK1 were used to knockdown its expression), and si1, but not si2, was effective at reducing the expression of NEK1 by >80% (Fig. 8 top. As expected, the overexpressed wt-NEK1 and NEK1-T141A were resistant to the si1. Note also that the T141A mutant migrated slightly faster than the overexpressed wt-NEK1, although the difference is not as dramatic as in Figure 6 for the dominant K_D-TLK1B mutants. It is possible that the overexpressed NEK1-T141A is phosphorylated at different compensatory sites in the absence of the important T141 site.

We thus treated the 3 cell lines (control, and overexpressing wt-NEK1 or T141A) with H2O2 for 4h, with and without pretreatment of one day with si1, and determined the cell cycle distribution with PI and FACS analysis (Fig. 8 bottom). Without knockdown of the endogenous NEK1, the control Hek293 cells treated with H2O2 displayed a modest increase in G1 cells and corresponding reduction in S-phase cells. In cells overexpressing wt-NEK1, the same effect was observed. In contrast, cells overexpressing NEK1-T141A displayed an increase in S-phase cells with a matching decrease of the G2/M population, suggesting that the G1 checkpoint is bypassed. Much more pronounced effects were seen in cells pre-treated with si1. Firstly, the parental Hek293 cells treated with H2O2 became prominently apoptotic and with a nearly complete loss of the S-phase population. In contrast, cells rescued with wt-NEK1 and treated with H2O2 displayed an increase in the G1 population, a reduction of cells in G2/M, and little change in the S fraction. A very different result was seen in cells expressing NEK1-T141A, which showed that most cells accumulated in S, and not in G1. The simplest explanation is that the reduced Chk1 activation in response to H2O2 in these cells, which we attribute to impaired NEK1 function, would lead to a bypass of the G1 checkpoint and rather activation of an intra-S phase checkpoint with the accumulation of DNA damage.

Discussion

The TLKs have been implicated in many diverse functions like DNA repair and the DDR (rev in[1]), but also aspects of transcription,[36,37] possibly mRNA splicing,[38] chromatin assembly by Asf1 interaction,[15,31] cell motility, and segregation of chromosomes.[20,39] However, identification of TLKs direct substrates has been frustratingly slow, and yielded just a few examples (rev in[1]). With even fewer targets identified using unbiased large screening methods[7,15,40] that were subsequently confirmed by direct interaction. Therefore, we have engineered a new screen to identify the proteomic targets of TLK1 by interrogating the Life Technologies Protoarray composed of 9000 full length human proteins, with purified native TLK1B. This resulted in identification of 165 targets, of which only 3 proteins (out of 3 tested) have been so far independently confirmed with coimmunoprecipitation assays. Notably, Asf1A or Asf1B, which were identified in a previous interaction (yeast 2-hybrid) screen,[15] were not obtained in our screen that is based on full length, native proteins. In contrast, Aurora Kinase, which was

previously identified in a yeast 2 hybrid screen from a C. elegans cDNA library,[40] was further supported as a strong interactor of TLK1 in our screen. Finally, Rad9, a protein which we identified as interacting with TLK1B in a phage display screen, was not present on the ProtoArray.

Because of our long-standing interest in the functions of TLKs in DNA repair and the DDR, we have chosen as our first candidate to investigate more thoroughly, NEK1. This kinase was implicated in the DDR, for interactions with ATR/ATRIP and checkpoint activation,[26,28] and more recently found to participate in HR-mediated repair through phosphorylation of Rad54.[30] Notably, we previously implicated TLK1 activity in regulation of ATR-mediated DDR [8] and in HR-mediated repair.[41] This sheds light on the plausible role of TLK1 in regulation of NEK1 activity and seemed a most logical avenue to pursue. Indeed, we found in this work that TLK1/1B interacts with and colocalizes with NEK1 in the nuclei at sites of DNA damage along with γH2AX in cells treated with doxorubicin. Furthermore, the interaction of TLK1 and NEK1 appears to be strengthened with DNA damage, and the kinase activity of NEK1 increases with doxorubicin but can also be suppressed with the TLK inhibitor, THD. We subsequently found that TLK1 phosphorylates NEK1 at T141, which stimulates its kinase activity. The phosphorylation of NEK1 by TLK1 may be cell cycle regulated, although we presently do not have available a phospho-specific antiserum to determine this directly. However, the NEK1 kinase activity is regulated during the cell cycle, and matches the reported cell cycle dependent activity of TLK1[21,22] suggesting a possible coordination of these 2 kinases. Notably, expression of the NEK1-T141A that cannot be phosphorylated by TLK1 resulted in a defect in the cell cycle checkpoint in response to treatment with H2O2. In these cells, the G1 checkpoint was largely abrogated, and cells accumulated in S-phase concomitant with the impairment of Chk1 activation. Since the activity of NEK1 has already been implicated in Chk1 activation, as shown by its loss in cells deficient in NEK1,[26,28] the simplest explanation for our results is that the TLK1-mediated phosphorylation of NEK1-T141 is a key determinant of NEK1 activation upstream of ATR and Chk1. Recently NEK1 was found to phosphorylate Rad54 at S572, and this activity was shown to promote the release of Rad51 during the completion of HR before entry into mitosis. While the authors demonstrate that Rad54 phosphorylation occurs in G2 and not during S phase arrest, it was not reported how that correlates with the activity of NEK1 under different damage conditions (either presence of DSBs or stalled replication forks, for instance). We have now shown that the activity of NEK1 is cell cycle-regulated and greatly increased in S and G2, which correspond well to the pattern of activity of TLKs.[21] We also noticed that another protein identified as interacting strongly with TLK1B is Rad54B, a paralog of Rad54 that performs very similar functions and also binds to Rad51,[42] presenting the possibility that TLK1, NEK1, Rad54, Rad54B, and Rad51 may all be present in a complex particularly at sites of DNA damage.

Materials and methods

Hydroxyurea (Catalog No. H8627), Doxorubicin hydrochloride (Catalog No. D1515), Thioridazine hydrochloride (Catalog No. T9025), KU-55933 (Catalog No SML1109) and G418 disulfate salt (Catalog No. A1720) were purchased from Sigma. Dulbecco's modified Eagle's medium (DMEM) was obtained from *Life Technologies* (Catalog No. 12100–046), Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Catalog No. 900108).

Antibodies used in this study were: rabbit α-Actin (Ab1801, Abcam), rabbit α-Chk1 phospho-317 (AP3070a, Abgent), rabbit α-Chk1 phospho-345 (sc-17922, Santa Cruz Biotechnology), rabbit α-TLK1 (GTX102891, GeneTex), rabbit αNEK1 Antibody, A304–570A (Bethyl Lab), mouse monoclonal α Nek1 (E-10) (sc-398813 Santa Cruz Biotechnology), mouse monoclonal Anti-polyHistidine antibody (H1029, SIGMA), rabbit αATR antibody (A300–138A, Bethyl Lab), rabbit αpATR (phospho Thr1989) antibody (GTX128145, GeneTex), donkey α-goat IgG-HRP (sc-2020, Santa Cruz Biotechnology), α-rabbit IgG-HRP (7074S, Cell Signaling), α-mouse IgG-HRP (7076, Cell Signaling), mouse α- H2A.X phospho-139 (05–636, Millipore).

Protein-Protein interaction assays (ProtoArray)

This was a service provided by Life Technologies. Briefly, all arrays were blocked with blocking buffer (50 mM HEPES, 200 mM NaCl, 0.08% Triton X-100, 25% glycerol, 20 mM reduced glutathione, 1.0 mM DTT, 1X Synthetic Block) at 4°C for 1 hour in 4-well quadriPERM trays (Greiner) with gentle orbital shaking. Arrays were rinsed once in probe buffer (1X PBS, 0.1% Tween-20, 1X Synthetic Block) before incubation with probe solution. The probe, biotinylated TLK1 protein, was provided by LSUHSC and diluted to concentrations of 5 ng/μl and 50 ng/μl in probe buffer. The negative control array was incubated with probe buffer only. The positive control array was incubated with 50 ng/ml biotinylated V5-epitope tagged yeast calmodulin kinase diluted in probe buffer. Approximately 120 ml of each probe solution was pipetted onto the arrays and incubated under a Lifterslip™ (Thermo Scientific). All arrays were incubated at 4°C for 90 minutes and then washed in probe buffer (5 × 5 ml washes, 5 minutes each). All arrays were incubated with 5 ml of a solution of AlexaFluor®647-conjugated streptavidin (Life Technologies, 1 mg/ml) with gentle shaking in 4-well quadriPERM trays at 4°C for 90 minutes. After incubation, the arrays were washed as described above and quickly rinsed in water to remove residual salts from the surface of the array. Arrays were then immediately placed in a slide holder and spun in a table top centrifuge equipped with a microplate rotor at 1000 RPM for 2 minutes to dry. Arrays were then scanned, as described below.

Data acquisition and analysis

Arrays were scanned using an Axon 4000B fluorescent microarray scanner (Molecular Devices). GenePix 6.0 software was used to overlay the mapping of human proteins in the array list file to each array image using the irregular feature option. After aligning each of the 48 subarrays using spots from the AlexaFluor®-conjugated and murine antibodies printed in each subarray, pixel intensities for each spot on the array were determined from the software and saved to a text file formatted for

use in GenePix, the GenePix Result file (.gpr filename extension). These files can be opened in text editing or spreadsheet programs.

Data was processed using Life Technologies' proprietary ProtoArray® Prospector software. ProtoArray® Prospector includes a series of algorithms specifically designed to analyze data resulting from protein-protein interaction profiling studies, with the goal of identifying specific protein-protein interactors. Significance call queries were performed by analyzing the Prospector output in Microsoft Excel to identify hits on each protein array.

Cell culture

HEK293 cells, obtained from ATCC repository, were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2.

siRNA, plasmids, transfections

Transfections of siRNA were performed using Lipofectamine 3000 (InVitrogen). The 2 siRNAs were made by Dharmaocon: NEK1 siRNA1: gcaaatgtctttaggttga; NEK1 siRNA2: acataagcttca aggacaa. The Nek1 mammalian expression vector was purchased from Origene (MR216282). The entire ORF or N-terminus (aa 1–480) and C-terminus (aa 482–1259) were subcloned in the pET30 bacterial expression vector (Novagen). The NEK1-T141A mutated plasmids were obtained by site-directed mutagenesis using QuickChange (Agilent).

