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GMCL1 Controls 53BP1 Stability and Modulates Taxane Sensitivity

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eLife Assessment

This study identifies 53BP1 as an interaction partner of GMCL1 (a likely CUL3 substrate receptor). The study proposes a novel mechanism by which cancer cells evade the mitotic surveillance pathway through GMCL1-mediated degradation of 53BP1, leading to reduced p53 activation and paclitaxel resistance. These data are the most **useful** aspect of the study, but the data supporting the authors' conclusions as to the clinical relevance of the study are **inadequate**. The authors have not taken relevant data about the clinical mechanism of taxanes into account.

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Abstract

Mitotic surveillance pathways monitor the duration of mitosis (M phase) in the cell cycle. Prolonged M phase, caused by spindle attachment defects or microtubule-targeting drugs triggers formation of the ternary “mitotic stopwatch pathway” complex (MSP) consisting of 53BP1, USP28, and p53. This complex stabilizes p53, leading to cell cycle arrest or apoptosis in daughter cells. In cancers that are resistant to paclitaxel, a microtubule-targeting agent, cells bypass mitotic surveillance activation, allowing unchecked proliferation, although the underlying mechanisms remain poorly understood. Here, we identify GMCL1 as a key negative regulator of MSP signaling. We show that 53BP1 physically interacts with GMCL1, but not its paralog GMCL2, and we map their interaction domains. CRL3^{GMCL1} functions as a ubiquitin ligase that targets 53BP1 for degradation during the M phase, thereby reducing p53 accumulation in daughter cells. Depletion of GMCL1 inhibits cell cycle progression upon release from prolonged mitotic arrest, a defect that is rescued by co-silencing 53BP1 or USP28. Moreover, GMCL1 depletion sensitizes cancer cells to paclitaxel in a p53-dependent manner. Together, our findings support a model in which dysregulated CRL3^{GMCL1}-mediated degradation of 53BP1 prevents proper MSP function, leading to p53 degradation and continued proliferation. Targeting GMCL1 may therefore represent one possible avenue for addressing paclitaxel resistance in cancer cells with functional p53.

Introduction

Mitosis is orchestrated by several intracellular signaling pathways to ensure proper cell division while maintaining genomic integrity. Errors during cell division, including chromosome mis-segregation or spindle defects, could lead to changes in chromosome content, producing aneuploid or polyploid progeny cells, which could be detrimental during development or lead to oncogenesis¹⁻³. Therefore, cells have evolved quality control mechanisms to ensure proper cell division during M phase. One such surveillance mechanism is known as the Mitotic Stopwatch Pathway (MSP). Foundational work from the Sluder lab in 2010⁴ first demonstrated a p53-dependent G1 arrest following prolonged mitosis. This was later expanded by three key studies published in 2016, which identified USP28 (ubiquitin-specific protease 28) and 53BP1 (p53-binding protein 1) as critical components of the pathway⁵⁻⁷. During prolonged mitosis without centrosome loss, a ternary complex of 53BP1, USP28, and p53 forms and persists into the G1 phase, where it induces p21 transcription and enforces p53-dependent cell cycle arrest. In this pathway, 53BP1 and USP28's deubiquitinase activity are required for p53 stabilization⁸⁻¹⁰.

Proper mitotic arrest is critical for the efficacy of microtubule-targeting therapies such as taxanes (*e.g.*, paclitaxel and docetaxel), which disrupt spindle formation and chromosome segregation. Pharmacological disruption of mitosis can induce cell death¹¹, as observed with paclitaxel treatment¹². Clinically, however, the effectiveness of taxanes is often limited, as many cancers develop resistance, including metastatic breast, ovarian, and non-small cell lung cancers¹³. This resistance is frequently associated with loss of MSP activity, for example due to defective p53 signaling¹⁴⁻¹⁶. These observations underscore the urgent need to further elucidate the mechanisms underlying paclitaxel resistance in cancer.

Human germ cell-less protein-like 1 (GMCL1) is a putative substrate receptor of one of many CUL3-RING ubiquitin ligases (CRL3s). However, to date, the biological role of GMCL1 and its substrates have remained uncharacterized. *GMCL1* received its namesake from its *Drosophila melanogaster* homolog, *GCL* (Germ Cell-Less), which plays essential roles in early embryonic development and germ cell determination^{17,18}. The role of *GMCL1* in germ cell development appears to have been evolutionarily conserved as loss of *GMCL1* expression in mice has been shown to cause defects in meiosis and spermiogenesis¹⁹, and altered *GMCL1* expression was functionally associated with human asthenozoospermia²⁰. Although *GMCL1* homologs have been primarily associated with germ cell biology, several databases (*e.g.*, GTEx²¹ and GENT2²²) indicate that *GMCL1* is expressed in somatic cells as well, suggesting broader biological functions beyond germ cell development. In contrast, the *GMCL1* paralog *GMCL2* is specifically expressed in germ cells.

