Reply to Editor:

We thank the editors for the comments provided and the following modifications have been made to accommodate the concerns raised. We hope the advances described herein could serve to facilitate the continuing efforts to improve cancer therapy globally.

(1) Science Editor

1. Scientific quality: The manuscript describes a frontier of the tumor-specific lytic path 'hyperploid progression-mediated death'. The topic is within the scope of the WJCO.

(1) Classification: Grade B

(2) Summary of the Peer-Review Report: The authors reported a very interesting topic. The manuscript well-written and clearly described. But some points of view in the article need to be further supported by more discussion with relevant published evidence and/or other data to make the review more integrated and substantial. The questions raised by the reviewer should be answered;

Specific Comments to Authors: This manuscript describes that clarification of Tumor-Specific Lytic Path might cast light on resolving the side effects of cytotoxic drugs of cancer therapy by targeting RB/p53-mutant. Firstly, the authors summarized the current status of chemoprevention and chemotherapy for various of cancers including colon cancer, lung cancer, breast cancer and head/neck cancer, introducing the mainline drugs used nowadays. Then the unresolved issue of side effect for the related drugs was discussed, including the chemoprevention and chemotherapy drugs. These issues limited the dose escalation and prolongation during treatment, impeding the cure of cancer. After that, the authors pointed out that the anti-tumor mechanism of cytotoxic drugs may be different from the mechanism underlying their side effects. Taking Taxol as an example, the authors explained how the lytic path would provide a mechanistic framework for developing cytotoxic drugs devoid of side effects by conferring tumor specificity at the genetic level. The topic is very interesting. The manuscript well-written and clearly described. But some points of view in the article need to be further supported by more discussion with relevant published evidence and/or other data to make the review more integrated and substantial.

1. Considering the authors pointed out that side effect was one of the most important obstacle against cytotoxic drug application in cancer cure, more reference need to be reviewed in section 5 of this manuscript. about the current research on developing cytotoxic drugs based on RB or p53 genetic mutants to avoid side effects.

The following text has been added to describe current research on developing cytotoxic drugs based on RB or p53 genetic mutants to avoid side effects.

Current pharmacological approaches targeting RB or p53 mutants

In the case of p53, its mutant form may impart oncogenic properties to the affected cell. Nevertheless, various molecularly targeted approaches for treating p53-mutant cancers have

been developed[56]. For RB, its loss is associated with enhanced efficacy for ionizing radiation therapy, chemotherapeutics such as cisplatin or adrimycin[57].

In 2018, Knudsen et al. of Thomas Jefferson University screened for therapeutic agents that may exhibit synthetic lethality with Rb loss. They found that drugs inhibiting CHK1 or PLK1 kinase exhibit greater lethality in RB mutant tumors. The finding raises the potential of treating triple-negative breast cancers with dysfunctional RB[58]. CHK1 encodes a serine/threonine kinase whose activation initiates cell cycle arrest in response to DNA damage during S, G2, and M phases. PLK1 (polo-like kinase 1) is a serine/threonine kinase that activates cdc25C, which in turn activates cyclin B/cdc2 complex through dephosphorylation, as well as anaphase-promoting complex to transit from G2 to M phase.

In 2019, Gomaa *et al.* of the University of Miami Miller School of Medicine showed that reconstituting the microRNA, miR-4715--3p, reduced aurora kinase A level in MKN45 gastric cancer cells, resulting in chromosome polyploidy and cell death. miR-4715--3p is an epigenetic regulator that downregulates aurora kinase A expression by binding to the 3'-untranslated region of its mRNA for degradation[59].

In the same year, Sun *et al.* of the National Cancer Institute demonstrated that genetically silencing INCENP (Inner centromere protein) in neuroblastoma cells induces polyploidy and apoptosis[60]. The INCENP gene encodes a key scaffolding component of chromosomal passenger complex consisting of Survivn, Aurora Kinase B, Borealin, and INCENP, which oversees proper alignment and segregation of chromosomes and cytokinesis during mitosis.