Cell cycle analysis

HEK293 cells were seeded in 6-well plates at a density of 2×10^5 cells/well and treated or not overnight with NEK1 siRNA. The next day cells were treated for 4 h with 0.2 mM H2O2. DU145 cells were seeded in 6 well plates and treated with 2mM Hydroxyurea (Sigma Aldrich H8627) for 8 hours, washed and released in fresh culture media for 6 hours, and then treated with 0.1 μg/ml Nocodazole (Sigma Aldrich M1404). 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, 3.8mM 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 phase (G1, S, G2, M) were determined by FACSDiva and Modfit modeling software.

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.

Immunoprecipitation

For co-immunoprecipitation (CoIP) experiment, cells were lysed in the lysis buffer (1% Triton X-100, 0.5% Na-Deoxycholate, 150 mM NaCl, 2.5 mM MgCl2, 1 mM EGTA, 1 mM EDTA, 20 mM Tris/HCl pH 8.0) supplemented with halt EDTA free protease and phosphatase inhibitors. Cell lysates were sonicated for 20 cycles using Diagenode Bioruptor. In total 0.3 mg of cell lysate was precleared with protein A/G Sepharose beads and then incubated overnight at 4°C with the indicated Ab's and then and tumbled with 30 μl of protein A/G Sepharose beads for 4 h at 4°C. After extensive washing in CoIP buffer, proteins were eluted by boiling treatment in 2X electrophoresis sample buffer before Western blotting analysis.

Mass spectrometric analysis of phosphopeptides

The sample preparation for mass spectrometry was described previously ([43]). Briefly, the peptide samples will be obtained after digestion by Promega trypsin, and then analyzed using LC-MS analysis (nanoAcquity UPLC-Synapt HDMS, waters corporation, MA). Mass spectra will be acquired in positive ion time-of-flight (TOF) V-mode. Data were acquired using MassLynxTM 4.1 software (waters corporation, MA) in an automatic data dependent acquisition mode. MS-TOF scans were acquired from m/z 300 to m/z 1500, and up to 3 precursor ions were selected for subsequent MS/MS scans from m/z 100 to 1500 using charge state recognition promote fragmentation. Mass spectrometry data was processed using PEAKs Studio 7.5 software (Bioinformatics Solutions Inc., Canada).

Kinase assay

For IP'd NEK1, these were as described in.[9] For assays with recombinant proteins, typical reactions contained 1 μg of kinases in 20 μl, unless otherwise specified in the figure legend.

Disclosure of potential conflicts of interest

The authors declare that no conflict of interest exists.

Acknowledgments

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

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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, suggesting the existence of a 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 cells cultured with bicalutamide. Moreover, THD significantly inhibited the growth of several PCa cells *in vitro* (including androgen independent lines). 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. 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).

SIGNIFICANCE

We target 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.

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 anti-androgens. 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 pro-survival 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 [5]). Then, after a quiescent period of ADT adaptation of 2-3 weeks, androgen independent (AI) colonies begin to form [6]. 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.

In this work we show that in LNCaP cells increased expression of TLK1B following ADT (part of a pro-survival adaptive response to mTOR activation) leads to activation of the NIMA-related kinase, NEK1 [7], which in turn activates ATR and the DDR [8, 9]. We then show that inhibition of the TLK1/NEK1 axis with thioridazine (THD), a rather specific inhibitor of TLKs [10], suppresses the DDR and cell cycle arrest elicited by ADT and causes unencumbered replication-induced DNA damage, leading to apoptosis in tissue culture and in mouse xenografts models.

THD was previously shown to improve therapeutic response in combination with DNA damaging agents [10-14]. Furthermore, THD was shown to cause growth inhibition of ovarian cancer cells via suppression of AKT activity [15] 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 [16], 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 pro-survival selection mechanism that can be optimally targeted.

MATERIALS AND METHODS

List of Antibodies

TLK1 antibody [N2C2], Internal-Genetex- Cat. #GTX102891
4EBP1 Monoclonal Antibody (E.992.6)-Cat. # MA5-15005
Rabbit NEK1 Antibody -Bethyl Lab- Cat. #A304–570A
Purified custom pNek1 antibody- 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
Phospho-Akt (Thr308) Antibody – CST-Cat.#9275
Phospho-Akt (Ser473) Antibody-CST-Cat. #9271
Anti-PCNA Antibody– Millipore – Cat# MAB424
Phospho-Chk1 (Ser317) Antibody-CST-Cat. #2344
Rabbit alpha pATR (phospho Thr1989) antibody- GeneTex- Cat. #GTX128145
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)

Data Mining:

Data mining for NEK1 expression was done through freely available cBioPortal (http://www.cbioportal.org) tool, as described in [17]. mRNA expression data from the Prostate Cancer Metastatic dataset and CRPC dataset were used with Z-score threshold of ±2. Genetic alterations in percent mRNA up-regulation and amplification were taken into account. PCAWG data set was also used for NEK1 expression.

Cell lines

All PCa cell lines were obtained from the ATCC 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 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. Mice were treated with THD (dissolved in sterile water - 2.5 mg/ml) at dosages similar to those reported previously in non-cancer 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 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.

Quantitation of TLK1 transcript variant 3 (qRT-PCR).

Total RNA was extracted from LNCaP cells (0.5X 10^6 cells) using the QIAGEN RNeasy MiniKit (Cat No- 74104). Purity was confirmed with NanoDrop, A260/A280= 2.1 (LNCaP). 500 ng of total RNA from each sample was used to prepare First strand cDNA using New England Biolabs, Protoscript II First Strand cDNA Synthesis Kit (Cat No- E6560S). For optimal results we used 50 ng cDNA and qRT-PCR was performed in a Bio-Rad CFX96 PCR machine using BioRad SYBR Green Supermix (Cat No- 1708880) and TLK1(NM_12290) primers from IDT (PrimeTime qPCR Primer Assays).

TLK1B - Primer 1: GCAACTCCAGTAAATCTAGCTTCC.
TLK1B - Primer 2: GTCCCTGCTGAATCACACG.]
Results were normalized for GAPDH (NM_001256799.2-F / R).

RESULTS

Androgen Deprivation results in translational increase in TLK1B expression. The Tousled Like kinases (TLK1 and TLK2) function in several processes including chromatin assembly, replication, transcription, repair, and chromosome segregation (rev. in [19]). 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 [20]. TLK1B is similar to TLK1 but lacks the first 169 aa. 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 [21]. 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 (Fig. 1A and Sup. Fig. 1A, B). Consistent with this mechanism, the phosphorylation of 4EBP1 increases in cells incubated in charcoal-stripped-serum (CSS), and this is suppressible with rapamycin (Fig. 1B). 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 [22]. 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 [9]. It seemed possible that a similar pathway becomes activated upon AR inhibition by ADT, leading to a pro-survival 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% patients with Metastatic Prostate Cancer. Finally, Nek1 is upregulated in PCa patients in PAN-CANCER analysis of whole genome Dataset (Fig. 1C-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. 1F). This analysis revealed that Nek1 is ubiquitously expressed and is phosphorylated at T141 in all cells, including PC3 that do not express TLK1 [23] 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 Fig.1G, 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 Fig. 2D. Moreover, treatment with THD of LNCaP overexpressing wt-Nek1 resulted in greatly reduced signal of pNek1 (Fig. 2E), whereas in cells overexpressing the T141A mutant, the pAb appeared to detect only the endogenous Nek1 (compare with the similar signal of the control LNCaP cells)

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-responsive 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 three weeks. At a concentration of 3-9 μM THD, which inhibits growth only 50% in androgen containing medium (FCS), few or no AI colonies emerged (Fig. 2A and B). In addition, after 4 weeks with bicalutamide+3 μM THD the remaining cells became

apoptotic and lifted from the plate – see example in 2B right panel, where we compare a colony without (top) and with THD treatment (bottom). On the other hand, THD has been reported to have anti-cancer activity, although the precise mechanisms for this have not been elucidated. We thus also tested a panel of androgen-insensitive 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. 2C). 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 anti-cancer agent, or it is possible that the TLK1/Nek1 axis remains critical even for CRPC cells. 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.

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, we analyzed the cell cycle distribution of LNCaP cells treated with bicalutamide, THD, or in combination for 24h to determine if there is cell cycle arrest or induction of apoptotic - sub-G1 cells (Fig. 3A). As expected, treatment with bicalutamide alone resulted in accumulation of cells in G1, 73% vs 56 (untreated). In contrast, THD (10 μM) did not induce accumulation of cells in G1, but it did show some toxicity, as indicated by an increase in sub-G1 cells. However, the most dramatic effect was seen in the combination treatment, which resulted in 3-times more apoptotic cells than untreated control, while THD also reduced the accumulation of cells in G1 that are seen with bicalutamide alone. This result can well explain the fact that AI cells are unlikely to form in these conditions (see Fig. 2A, B).

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 (Fig. 3B, C). Treatment with bicalutamide resulted in the anticipated increase in pChk1 (Fig. 3B), 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 he TLK1>NEK1>ATR>Chk1 pathway as we previously showed [18]. Bicalutamide also caused an increase in pH2AX (Fig. 3C), 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]. 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. 3B), 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

[16]. Indeed, there was a strong increase in p21 expression in the combination treatment group (Fig. 3B). P27 was also slightly increased in the THD treated groups.

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 anti-androgens halt the progression of LNCaP xenografts for some time (2-3 weeks), while typically the subsequent re-growth 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 Fig. 4A-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 (Sup. Fig. 2D), consistent with the mechanistic results previously shown. Interestingly, treatment with THD alone resulted is a similar temporary suppression of tumor growth (*** $p<0.005$), showing that THD can have anti-tumor 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 Fig. 4B-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 Fig. 4D-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 three 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 4F), 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. 4G) and decreases Ki67 (Fig. 5A) and Proliferating Cell Nuclear Antigen (PCNA) expression (Sup. Fig. 2C), which are markers of proliferation. The combination treatment also induces apoptosis (as shown by cleaved PARP [Fig.5B] and cleaved Caspase 3 [Fig.5C]) and results in increased DNA damage, as indicated by γH2AX staining (Fig.5D), which can be attributed to a failure to properly activate the DDR. Representative tumor sections H&E stained are shown in Sup. Fig. 2B. In addition, and consistent with what was reported for treatment of ovarian cancer cells with THD [15], tumors isolated from mice treated with THD and in combination showed a decrease in pAKT (Fig. 4F), an event that was linked to decreased viability of ovarian cancer cells [15]. 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 (Sup. Fig. 2A), 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 two 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, 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. 6B). 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 experiments, where all the LNCaP-T141A tumors isolated from mice treated with bicalutamide were clearly smaller than those in the untreated group (Fig. 6, B-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. 6E).