Here, we present studies that clarify the role of *GMCL1* in somatic cells. We show that, similar to its *D. melanogaster* counterpart²³, *GMCL1* functions as a CRL3 substrate receptor. Furthermore, we identify 53BP1 as a *bona fide* substrate of CRL3^{GMCL1} and demonstrate that its levels are regulated by *GMCL1* during prolonged mitotic arrest. By reducing mitotic 53BP1, *GMCL1* inhibits the function of the USP28-p53-53BP1 mitotic stopwatch complex and limits p53 transmission to daughter cells. Based on these findings, we propose that *GMCL1* inhibition may represent a potential novel approach to overcoming paclitaxel resistance in cancer cells with functional p53.

Results

Identification of 53BP1 as an interactor of GMCL1

We have shown that the *GMCL1* ortholog in *D. melanogaster*, *GCL*, is a substrate recognition subunit of a CRL3 complex that is active specifically in mitosis²³. Therefore, we predicted the human *GMCL1* to also behave as a CRL3 substrate receptor. *GMCL1* contains a BTB (Broad-Complex, Tramtrack, and Bric-à-brac) and a BACK (BTB and C-terminal Kelch) domain (Supplementary Figure 1A²⁴), consistent with other CRL3 receptors. On its C-terminus, *GMCL1* also contains a GCL domain (residues 379-515), which is distinct from Kelch domains commonly found in other CUL3 substrate receptors. This GCL domain is predicted to form a β -sandwich characterized by two opposing anti-parallel β -sheets, each made up of four β -strands^{23,24}.

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To investigate the role of GMCL1 in somatic cells, we used immunoprecipitation followed by mass spectrometry (IP-MS) to identify binding partners of GMCL1. Proteomics studies were performed by expressing and purifying the following FLAG-tagged proteins: (i) wild-type GMCL1 (GMCL1 WT), (ii) GMCL1 E142K (GMCL1 EK), which carries a mutation in the BTB domain that is predicted to disrupt its interaction with CUL3, and (iii) GMCL1 BTB/BACK-only (GMCL1 BBO) that lacks the GCL domain (Figure 1A-C [↗](#)). IP-MS analysis identified 1,765 potential binding partners that specifically interact with GMCL1 *via* its C-terminal domain. Using SAINT scores > 0.70 and FDR < 5%, this list was refined to 9 proteins that showed significant interaction with GMCL1 WT and GMCL1 EK, but not GMCL1 BBO, nominating 53BP1 as the most enriched protein (Figure 1B [↗](#), Supplementary Table 1). This selective enrichment suggests that the C-terminal, “MATH-like” GCL domain of GMCL1 is critical for its interaction with binding partners, including 53BP1. The interaction between GMCL1, and endogenous 53BP1 and CUL3 was validated using immunoprecipitations (IPs) followed by immunoblotting (Figure 1C, D [↗](#)).

To further study the direct interaction between GMCL1 and 53BP1, we used AlphaFold 3²⁷ to locate the positions of contact residues at their predicted binding interface. Consistent with our initial IP-MS experiment, AlphaFold 3 model predicted that the C-terminal domain of GMCL1 would interact with 53BP1. Based on the predicted GMCL1-53BP1 complex structure, we identified Arg 433, which appears to be a solvent-exposed residue in GMCL1’s GCL domain that could interact with 53BP1 without impeding GMCL1 binding with CUL3. Thus, we generated the GMCL1 R433A (GMCL1 RA) point mutant and tested its binding to 53BP1 upon IP. As anticipated, compared to WT GMCL1, the R433A mutation completely abolished the binding of GMCL1 to 53BP1, but did not impact GMCL1’s binding to CUL3 (Figure 1D [↗](#)).

To determine which region of 53BP1 mediates its binding to GMCL1, we mapped the predicted GMCL1-binding site on 53BP1 and identified a conserved IEDI amino acid sequence within the Minimal Focus Forming region (MFF) of 53BP1²⁸ (Supplementary Figure 1B-E [↗](#)). Through a series of IPs, we demonstrate that a 53BP1 mutant either lacking the MFF region or containing the IEDI-to-AAAA mutation within this region lost its interaction with CRL3^{GMCL1}, suggesting that this conserved IEDI sequence within the 53BP1 MFF region forms a critical degron recognized by GMCL1 (Figure 1E [↗](#)). To examine the interaction between endogenous GMCL1 and endogenous 53BP1, we used CRISPR-Cas9 to knock in a FLAG tag at the C-terminus of *GMCL1*.