Additionally, Zheng *et al.* of the University of Texas M. D. Anderson Cancer Center showed that treating lung cancer cells with a highly selective TTK inhibitor, CFI-402257, causes aneuploidy and apoptosis[61]. TTK (also called Mps1) or tyrosine threonine kinase is a component of mammalian spindle assembly checkpoint, which is integral to maintaining chromosome integrity.

In 2020, Serrano *et al.* of the Lund University in Sweden reported that inhibiting Mps1 kinase (mitotic kinase Monopolar Spindle 1) induces hyperploid progression mediated death in neuroblastoma cells. The mechanism of death in Mps1 inhibited cells involves transiting through polyploidization/aneuploidization before the onset of mitotic catastrophe[62].

2. The author considered the TSLP 'hyperploid progression mediated death (HPMD)' as tumor specific pathway and took Taxol induced chromosomal aneuploidy as an example. Recently, depletion of microtubule-associated protein ATIP3 (AT2 receptor-interacting protein 3) was approved to induce aneuploidy and sensitizes breast cancer cells to taxanes. As always, combination therapy plays important role in reducing side effect and improve therapy efficacy, therefore this part should be included in the manuscript to make the discussion stronger.

The following text has been added to address the point of developing a combination therapy to reduce side effects and improve therapy efficacy.

Combination therapy targeting RB or p53 mutants

In 2010, Georgieva et al. of Charité-Universitätsmedizin Berlin in Germany determined that combining the aurora kinase inhibitor ZM447439 significantly improves the antiproliferative effects of the chemotherapeutic drug cisplatin and streptozocin. For the study, the authors employed gastroenteropancreatic neuroendocrine tumor cells.

In 2017, Herudkova et al., of the Czech Academy of Sciences in the Czech Republic examined the effect of combining CHK1 kinase inhibitor SCH900776 with the DNA crosslinking drug cisplatin or platinum(IV)-LA-12 complexes in treating colon cancer. In p53 or p21 deficient cells, the combination therapy increased mitotic slippage, leading to polyploidy. Further, the delayed death caused by the drug combination in p53 deficient cells was accelerated by p21 deficiency[63].

In the same year, Bressy *et al.* of Université Paris-Saclay in France assessed the therapeutic efficacy of combining oncolytic adenovirus containing delta-24 deletion in the E1A gene with valproic acid, a histone deacetylase inhibitor for colon carcinoma. Previously, it was shown that E1A interacts with Rb; thus, the recombinant virus may selectively replicate in Rb-deficient cancer cells but not in normal cells expressing wild-type Rb. The co-treatment led to polyploidy with increased H2AX phosphorylation indicative of DNA damage, as well as elevated cell death[64].

In 2018, Kawakami *et al.* of the University of Texas M. D. Anderson Cancer Center reported that treating with CFI-400945, which inhibits Polo-like kinase 4 regulating centriole duplication, causes polyploidy and mitotic defect, resulting in the death of lung cancer cells. Further, it was shown to cooperate with the Cdk2 inhibitor seliciclib[65].

In 2019, Gong *et al.*. of Eli Lilly and Company showed that cell cycle inhibitors targeting aurora kinase B exhibit synthetic lethality with RB mutant. LY3295668 is an inhibitor of Aurora

kinase but exhibits with >1000-fold selectivity against Aurora kinase B. Further, prolonged treatment was possible due to little toxicity against bone marrow[66].

A recent report in 2020 by Liu et al.of Kaohsiung Medical University in Taiwan showed that treating non-small-cell lung cancer cells with 4-HPPP (4-[4-(4-hydroxyphenoxy)phenoxy]phenol) caused polyploidy-specific cell death[67]. The treatment resulted in cellular aneuploidization accompanied by the activation of double-strand DNA break markers such as ATR (Ataxia-telangiectasia-mutated and Rad3-related) and gamma-H2AX. Previously, the phenoxyphenol derivatives have been suggested to sensitize the lung cancer cells to the topoisomerase inhibitor camptothecin by reducing the apoptosis-inducing threshold[68].