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 [10], 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, we propose that 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 cells like LNCaP with ADT results in activation of the DDR via ATR or ATM [3]. We have now 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 particular there was an increase in markers of apoptosis, like cleaved Caspase 3 and PARP, and presence of numerous pH2AX-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 Fig. 7).

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 [8, 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 [9]. 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, 16], 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 pro-survival 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed

Authors' Contribution
Conception and design: V. Singh, P. Jaiswal, I. Ghosh, H. Koul, X. Yu, A. De Benedetti.
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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.
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FIGURE LEGENDS

Fig. 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 4h time-course. **B**. **The phosphorylation of 4EBP1** was monitored in parallel via its change in mobility, and where indicated, rapamycin (20 nM) was added to inhibit mTOR (see also Sup. Fig.1A). **C-E. Nek1 expression and Gene Amplification** in PCa samples. **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 Cance**r. **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).

Fig. 2. A-B. 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. **B**. An example of crystal violet-stained plate from which the quantitation in panel A was derived. Here the cells AI colonies in LNCaP cells incubated with increasing concentrations of THD were scored after 3 weeks. After one month, a typical AI colony is shown on the right (top) without THD, or with addition of 3 μM THD (right bottom). **C. Percent of colonies treated with different conc. of THD in different PCa cell lines with increasing concentrations of THD. D-E. Phosphorylation of Nek1 in parental LNCaP cells and overexpressing Nek1 (wt) and T141A mutant after incubation with bicalutamide +/- THD.**

Fig. 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 24h for cell cycle analysis of PI-stained cells. **B-C. 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.

Fig. 4. A-E. Combination bicalutamide and THD suppresses growth of LNCaP xenografts. A. Time course of tumor growth LNCaP cells xenografts in 4 treatment groups. Treatment started after two 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.

Fig. 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).

Fig. 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 two 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.

Fig. 7. Model for the combination of ADT and inhibition of the TLK1-Nek1, DDR axis with bicalutamide and THD, culminating in abrogation of the checkpoint and then cell death.

Supplementary Fig. 1

S1A. Normalized expression of TLK1B splice variant. LNCaP cells were shifted for 24h to charcoal-stripped-serum (CSS) or maintained in FCS. After preparation of cDNA the abundance of TLK1B transcript was determined relative to GAPDH mRNA. The Cq mean was 26.4 for TLK1B and 23.8 for GAPDH.

S1B. **ADT-induces a translational increase in TLK1B.** LNCaP cells were shifter for 16h to charcoal-stripped-serum (CSS) containing medium, and where indicated supplemented with rapamycin.

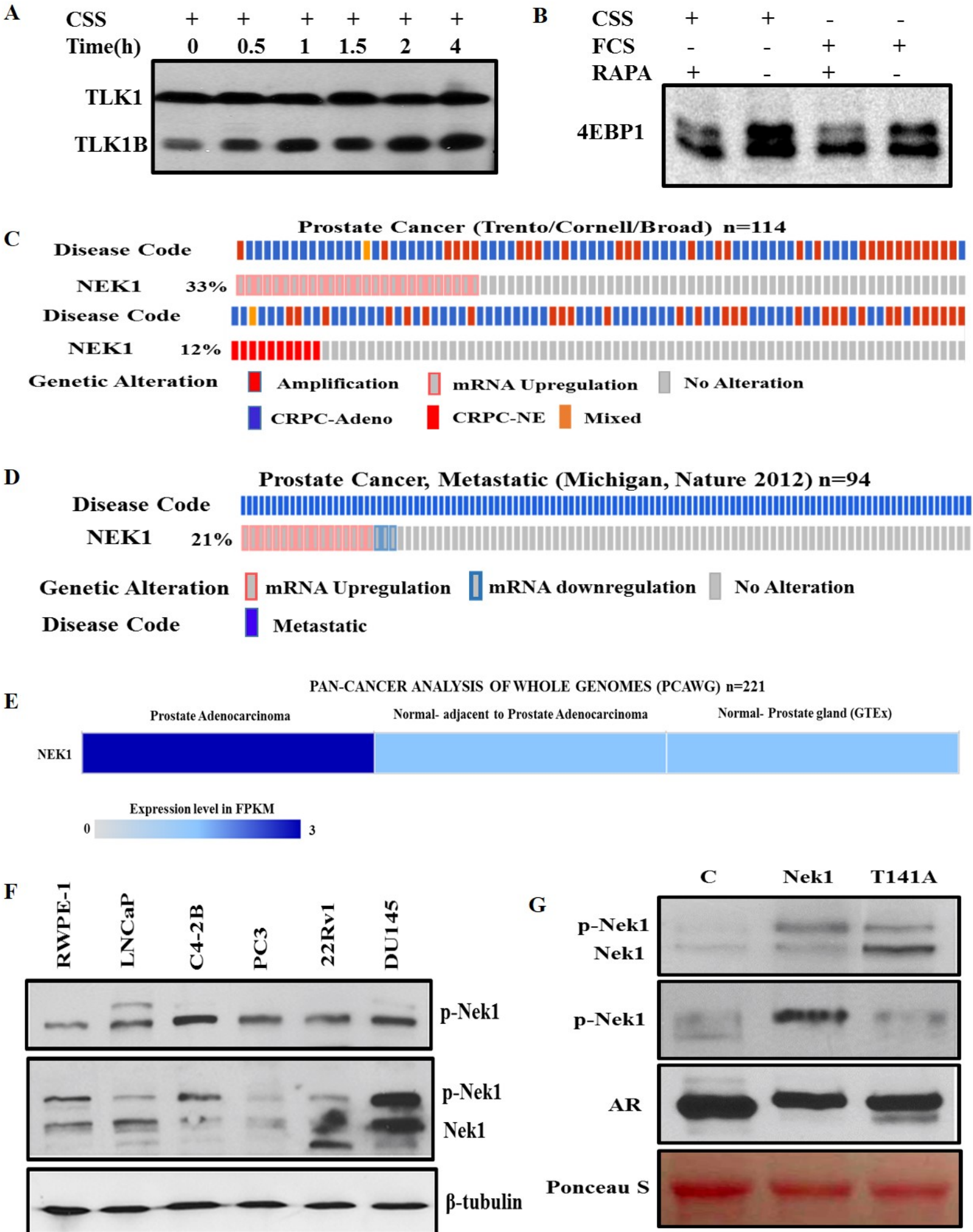
Supplementary Fig. 2

S2A. Body weights (LNCaP xenografts) do not differ statistically in mice treated with bicalutamide and/or THD.

S2B. Representative H&E-stained section of tumors from mice from the four groups. Note that in the THD+bicalutamide tumors there appear to be gaps in the tumor tissue and many cells look shrunk.

S2C. Representative tumor sections stained for PCNA.

S2D. Tumors from mice treated with bicalutamide display an increase in TLK1B proteins.