Immunoprecipitation experiments confirmed that endogenous GMCL1 interacts with both endogenous CUL3 and 53BP1 (Figure 1F [↗](#)).

Finally, we sought to determine whether GMCL2, a GMCL1 paralog, is also able to interact with 53BP1. Immunoprecipitation of FLAG-tagged GMCL1 or GMCL2 from HEK293T cells revealed that while GMCL1 binds to 53BP1, GMCL2 does not (Supplementary Figure 1F [↗](#)), suggesting that GMCL1 and GMCL2 have distinct functions. Overall, our results suggest that GMCL1 is a CRL3 substrate receptor that interacts with 53BP1.

53BP1 is a *bona fide* substrate of GMCL1

To investigate whether GMCL1 regulates 53BP1 stability, we generated *GMCL1* knock-out (KO) cells using CRISPR Cas-9²⁹ and compared 53BP1 levels between *GMCL1* WT and two *GMCL1* KO clones. RT-PCR analysis confirmed the loss of *GMCL1* mRNA expression in the KO clones (Supplementary Figure 1G [↗](#)). We found that 53BP1 levels were significantly increased in our *GMCL1* KO cells

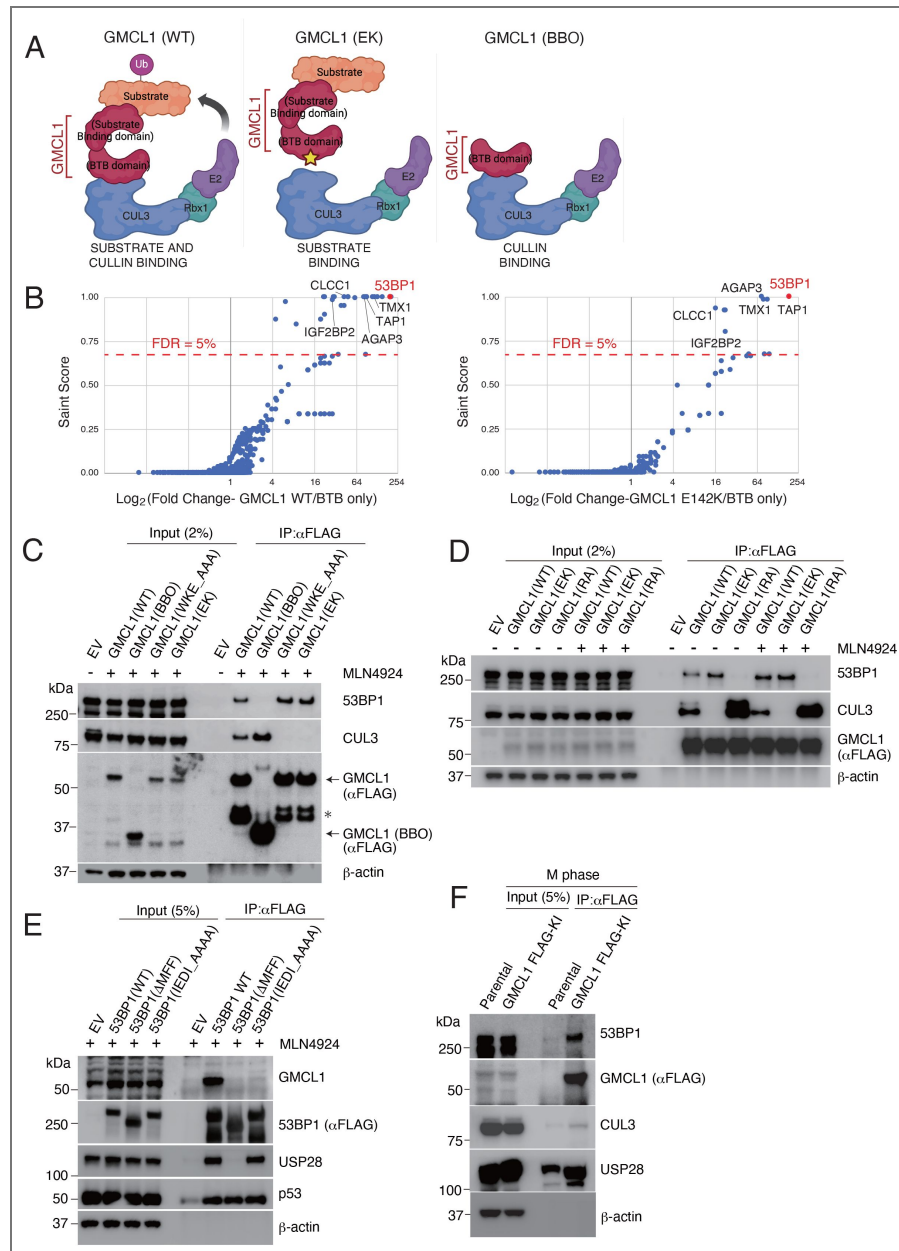


Figure 1. Identification of 53BP1 as a GMCL1 interactor

A) Schematics for the immunoprecipitation-mass spectrometry (IP-MS) workflow using wild-type GMCL1 (GMCL1 WT) and mutants (GMCL1 EK and GMCL1 BBO). Color coding: Red, GMCL1; orange, putative substrates/interacting partners; blue, CUL3; green, RBX1; purple, E2 ubiquitin-conjugating enzyme. B) HEK293T cells were transfected with FLAG-GMCL1 WT, FLAG-GMCL1 EK, or FLAG-GMCL1 BBO. After 24 hours, FLAG-tagged proteins were immunoprecipitated and analyzed by MS/MS. Left panel: proteins enriched with GMCL1 WT vs. BBO; right panel: proteins enriched with GMCL1 EK vs. BBO. Significant interactors were identified using SAINT scores > 0.70 and FDR < 5%. C) HEK293T cells transfected with empty vector (EV), FLAG-GMCL1 WT, FLAG-GMCL1 BBO, FLAG-GMCL1 WKE_AAA (broadly disrupts the binding to CUL3) and FLAG-GMCL1 EK were treated with MLN4924 (3h). 