In the same year, Jemaa *et al.* of Lund University in Sweden described that treating tetraploid colon cancer cells with PLK1 inhibitor caused mitotic slippage, followed by apopotis[69]. Further, combining PLK1 inhibitor with vincristine or colchicine resulted in greater lethality, demonstrating a synergistic effect.

3. The references cited in this manuscript are rather old. I understand that the author integrated some of his own works in this manuscript, and of course they are really important and great work, however, as a review, more updated information need to be included.

We have now added the following updated information that provides the latest advances regarding the molecular biology of Rb tumor suppressor protein and the continuing global pharmaceutical works concerning the development of novel anticancer drugs based on 'hyperploid progression mediated death' to selectively target Rb or p53 mutant human cancers.

Universal inactivation of the Rb pathway in human cancers

The genetic basis of human cancers was elucidated through the identification of the prototypic tumor suppressor gene RB, whose inactivation predisposes to the development of retinoblastoma[27]. The underlying genetic mechanism was proposed by A. Knudsen, who suggested that its dominant pattern of inheritance ould be explained through the late inactivation of the remaining wild-type allele in a hereditary case harboring a mutant allele[28]. It also suggested that the sporadic cases may take a longer period to develop tumors due to the time it takes to acquire mutations in both wild-type alleles.

The retinoblastoma susceptibility gene was identified through molecular cloning, which was conducted in several laboratories[3,29,30]. The human RB gene consists of 27 exons and the

germline and somatic mutations may occur throughout its coding region as well as the promoter[6,31]. The delineation of the RB gene structure has significantly advanced our ability to manage retinoblastoma clinically through genetic diagnosis[32]. Subsequent investigations documented the occurrence of RB gene mutation in various human cancer types including breast cancer, prostate cancer, osteosarcoma, non-small cell lung cancer (>80%), and brain cancer[6,33,34,35].

Rb protein (also known as RB1) is a key component of mammalian DNA damage checkpoint that monitors the integrity of DNA and blocks cell cycle progression past the G1 (or S) phase in the event of DNA damage. The arrest at G1 is critical as it renders time to repair damaged DNA and avoid replicating mutated DNA. The step mediated by Rb represents a focal point where growth regulatory signals transduced through MAPK/ERK or PTEN/AKT pathway as well as mitogenic signals initiated by EGF receptor or ERBB2 (also known as Her2) converge to modulate cell proliferation--hence, giving rise to the concept that Rb represents the center of cell cycle control.

Nevertheless, the exact molecular mechanism through which Rb executes G1 arrest remains incompletely understood[36]. The binding of Rb to DNA-affinity columns suggested that Rb's intracellular function may be regulatory in nature, which was confirmed by its role in regulating gene transcription[4,37,38].

Critical insight into the Rb function was obtained upon uncovering its propensity to form oligomers through self-interaction[8,9]. The observation that cellular Rb exists as a dimer in the cell lysate confirmed its oligomerization potential[10]. A 3-dimensional model showing Rb protein dimer as observed in the asymmetric crystal unit for Rb^{PL-P} is shown (Figure 2). The crystal structure (2.0 Å) of an Rb construct containing 'pocket domain' with the phosphoserine-mimetic (S608E) and a shortened RbPL (large loop within the pocket domain) was solved by Burket *et al*. via crystallizing Rb^{PL-P} (representing Rb380–787Δ616–642/S608E/S612A/S780A) that binds $E2F^{TD}$ (transactivating domain of E2F) with a lesser affinity, suggesting that the glutamate substitution mimics phosphorylated S608[39]. Rb's post-translational modification via phosphorylation, which was uncovered through the identification of cyclin-dependent kinase (CDK) recognition motifs in the Rb polypeptide sequence, was shown to negatively regulate Rb self-interaction[11]. These findings suggested that Rb may form a higher-ordered structure *in vivo* to execute G1 arrest.