A
CSS + + + + + +
Time(h) 0 0.5 1 1.5 2 4
TLK1
TLK1B
B
CSS + + - -
FCS - - + +
RAPA + - + -
4EBP1
C
Prostate Cancer (Trento/Cornell/Broad) n=114
Disease Code
NEK1 33%
Disease Code
NEK1 12%
Genetic Alteration
Amplification
mRNA Upregulation
No Alteration
CRPC-Adeno
CRPC-NE
Mixed
D
Prostate Cancer, Metastatic (Michigan, Nature 2012) n=94
Disease Code
NEK1 21%
Genetic Alteration
mRNA Upregulation
mRNA downregulation
No Alteration
Disease Code
Metastatic
E
PAN-CANCER ANALYSIS OF WHOLE GENOMES (PCAWG) n=221
Prostate Adenocarcinoma
Normal- adjacent to Prostate Adenocarcinoma
Normal- Prostate gland (GTEx)
NEK1
Expression level in FPKM
0
3
F
RWPE-1
LNCaP
C4-2B
PC3
22Rv1
DU145
p-Nek1
p-Nek1
Nek1
β-tubulin
G
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T141A
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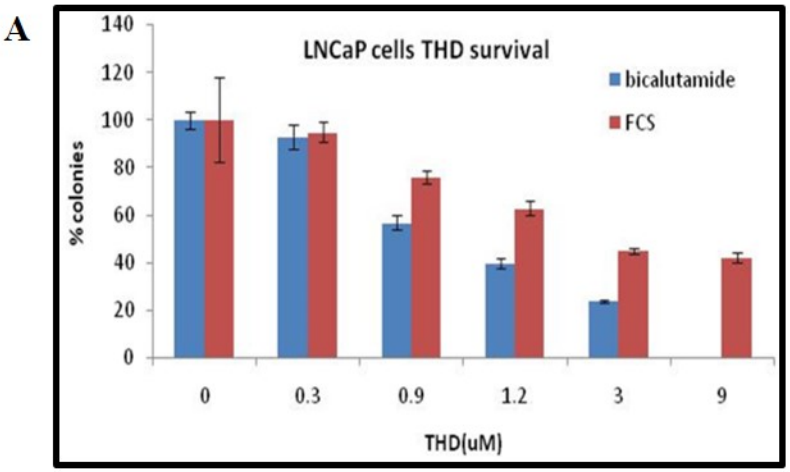
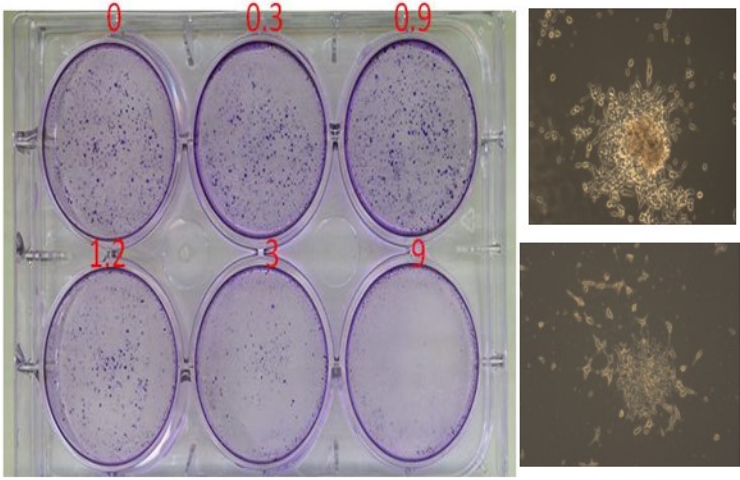
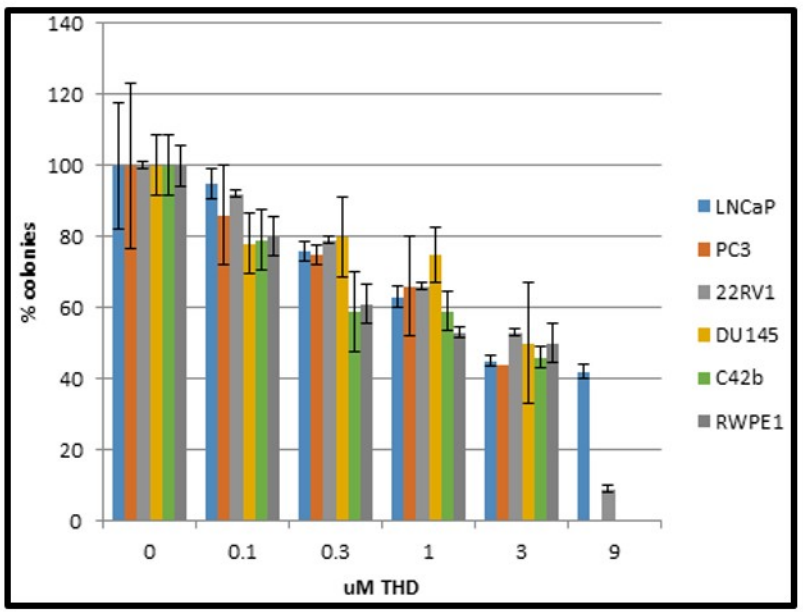
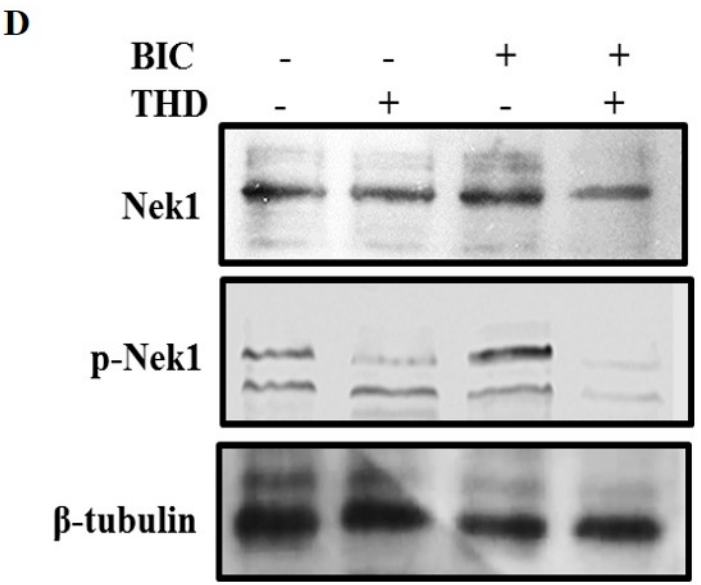
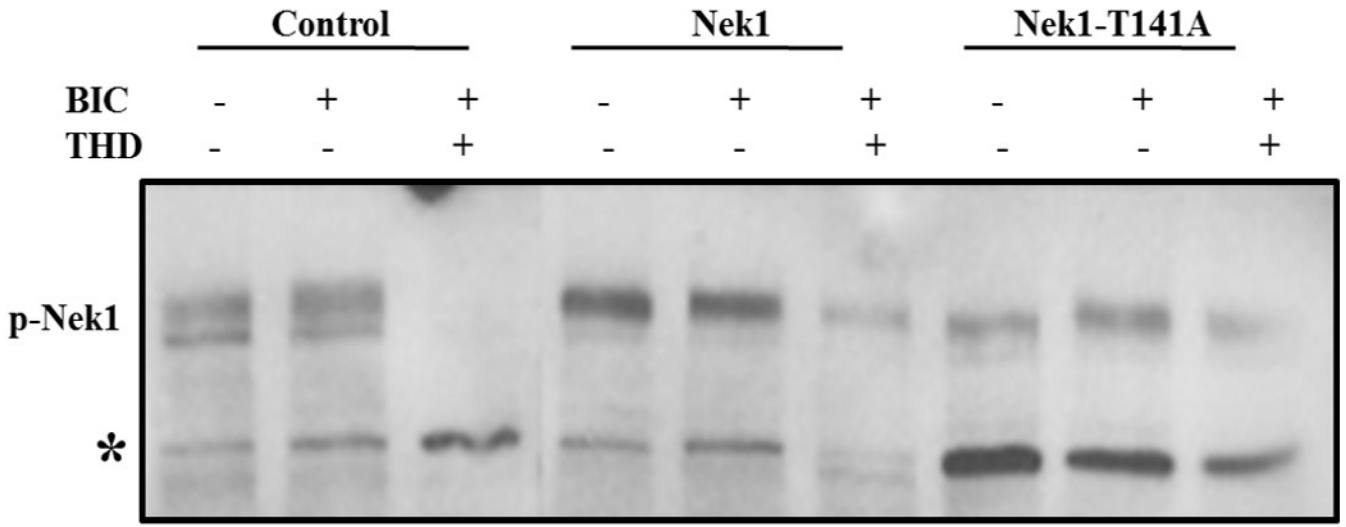

A
LNCaP cells THD survival
bicalutamide
FCS
% colonies
140
120
100
80
60
40
20
0
0
0.3
0.9
1.2
3
9
THD(uM)

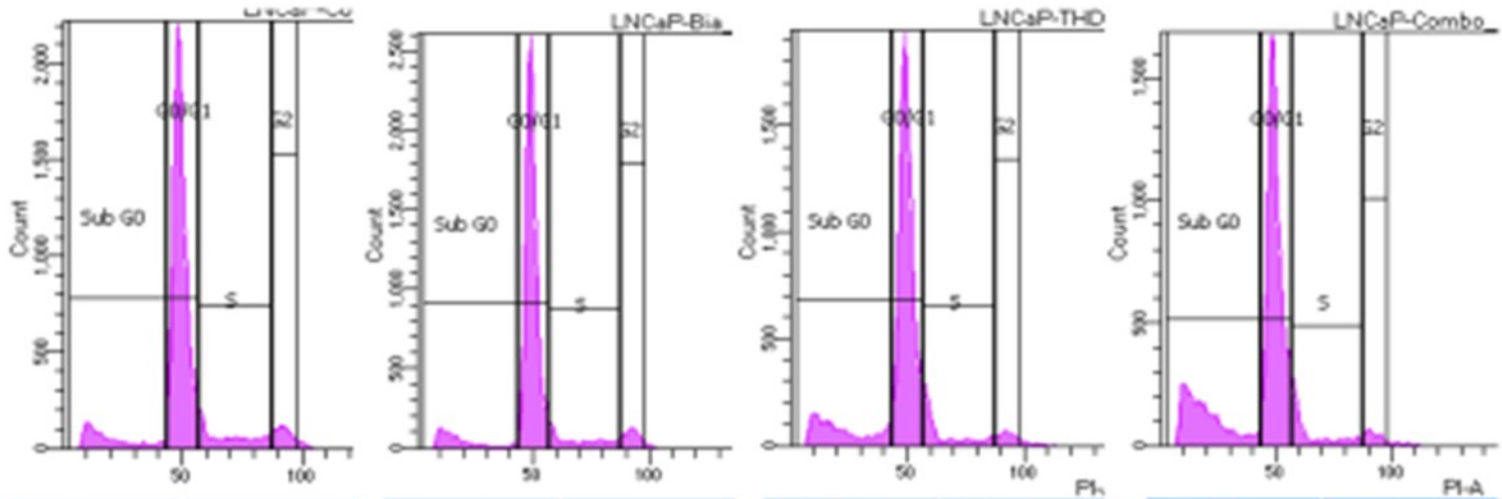

B
0
0.3
0.9
1.2
3
9


C
% colonies
140
120
100
80
60
40
20
0
0
0.1
0.3
1
3
9
uM THD
LNCaP
PC3
22RV1
DU145
C42b
RWPE1