53BP1 and CUL3 were immunoprecipitated with FLAG beads and analyzed by western blot. Asterisk indicates non-specific bands. This experiment was performed four times, and a representative blot is shown. D) HEK293T cells were transfected with EV, FLAG-GMCL1 WT, FLAG-GMCL1 EK, or FLAG-GMCL1 RA. FLAG immunoprecipitations were probed for 53BP1 and CUL3. This experiment was performed four times, and a representative blot is shown. E) HEK293T cells were transfected with EV, FLAG-53BP1 WT, FLAG-53BP1 ΔMFF and FLAG-53BP1 IEDI_AAAA. After MLN4924 treatment (3h), 53BP1 was immunoprecipitated and immunoblotted. This experiment was performed three times, and a representative blot is shown. F) M phase-synchronized GMCL1 FLAG knock-in HCT116 cells were collected. GMCL1 was immunoprecipitated using FLAG-beads and analyzed by immunoblotting.

during M phase. While the whole cell extracts (WCE) showed modest differences in GMCL1 levels between the *GMCL1* WT and KO clones, our fractionation experiments revealed that both 53BP1 and p53 mainly accumulated in the chromatin-bound fraction of *GMCL1* KO M phase cells and this increase did not correspond to a decrease in 53BP1 levels in the soluble fraction (Figure 2A [↗](#)).

To further explore the role of GMCL1 on 53BP1 stability, we stably reconstituted U2OS *GMCL1* KO cells with either GMCL1 WT or binding mutants (*i.e.*, GMCL1 EK and GMCL1 RA). Notably, the accumulation of 53BP1 in *GMCL1* KO cells was rescued only upon re-expression of GMCL1 WT in the chromatin fraction. In contrast, both 53BP1 and p53 levels remained high in *GMCL1* KO cells expressing either GMCL1 EK or GMCL1 RA (Figure 2B [↗](#), Supplementary Figure 2A [↗](#)), emphasizing the importance of GMCL1's ability to bind both CUL3 (through the E142 residue) and 53BP1 (through the R433 residue) to regulate 53BP1 levels. Of note, the R433A mutant was expressed at levels comparable to the WT protein. Interestingly, the E142K mutant showed reduced expression in mitotic cells, yet was the most abundantly expressed in asynchronous cells. Decreases in 53BP1 protein observed upon GMCL1 WT expression was not accompanied by an increase in the soluble fraction (Figure 2B [↗](#), Supplementary Figure 2A [↗](#)), indicating that the reduction in chromatin-associated 53BP1 is not due to re-localization.