The central role of Rb in regulating cell cycling stems from the post-translational modification of Rb by various cyclin-dependent kinases, which occurs progressively as cells transit from the G1 to the M phase. These include Cdk4 and Cdk6 in the G1 phase and Cdk2 in the S phase whose activities require associating with distinct cyclins. The activities of specific Cdks are further modulated through complexing with other factors such as $p14^{Art}$ and p16, with the latter representing a distinct tumor suppressor. The activity of Cdk2 is inhibited by $p21^{Cip1/Waf1}$, whose transcription is regulated by p53, which is mutated in nearly 50% of all human cancers. Additionally, the oncogenic proteins encoded by viruses such as papillomavirus and adenovirus target Rb to promote cell proliferation. As many as 200 cellular proteins may interact with Rb, including E2F regulating the transcription of genes involved in S phase activities that bind to the pocket domain. The above 'Rb pathway' is inactivated in nearly all human cancers.

Triggering of hyperploid progression mediated death in Rb mutant human cancer cells

Hyperploid progression occurs due to continued cell cycle progression without cytokinesis. Briefly, disruption of the mitotic spindle by antimicrotubule drugs activates spindle checkpoint, a component of M phase DNA damage checkpoint, to induce M arrest. After a transient M-arrest, the treated cells re-enter the cell cycle (due to mitotic slippage) without cytokinesis to eventually become re-arrested at a 'G1-like' phase in Rb-retaining cells. In cells lacking Rb, however, the treated cells continue with DNA replication, resulting in hyperploidy. Continued treatment with the antimicrotubule drug leads to the death of the hyperploid cells.

Experimental evidence for the triggering of hyperploid progression mediated death in RB-mutant human cancer cells is shown. WERI-1 is a human retinoblastoma cell line lacking both RB alleles[40]. Continued treatment of WERI-1 cells with the antimicrotubule drug nocodazole led to the death of hyperploid cells induced (Figure 3).

Triggering of hyperploid progression mediated death by antimicrotubule drugs

After the initial report by Hong *et al* in July of 1999[12], multiple other reports followed providing additional evidence for hyperploid progression mediated death. The first category of such reports used antimicrotubule drugs as the inducer.

In August of the same year, Casenghi *et al.* of the University of La Sapienza in Italy reported the propensity of K562 cells lacking p53 to undergo hyperploid progression before

dying following the nocodazole treatment[41]. In the report, polyploidization was confirmed via *in situ* hybridization using chromosome-specific pericentromeric probes. Their exit after a transient M arrest was confirmed by assessing the cyclin B1 or MPM-2 level and the re-initiation of DNA replication was detected by flow cytometric analysis of bromodeoxyuridine (BrdUrd)-incorporated cells.

In September of the same year, Verdoodt *et al.* of Vrije Universiteit Brussel in Belgium reported that a positive correlation exists between the extent of polyploidization induced by nocodazole and the level of apoptosis[42].

In 2001, Tsuiki *et al.* of Kumamoto University in Japan reported that U251MG cells containing mutant p53 undergo hyperploid progression (>4N or 8N peak appeared) before dying (sub-2N or 0-1N peak detected) as monitored by flow cytometry[43]. The authors also showed that enhancing hyperploid progression through the broad-range protein kinase inhibitor Staurosporine causes a greater extent of lethality in U251MG cells.

In the same year, Cassinelli *et al.* of Instituto Nazionale per lo Studio e la Cura dei Tumori in Italy also reported that treatment of p53-mutant human ovarian cancer IGROV-1/Pt1 cells, p53-deficient human prostate carcinoma P3 cells, or Saos-2 with Taxol or its analog IDN 5109 led to hyperploid progression and death[44].

In a separate report by Lanzi *et al.* of Instituto Nazionale per lo Studio e la Cura dei Tumori in Italy, the death of PC3 cells undergoing hyperploid progression following Taxol treatment was directly shown using the TUNEL/PI double-staining method[45].

In 2002, Landen *et al.* of Emory University reported that altering microtubule by Noscapine caused murine melanoma B16S9 cells to undergo hyperploid progression before dying.

In 2012, Qi *et al.* of Showa Pharmaceutical University in Japan showed that pseudolaric acid B (PAB) induces hyperploid progression, resulting in mitotic catastrophe before apoptosis in murine fibrosarcoma L929 cells[46]. PAB is a microtubule destabilizing agent found in the bark of Pseudolarix kaempferi Gorden (Pinaceae) tree in central China, which was used for treating fungal infection.