D
BIC - - + +
THD - + - +
Nek1
p-Nek1
β-tubulin


E
Control
Nek1
Nek1-T141A
BIC - + + - + + - + +
THD - - + - - + - - +
p-Nek1
*

A



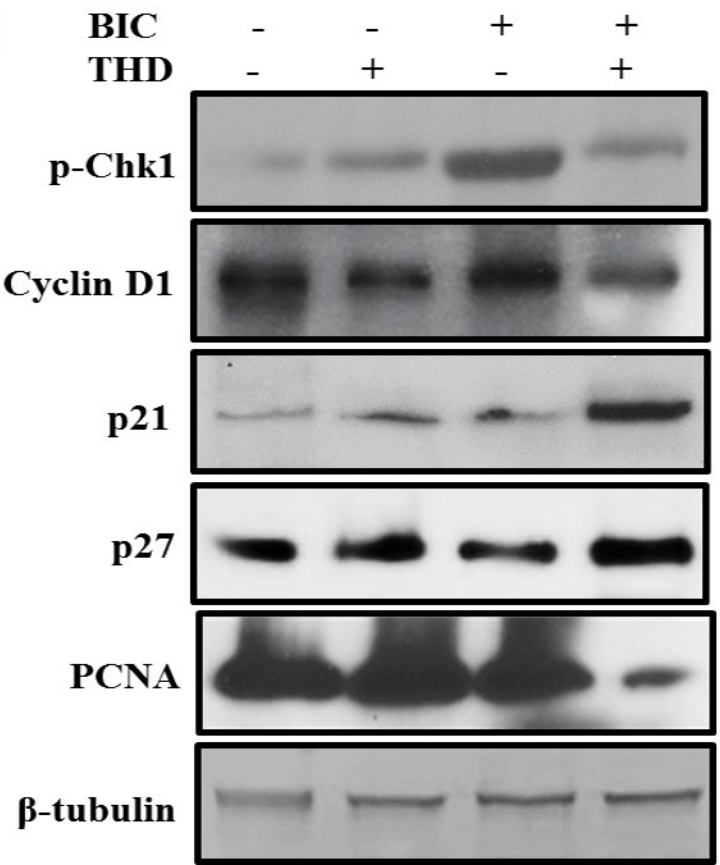
G0/G1	56
G2	4.5
S	8.9
Sub G1	9

G0/G1	72.6
G2	5.6
S	7.5
Sub G1	14.3

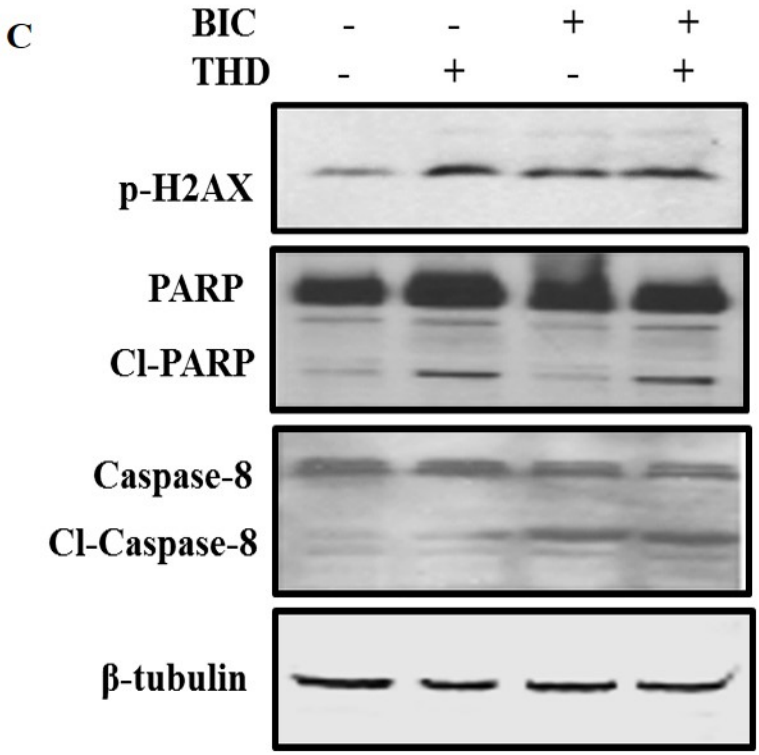
G0/G1	57.3
G2	2.9
S	7.5
Sub G1	22.3

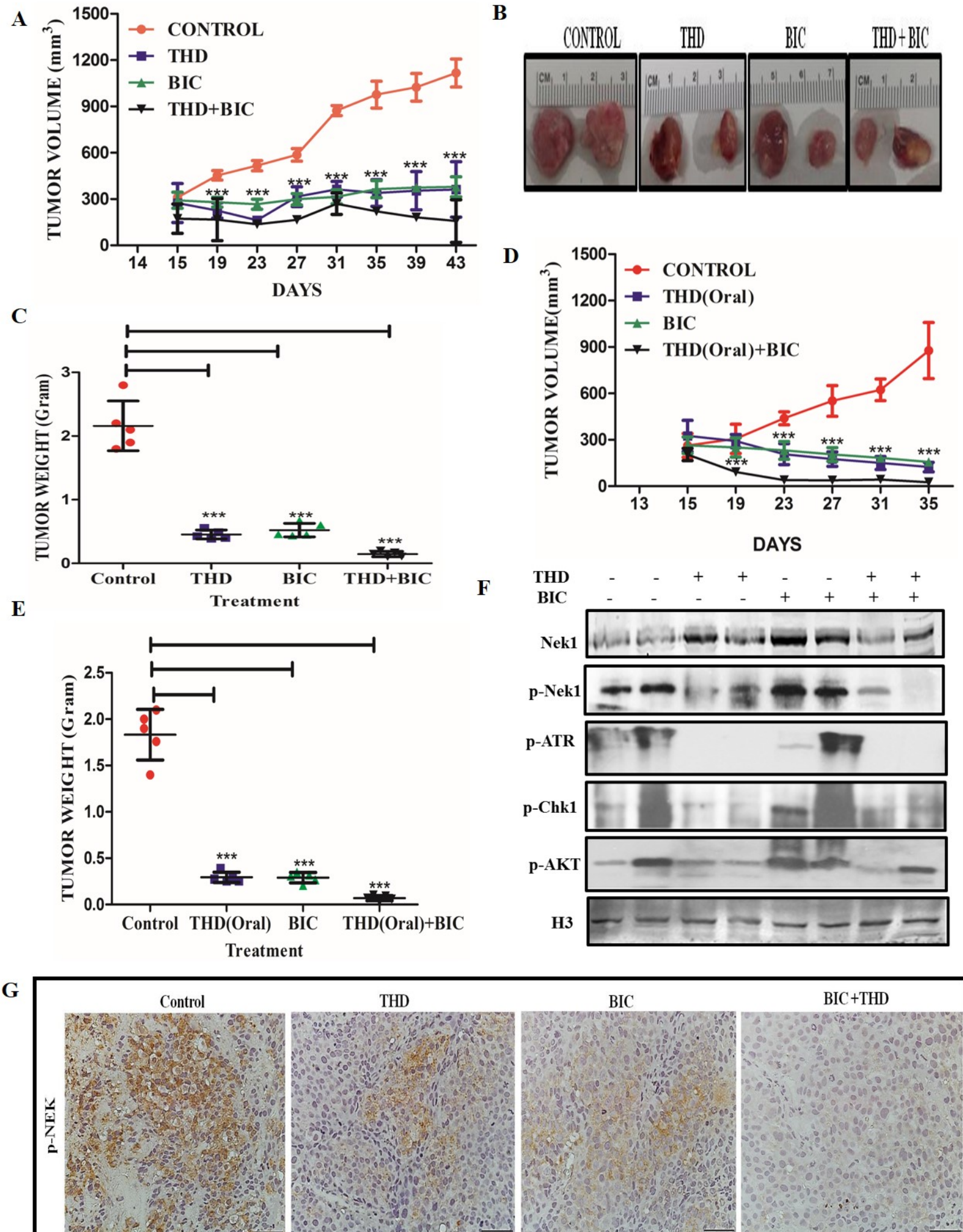
G0/G1	60.5
G2	3.2
S	8.2
Sub G1	28

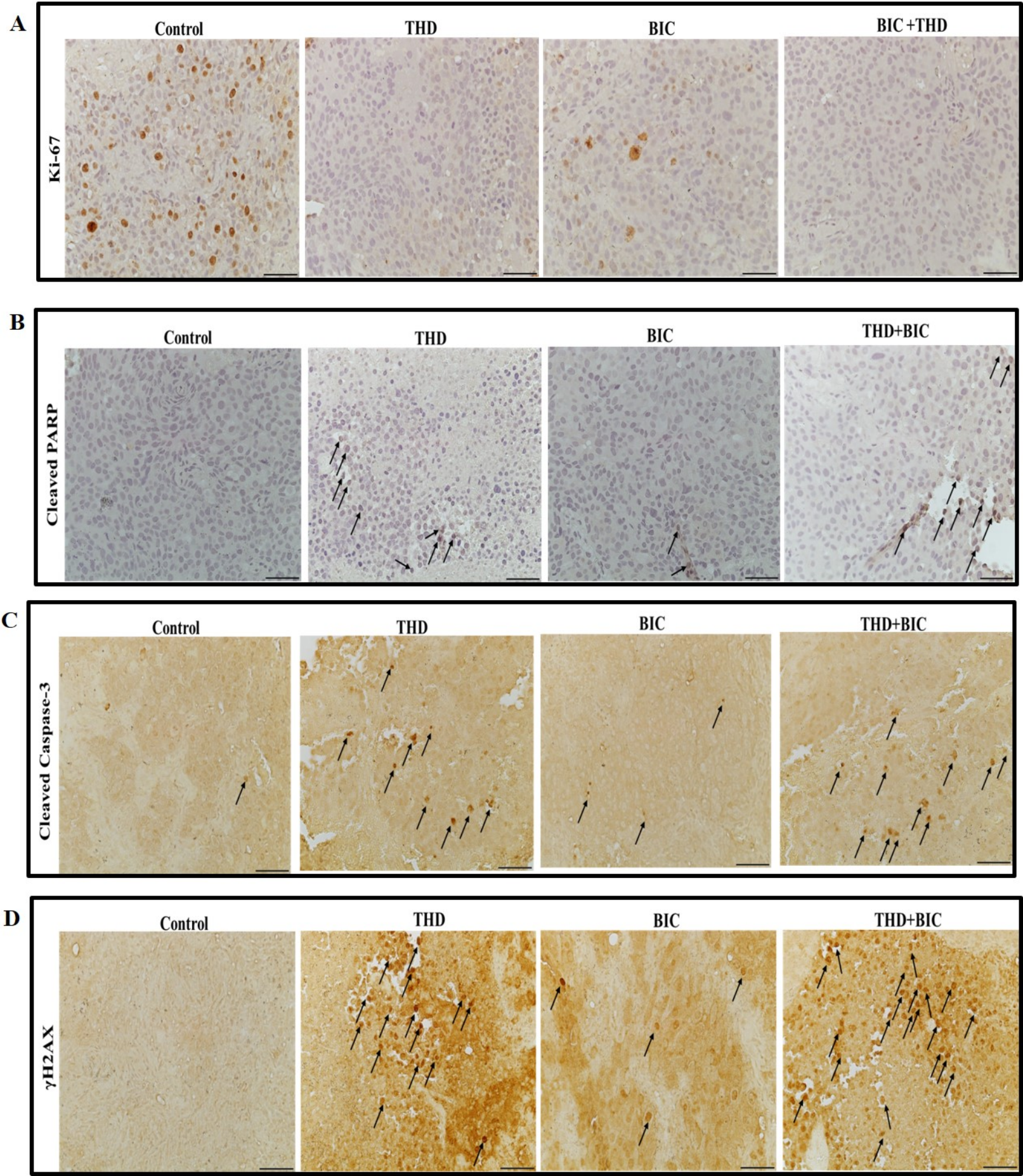
B