Next, we performed cycloheximide (CHX) to inhibit protein synthesis and to directly assess 53BP1 stability. In the chromatin-bound fraction, 53BP1 was more stable in *GMCL1* KO cells compared to cells rescued with GMCL1 WT (Figure 2C [↗](#)). To further support the direct role of GMCL1 in regulating 53BP1 turnover, we co-expressed FLAG-tagged Trypsin-Resistant Tandem Ubiquitin-Binding Entity (TR-TUBE), a construct composed of tandem ubiquitin-binding motifs³⁰, together with either GMCL1 WT or the E142K mutant in HEK293T cells. TR-TUBE efficiently pulled down endogenous ubiquitinated 53BP1, with a marked increase in the presence of GMCL1 WT, but not the GMCL1 E142K mutant (Figure 2D [↗](#) and Supplementary Figure 2B [↗](#)). Collectively, these findings indicate that GMCL1 promotes 53BP1 ubiquitination and subsequent degradation during prolonged mitosis. Consequently, GMCL1-deficient cells retain elevated levels of 53BP1 and p53 following mitosis stress.

GMCL1-regulation of MSP affects cell cycle progression in daughter cells

During mitosis, 53BP1 helps monitor centrosome integrity and mitotic progression through the MSP complex (53BP1-USP28-p53), transmitting this information into daughter cells¹⁰. To investigate the effects of GMCL1 regulation on the MSP complex, we first analyzed post-mitotic *GMCL1* KO daughter cells seven hours after release from prolonged nocodazole arrest. *GMCL1* KO daughter cells reconstituted with GMCL1 WT, exhibited low levels of 53BP1, p53, and p21, along with reduced expression of apoptosis-related genes (Supplementary Figure 2C, D [↗](#)). In contrast, cells reconstituted with either the E142K or the R433A mutant displayed persistently elevated levels of 53BP1, p53, and p21, accompanied by increased expression of apoptosis-related genes (Supplementary Figure 2C, D [↗](#)).

To assess how GMCL1 levels affect cell cycle progression following mitotic delays, we performed FACS analyses of EdU incorporation in hTERT-RPE1 cells. Cells subjected to extended mitotic arrest were released by mitotic shake-off into fresh medium. After 30 hours, significantly less cells were found in S phase in the knockdown population, compared to control cells (Figure 3A, B [↗](#) and Supplementary Figure 3A [↗](#)). Importantly, co-depletion of *GMCL1* with either *TP53BP1* (hereafter *53BP1*) or *USP28* abolished this G1 arrest, and cells proceeded into S phase at levels comparable to or even exceeding those of control cells, effectively rescuing the phenotype caused by *GMCL1* knockdown (Figure 3A, B [↗](#) and Supplementary Figure 3A [↗](#)).

To further support our model, we overexpressed GMCL1 in hTERT-RPE1 cells and monitored cell cycle re-entry after mitotic delay. GMCL1-overexpressing cells progressed into S phase more rapidly than control cells, with notable entry observed as early as 18 hours post-release (Figure 3C, D [↗](#) and Supplementary Figure 4B [↗](#)). Importantly, in the absence of anti-mitotic drug treatment,