Triggering of hyperploid progression mediated death by alternate therapeutics

The second category of reports used antimitotic agents other than antimicrotubule drugs as the inducer of hyperploid progression mediated death. In 2003, Ditchfield *et al.* of the University of Manchester and AstraZeneca Pharmaceuticals in England reported that ZM447439-treated HeLa cells undergo hyperploid progression and die[47]. ZM447439 is an inhibitor of Aurora B kinase.

In 2004, Harrington *et al.* of Vertex Pharmaceuticals in England reported that treatment of HeLa cells or human breast cancer MCF-7 cells with VX-680 (Tozasertib), which targets aurora kinase (see below) causes them to undergo hyperploid progression before dying[48]. The exit from M phase of VX-680 treated cancer cells were monitored by assessing the cyclin B1 level. DNA replication occurring without the prior cytokinesis, leading to >4N cells, was also described.

In the same year, Gizatullin *et al.* of Dana Farber Cancer Institute at Harvard Medical School reported that VX-680 triggers hyperploid progression before death in human non-small cell lung cancer A549 cells with a deficient level of p53-induced $p21^{cip1/waf1}$. They also were able to document that cells undergoing hyperploid progression were dying via apoptosis[49].

In 2005, Tao *et al.* of Merck Research Laboratories reported that treating human ovarian cancer A2780 cells with KSP-IA causes hyperploid progression and death[50].
KSP-IA is an inhibitor of Eg5, a member of the kinesin-5 family that plays a critical role in chromosome segregation by maintaining spindle bipolarity. Eg5 functions as a molecular motor that slides along microtubule tracks within cells.

In 2006, Dijkhuis *et al.* of University Medical Center Gronigen in the Netherlands reported that treating with PDMP (D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol) sensitizes neuroblastoma cells to paclitaxel by triggering hyperploidy[51]. PDMP is an inhibitor of glucosylceramide synthase that suppresses sphingolipid biosynthesis.

In the same year, Chin *et al.* of the DNAX Research Institute of Molecular and Cellular Biology Research Institute reported that treating M checkpoint-suppressed HeLa cells with Monasterol causes hyperploid progression and death[52]. Monasterol is an inhibitor of kinesin-5.

In 2007, Hsieh *et al.* of New York Medical College reported that treatment of human prostate cancer PC-3, CWR22Rv1, or DU-145 cells with Reversine causes polyploidy via suppressing cyclin B or Cdk1, resulting in growth arrest.

In 2008, D'Alise AM *et al.* of the European Institute of Oncology in Italy reported that the synthetic purine Reversine inhibits the Aurora kinase and induces hyperploid progression and death in human colon cancer HCT116 cells[53].

In the same year, Hauf *et al.* of Research Institute of Molecular Pathology in Austria reported that treating HeLa cells with Hesperadin, which also targets aurora kinase, causes them to undergo hyperploid progression before dying. Flow cytometry showed the emergence of hyperploidy peak (8N and 16N) preceding the appearance of dead cells[54].

In 2018, Cheng et al. of Peking University in China demonstrated that the treatment of human renal carcinoma cells with Reversine led to polyploidy formation and caspase-dependent cell death[55].

(3) Format: There is 1 figure. A total of 28 references are cited, including no references published in the last 3 years. There are no self-citations.

We now provide a total of 3 figures.

The 2 new figures added describe data for the triggering of hyperploid progression mediated death in RB-mutant human retinoblastoma cells and the structural analysis of the Rb protein.