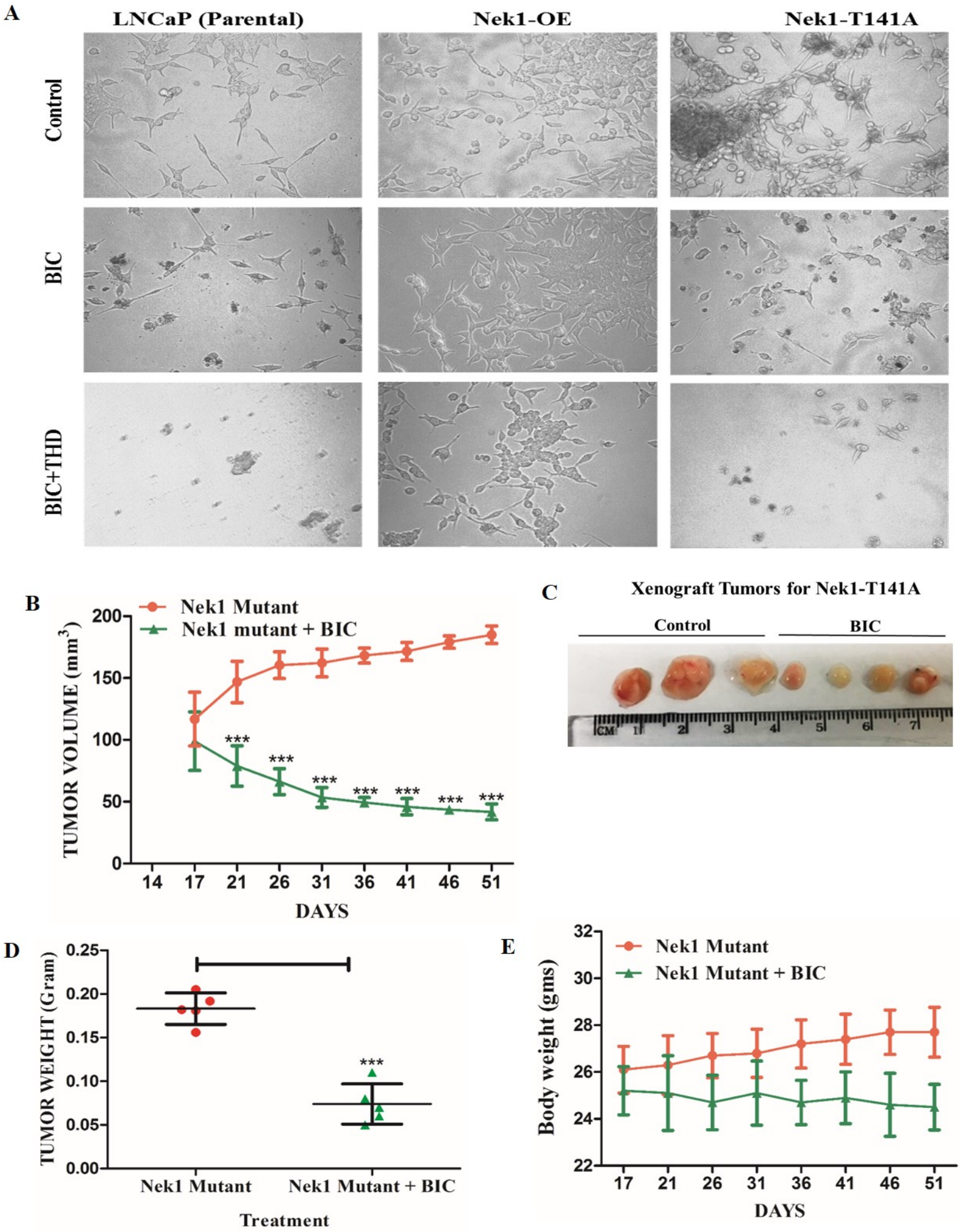


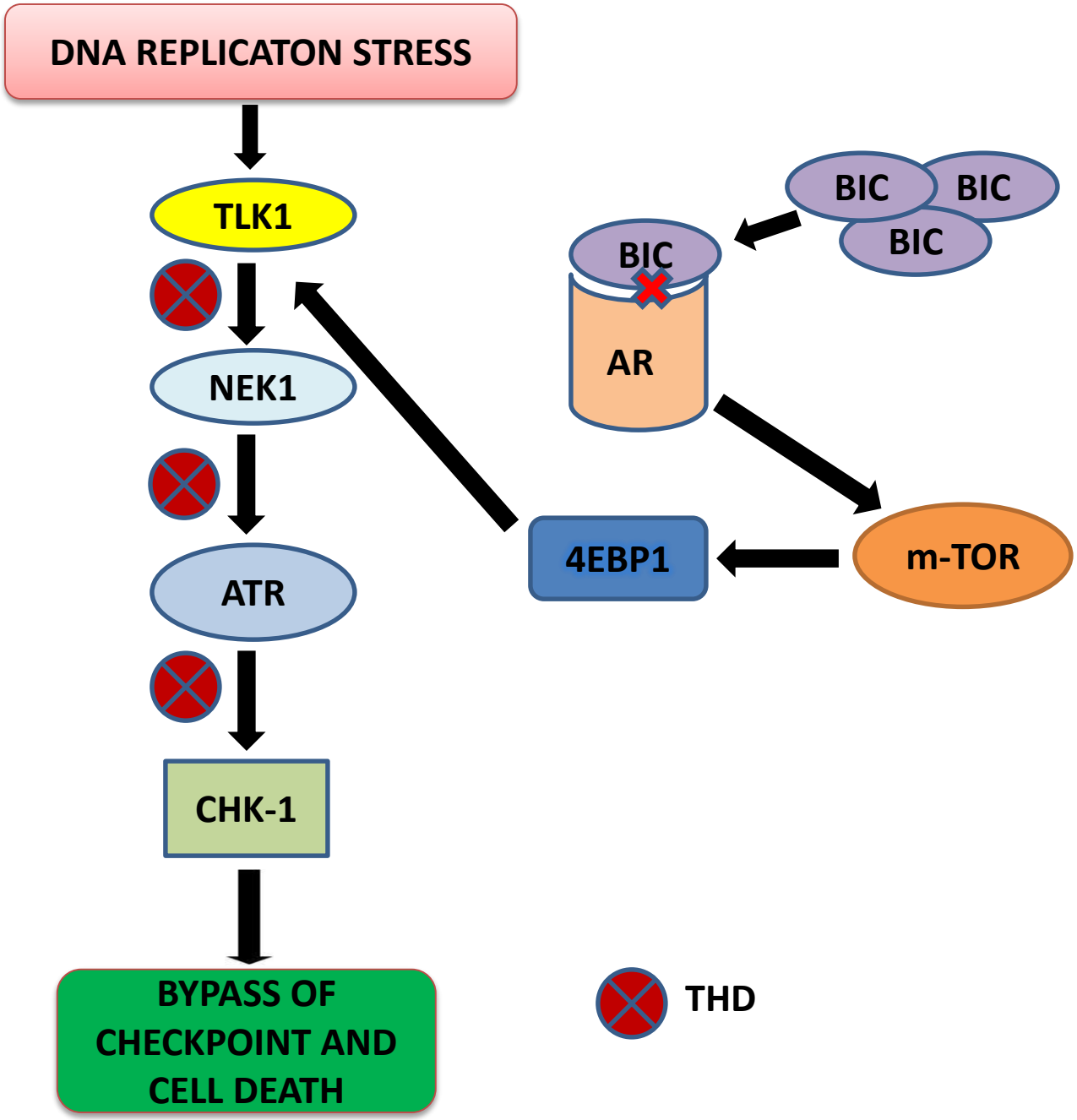
C



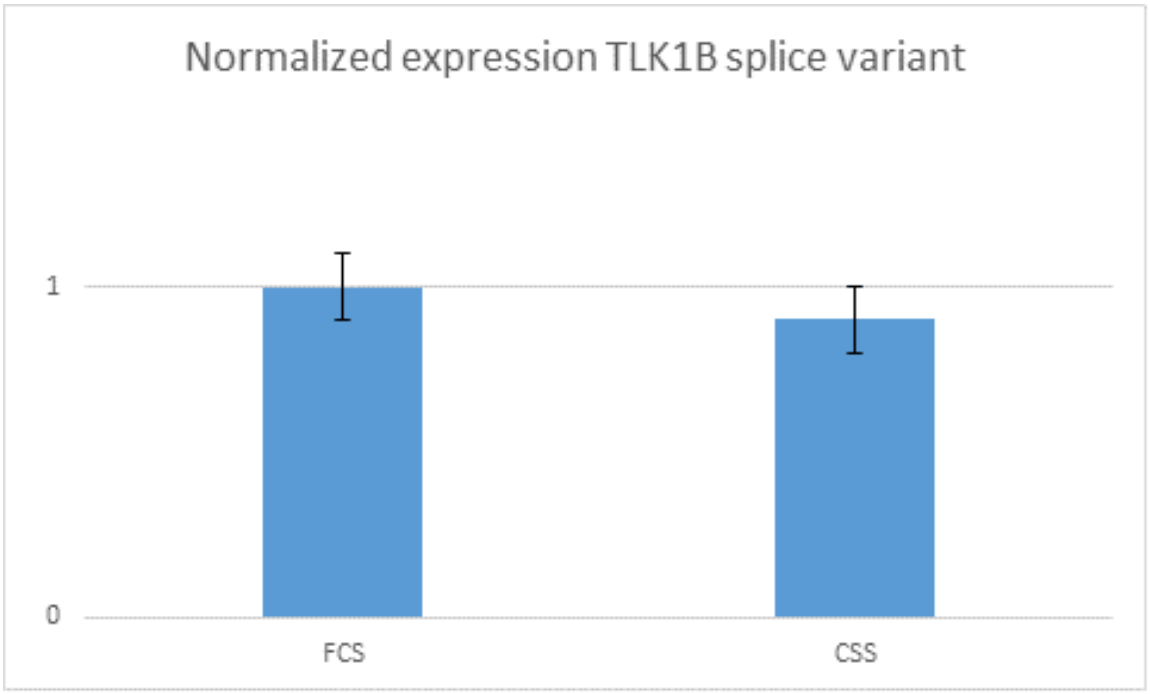

A
TUMOR VOLUME (mm3)
CONTROL
THD
BIC
THD+BIC
DAYS
B
CONTROL
THD
BIC
THD+BIC
C
TUMOR WEIGHT (Gram)
Control
THD
BIC
THD+BIC
Treatment
D
TUMOR VOLUME(mm3)
CONTROL
THD(Oral)
BIC
THD(Oral)+BIC
DAYS
E
TUMOR WEIGHT (Gram)
Control
THD(Oral)
BIC
THD(Oral)+BIC
Treatment
F
THD
BIC
Nek1
p-Nek1
p-ATR
p-Chk1
p-AKT
H3
G
Control
THD
BIC
BIC+THD
p-NEK