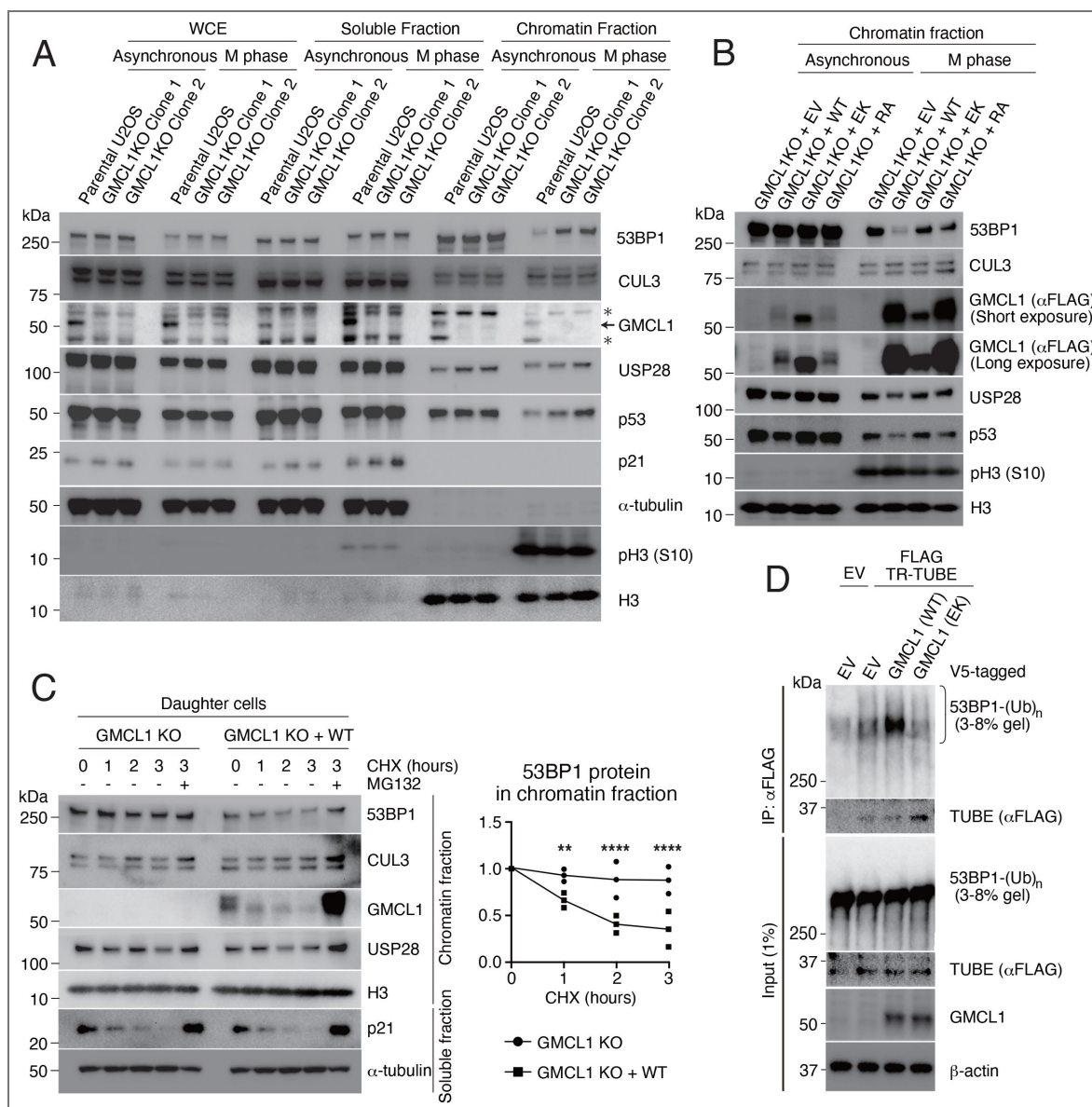


Figure 2. GMCL1 targets 53BP1 for degradation during M phase

A) Asynchronous or M phase-synchronized parental or *GMCL1* knockout (KO) U2OS cells were collected. Whole-cell extracts (WCE) were prepared using RIPA buffer, and other lysates were fractionated into soluble and chromatin-bound fractions for immunoblotting. Arrow indicates GMCL1-specific bands. Asterisk indicates non-specific bands. B) Stable U2OS cell lines expressing EV, FLAG-GMCL1 WT, FLAG-GMCL1 EK, or FLAG-GMCL1 RA in a *GMCL1* KO background were synchronized into M phase and fractionated. Immunoblots show the chromatin fraction. C) M phase synchronized FLAG-GMCL1-expressing U2OS cells were collected by mitotic shake-off and cultured in fresh FBS-containing medium for 7 h. G1 phase daughter cells were treated with CHX for the indicated time points, fractionated into soluble and chromatin-bound fractions, and analyzed by immunoblotting. Immunoblots show the chromatin fraction. Independent experiments were performed in triplicate. Differences between KO and KO+WT were tested by two-way ANOVA followed by a Bonferroni test (** $p < 0.005$, **** $p < 0.0001$). D) HEK293T cells were transfected with EV, V5-GMCL1 WT, or V5-GMCL1 EK, together with FLAG-TR-TUBE where indicated. FLAG immunoprecipitates were probed for 53BP1 and GMCL1. To visualize ubiquitinated 53BP1, the 53BP1 blot was resolved on a 3-8% gel. The arrow indicates the band corresponding to TR-TUBE.