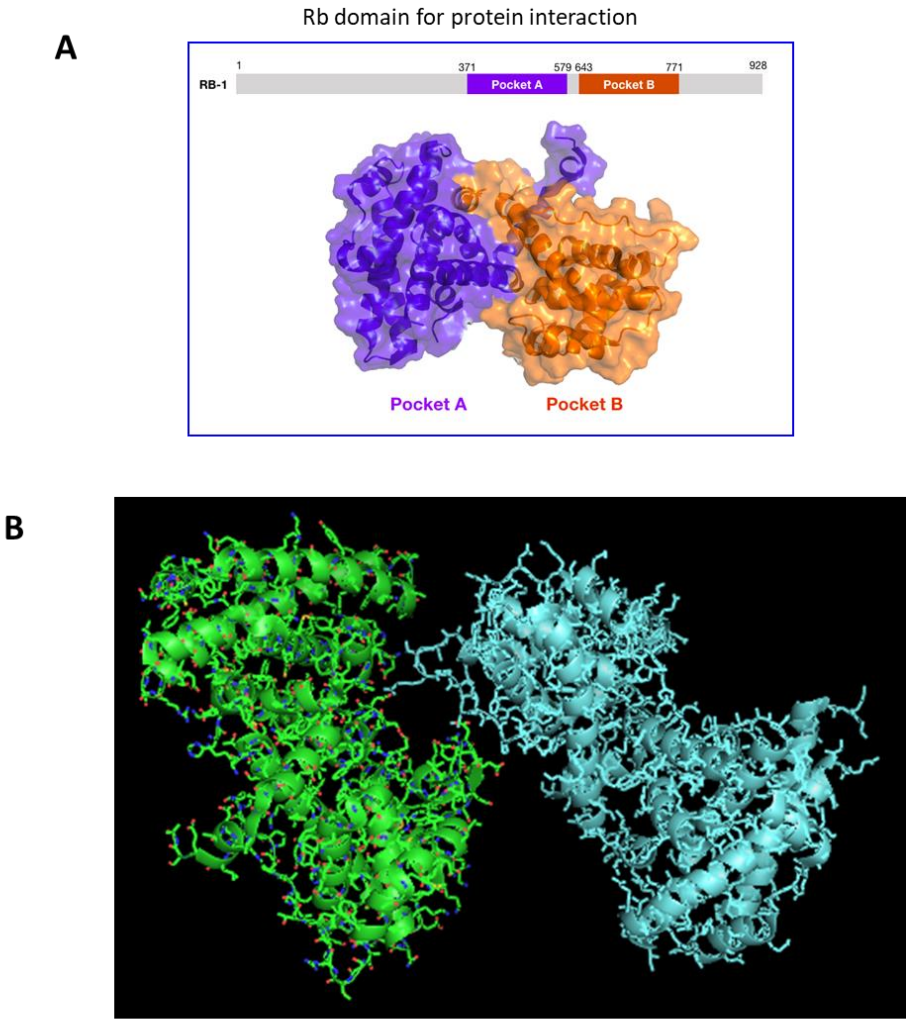

Fig 2

Figure 2.

Structural analysis of human RB protein. (A) 3D structure of the inactive retinoblastoma protein pocket domain of RB-1 (PDB ID: 4ELL). The model was generated using PyMOL Molecular Graphics System, Version 1.2r3pre, Schrödinger, LLC. (B) Structures (or structural model) of phosphorylated Rb. The model shows the protein dimer as observed in the asymmetric crystal unit for Rb^{PL-P}. The structural coordinates for PDB ID 4ELL were downloaded from the NIH protein database. PyMol was used to re-enter the image of the model as shown.