A
Control
THD
BIC
BIC +THD
Ki-67
B
Control
THD
BIC
THD+BIC
Cleaved PARP
C
Control
THD
BIC
THD+BIC
Cleaved Caspase-3
D
Control
THD
BIC
THD+BIC
γH2AX

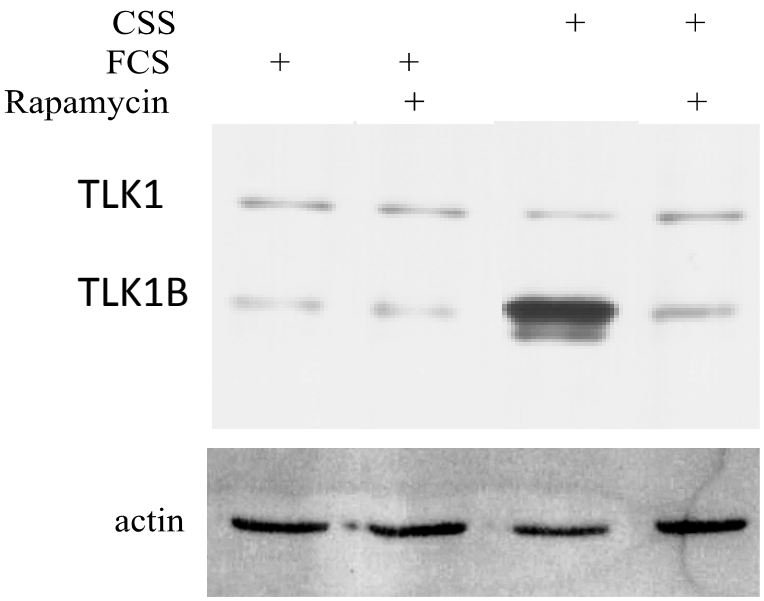

A
LNCaP (Parental)
Nek1-OE
Nek1-T141A
Control
BIC
BIC+THD
B
Nek1 Mutant
Nek1 mutant + BIC
TUMOR VOLUME (mm3)
200
150
100
50
0
***
14
17
21
26
31
36
41
46
51
DAYS
C
Xenograft Tumors for Nek1-T141A
Control
BIC
CM
1
2
3
4
5
6
7
D
TUMOR WEIGHT (Gram)
0.25
0.20
0.15
0.10
0.05
0.00
***
Nek1 Mutant
Nek1 Mutant + BIC
Treatment
E
Nek1 Mutant
Nek1 Mutant + BIC
Body weight (gms)
32
30
28
26
24
22
17
21
26
31
36
41
46
51
DAYS