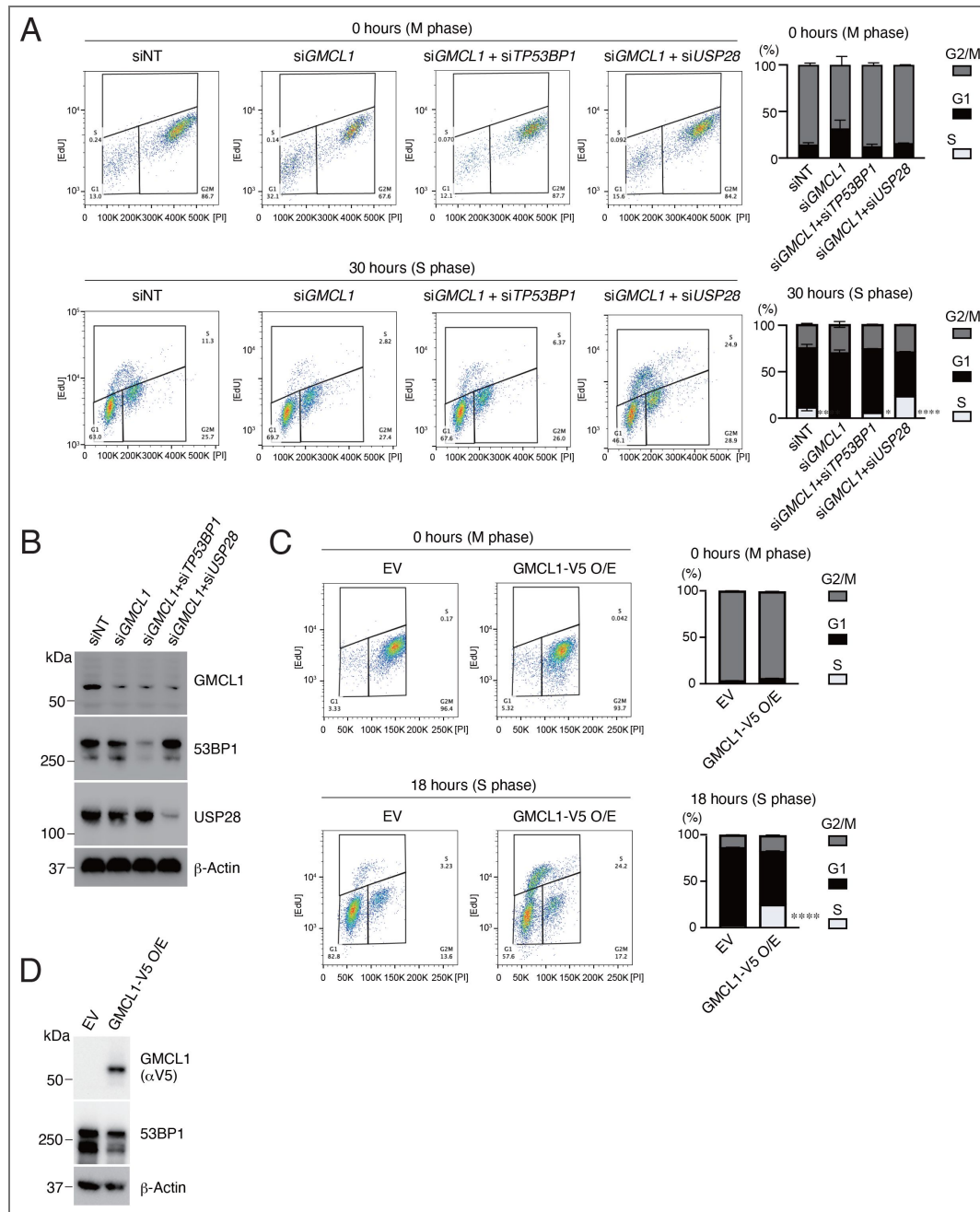


Figure 3. Cell Cycle Fate Determination of Daughter Cells Following Prolonged Mitosis

A) Dot plots and graphs show the proportions of RPE1 cells in S, G1, and G2/M phases at the indicated time points following mitotic shake-off. Cells were synchronized in mitosis by nocodazole treatment for 16 h and were subsequently released into fresh medium. Cell cycle distribution was determined by EdU pulse labeling and PI staining. EdU was added 1 h prior to each indicated time point. Cells had been transfected 48 h before the experiment with siNT, siGMCL1, or co-transfected with siGMCL1 and either siUSP28 or siTP53BP1. Error bars represent standard deviation. Differences among four groups were tested by one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$). B) Representative immunoblot showing the silencing efficiencies for panel (A). C) Dot plots and graphs show the proportions of RPE1 cells stably expressing either an empty vector or V5-GMCL1, in S, G1, and G2/M phases at the indicated time points following mitotic shake-off. Cells were synchronized in mitosis by nocodazole treatment for 16 h and were subsequently released into fresh medium. Cell cycle distribution was determined by EdU pulse labeling and PI staining. EdU was added 1 h prior to each indicated time point. Error bars represent standard deviation. Differences between EV and V5-GMCL1 were tested by a two-tailed Welch's t test (**** $p < 0.0001$). D) Representative immunoblot showing the over-expression of V5-GMCL1 for panel (C).