Tumor Specific Lytic Path is Dependent on Inactive RB

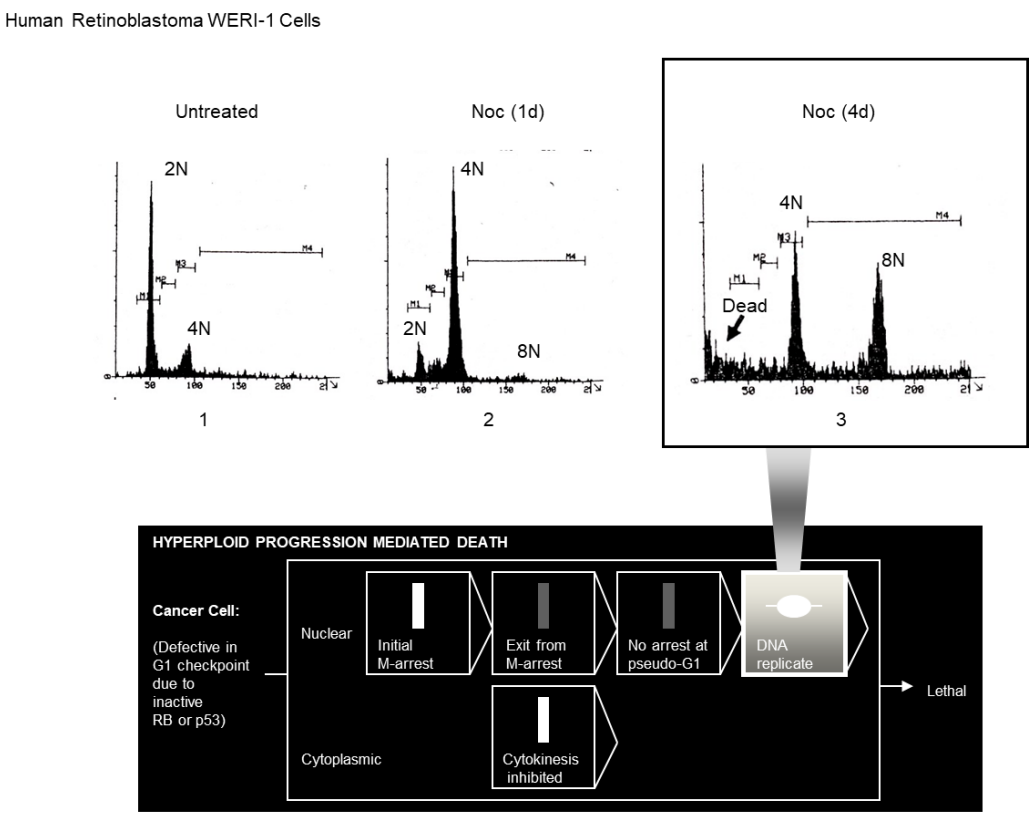

Fig 3

Figure 3.

Nocodazole triggers hyperploid progression mediated death in RB-mutant WERI-1 retinoblastoma cells. A DNA content flow cytometric histogram of WERI-1 cells continuously treated with nocodazole (Noc; 0.415 uM) for the duration (1d, 24 h; 4d, 96 h) is shown. Cells ($5x10^5$) were treated as indicated, harvested using trypsin and fixed in 70% ethanol. To determine DNA content per cell, propidium (100 Kunitz units/ml) and RNase A (50 ug/ml) were added and assayed using Beckton Dickinson FACS. Persistent treatment with nocodazole led to the death of hyperploid cells induced.

In the revised manuscript we now provide a total of 69 references with 25 references published within the last 3 years including 2017.

The newly added references are the following:

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2. Language evaluation: Classification: Grade B.

The manuscript has now been proofread and polished by Klaus Linse, a native speaker who is listed as a co-author for contributing computer-based molecular modeling data of human Rb protein.

3. Academic norms and rules: The authors did not provide the signed Conflict-of-Interest Disclosure Form and Copyright License Agreement. No academic misconduct was found in the CrossCheck detection and Bing search

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4. Supplementary comments: This is an invited manuscript. The topic has not previously been published in the WJCO.

5. Issues raised: (1) The authors did not provide original pictures. Please provide the original figure documents. Please prepare and arrange the figures using PowerPoint to ensure that all graphs or arrows or text portions can be reprocessed by the editor; and (2) the reference's number should be put in a square brackets, then make it superscript..

We now include 3 original pictures in PowerPoint format that can be reprocessed by the editor.

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6 Re-Review: Required.

7 Recommendation: Conditional acceptance.

(2) Editorial Office Director:

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I have reviewed the Peer-Review Report, the full text of the manuscript and the relevant ethics documents, all of which have met the basic publishing requirements, and the manuscript is conditionally accepted with major revisions. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report and the Criteria for Manuscript Revision by Authors. Before final acceptance, authors need to correct the issues raised by the editor to meet the publishing requirements.

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