DNA REPLICATON STRESS
TLK1
NEK1
ATR
CHK-1
BYPASS OF CHECKPOINT AND CELL DEATH
BIC
BIC
BIC
BIC
AR
4EBP1
m-TOR
THD

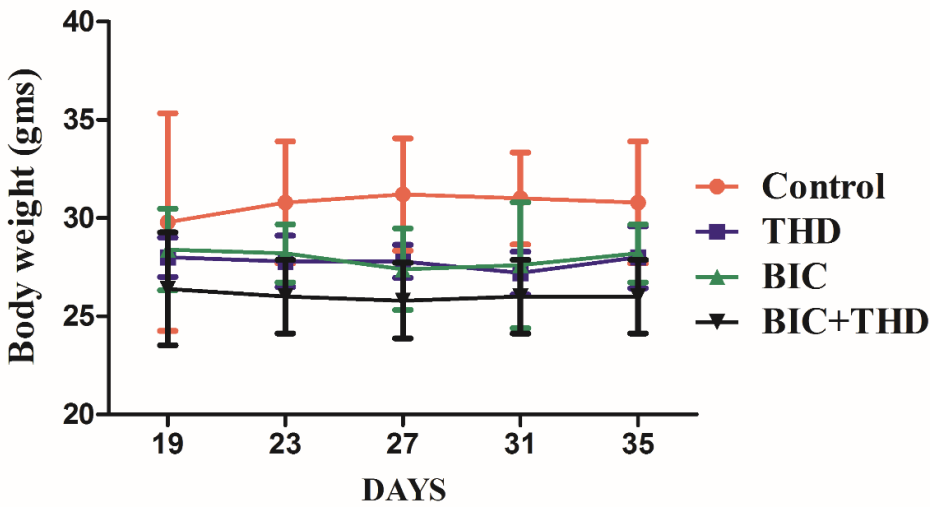
S1A


Normalized expression TLK1B splice variant
1
0
FCS
CSS

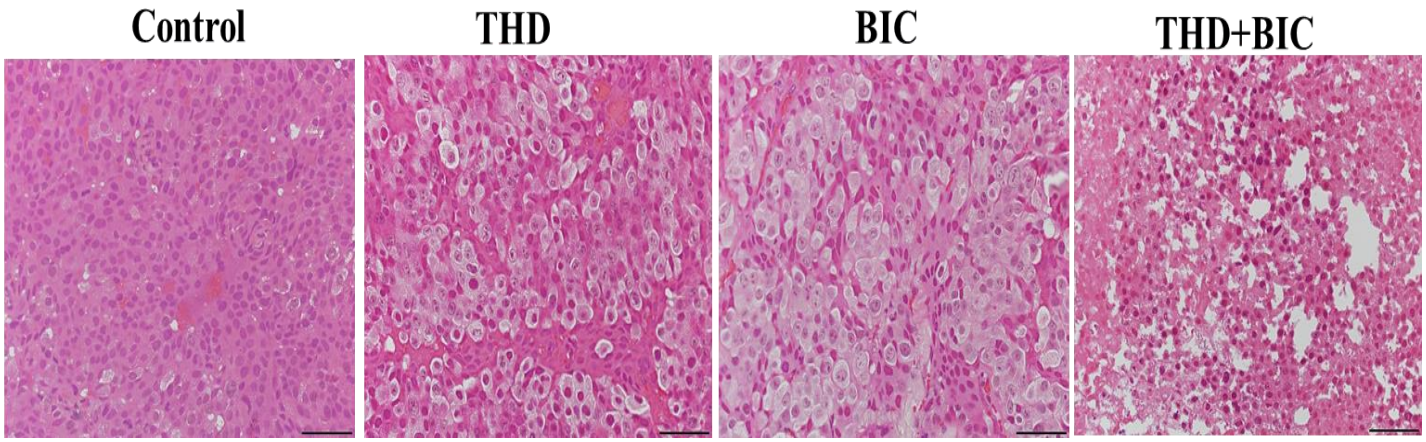
S1B


CSS + +
FCS + +
Rapamycin + +
TLK1
TLK1B
actin

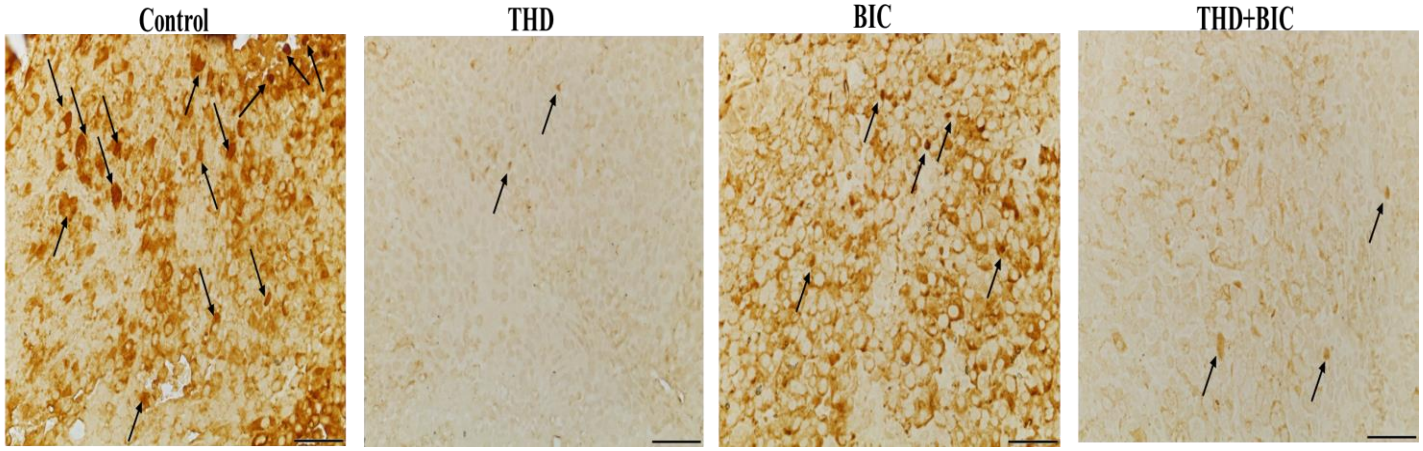
S2A



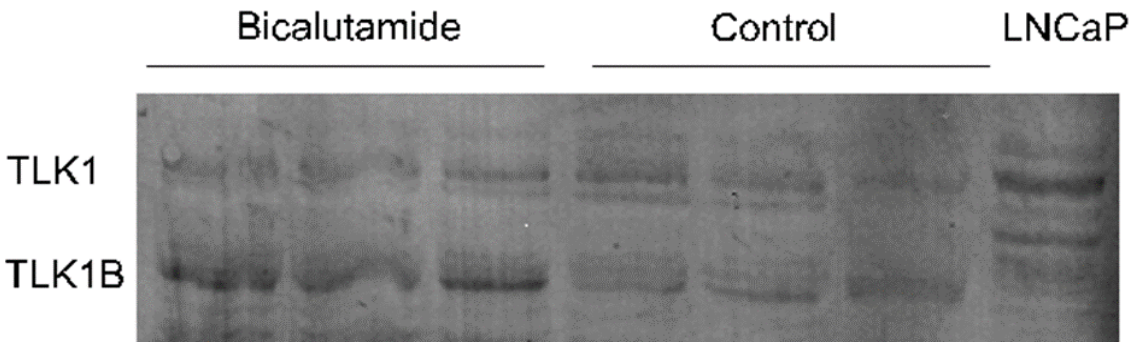
S2B



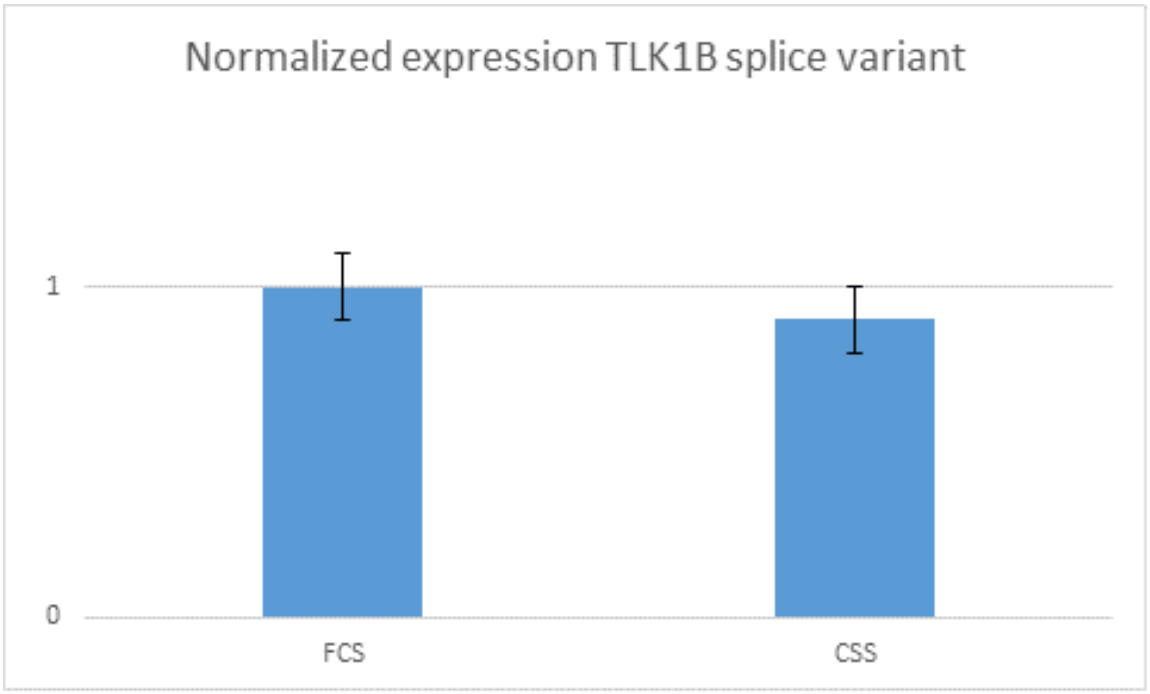
S2C



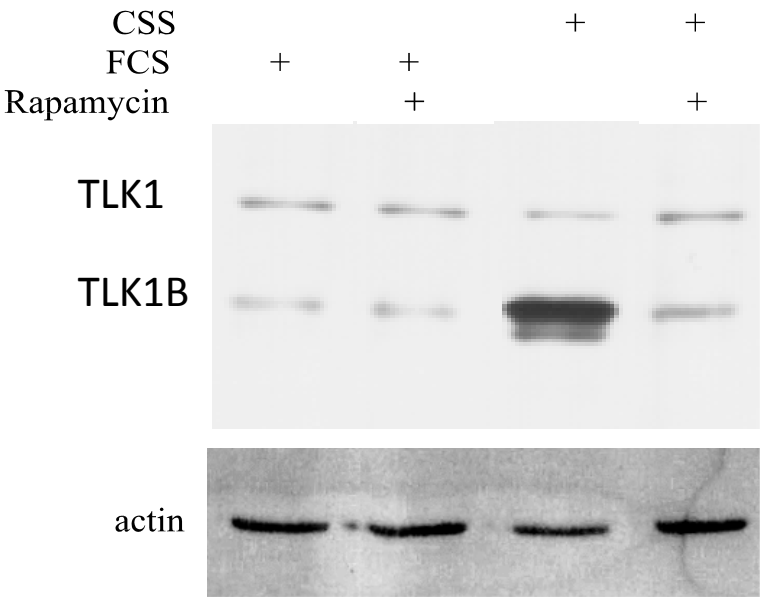
S2D



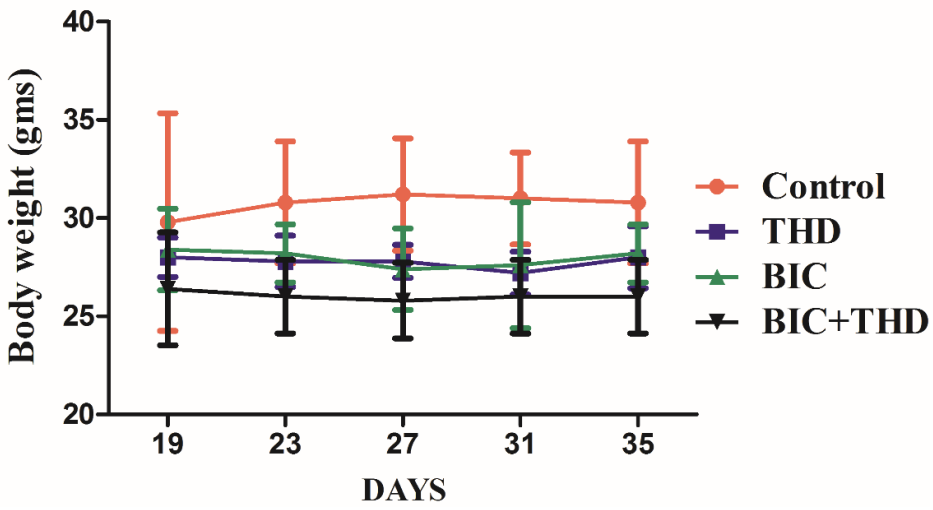
S1A


Normalized expression TLK1B splice variant
1
0
FCS
CSS

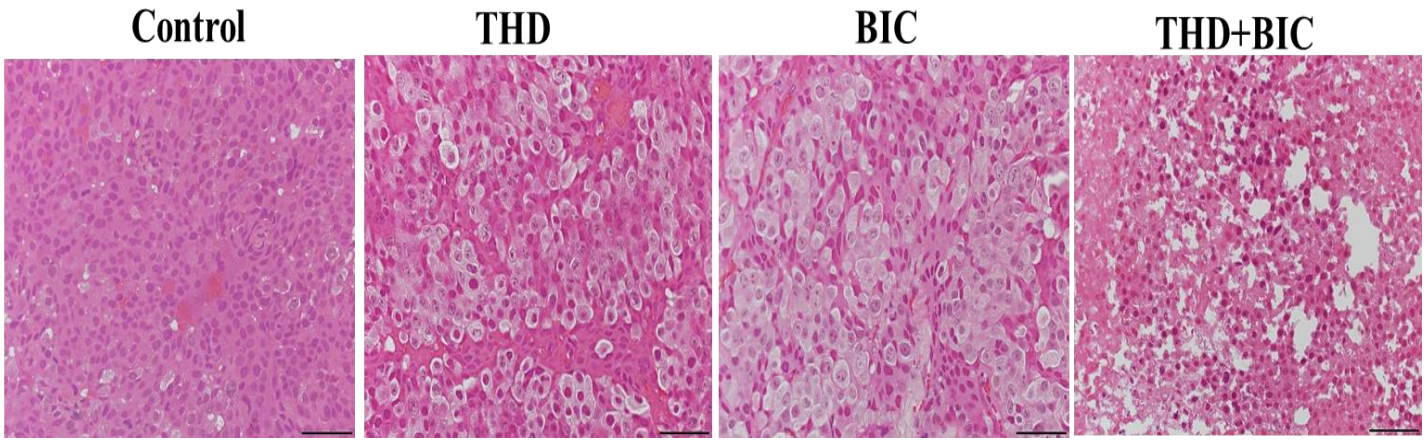
S1B


CSS + +
FCS + +
Rapamycin + +
TLK1
TLK1B
actin

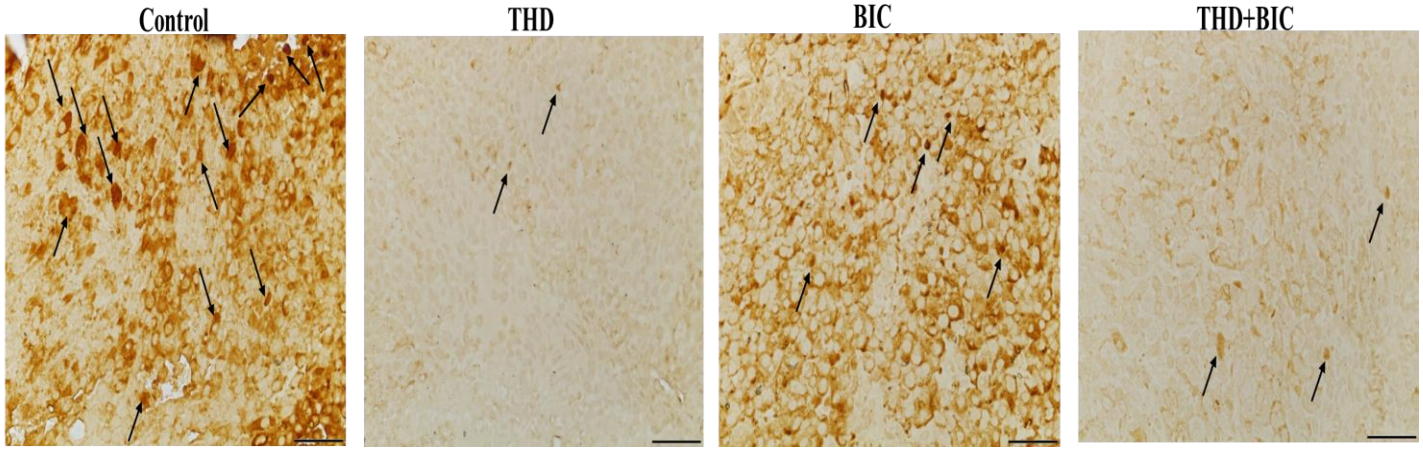
S2A



S2B



S2C



S2D