GMCL1 KO cells exhibited no significant changes in baseline cell cycle profiles, regardless of reconstitution with WT or mutant *GMCL1*, compared to parental cells (Supplementary Figure 3C).

Together, these findings identify *GMCL1* as a key regulator of cell fate under mitotic stress by acting upstream of the USP28-p53-53BP1 axis to influence cell cycle re-entry dynamics.

GMCL1 modulates taxane resistance in cancer cells

Taxanes, including paclitaxel, docetaxel, and cabazitaxel, are widely used chemotherapeutics that stabilize microtubules by preventing depolymerization³¹. However, resistance to taxanes commonly emerges through multiple mechanisms^{13,32,33}, including activation of pro-survival pathways such as destabilization of the 53BP1-USP28-p53 complex. Given our finding that *GMCL1* controls 53BP1 stability during prolonged mitosis, we sought to investigate whether *GMCL1* expression is associated with taxane resistance and 53BP1 protein abundance in cancer cells. To this end, we leveraged the PRISM (profiling relative inhibition simultaneously in mixtures) repurposing dataset, which quantifies the proliferation-inhibitory effects of 4,518 compounds across 578 cancer cell lines³⁴, and integrated this data with DepMap proteomic and transcriptomic profiles (<https://depmap.org>). Since *GMCL1* protein levels were not quantified in the dataset, we used *GMCL1* mRNA expression as a surrogate to assess its association with resistance to taxanes (Figure 4A). Interestingly, we found that five cancer types (i.e., endometrial, breast, kidney pancreas, and upper aerodigestive tract cancers) with high levels of *GMCL1* mRNA exhibited significant resistance to paclitaxel, cabazitaxel, and/or docetaxel (Figure 4B). In contrast, cell lines derived from 17 other cancer tissues with high *GMCL1* mRNA expression did not show such significant correlation (Supplemental Figure 4A). Across multiple cancer types (Supplemental Figure 4B), we observed that in lung cancer cells with wild type p53, high *GMCL1* expression combined with low 53BP1 levels was associated with significantly increased resistance to cabazitaxel and paclitaxel compared with cells showing low *GMCL1* expression and high 53BP1 levels (Figure 4C). In contrast, this relationship was absent in p53-mutant lung cancer cells, where *GMCL1* status did not correlate with taxane resistance (Figure 4C).

To verify the impact of *GMCL1* levels on paclitaxel sensitivity, we performed cell viability and apoptosis assays using cells with wild type or mutant p53. Paclitaxel treatment was chosen to mimic the conditions reported in DepMap. In p53 wild-type cells (MCF7 and U2OS), paclitaxel treatment led to a significant reduction in cell viability and an increase in apoptosis in *GMCL1*-depleted cells compared to cells transfected with non-targeting control siRNA (Figure 5A-D). However, *GMCL1* knockdown did not affect cell viability or apoptosis in paclitaxel-treated cells with inactivated p53 (HeLa and HEC-1-A, respectively) (Figure 5E-H). Importantly, in hTERT-RPE1 cells, the reduction in cell viability and increase in apoptosis seen upon paclitaxel treatment in *GMCL1* knockdown cells were rescued by simultaneous knockdown of either 53BP1 or USP28 (Figure 5I, J). These observations are consistent with the results in Figure 4 and suggest that paclitaxel resistance may, at least in part, be influenced by *GMCL1* through the USP28-p53-53BP1 complex. Specifically, high *GMCL1* expression appears to promote 53BP1 degradation, which in turn helps maintain lower p53 levels and reduces paclitaxel-induced cell death in cells with functional p53.

Discussion

We identify *GMCL1*, a previously uncharacterized human CRL3 substrate receptor, as a regulatory component of the mitotic surveillance pathway. Specifically, we found that *GMCL1* interacts with and mediates the degradation of 53BP1 during prolonged arrest in M phase. While 53BP1 is well known for its role in double-strand break (DSB) repair via non-homologous end joining (NHEJ)³⁵, it also participates in the so-called mitotic stopwatch composed of the 53BP1-USP28-p53 complex that stabilizes p53 during prolonged mitotic arrest^{5-7,10}. We showed that *GMCL1* controls the levels of 53BP1 and, consequently, those of p53 in mitotic cells, thereby influencing p53 transmission to daughter cells (see model in Figure 5K).

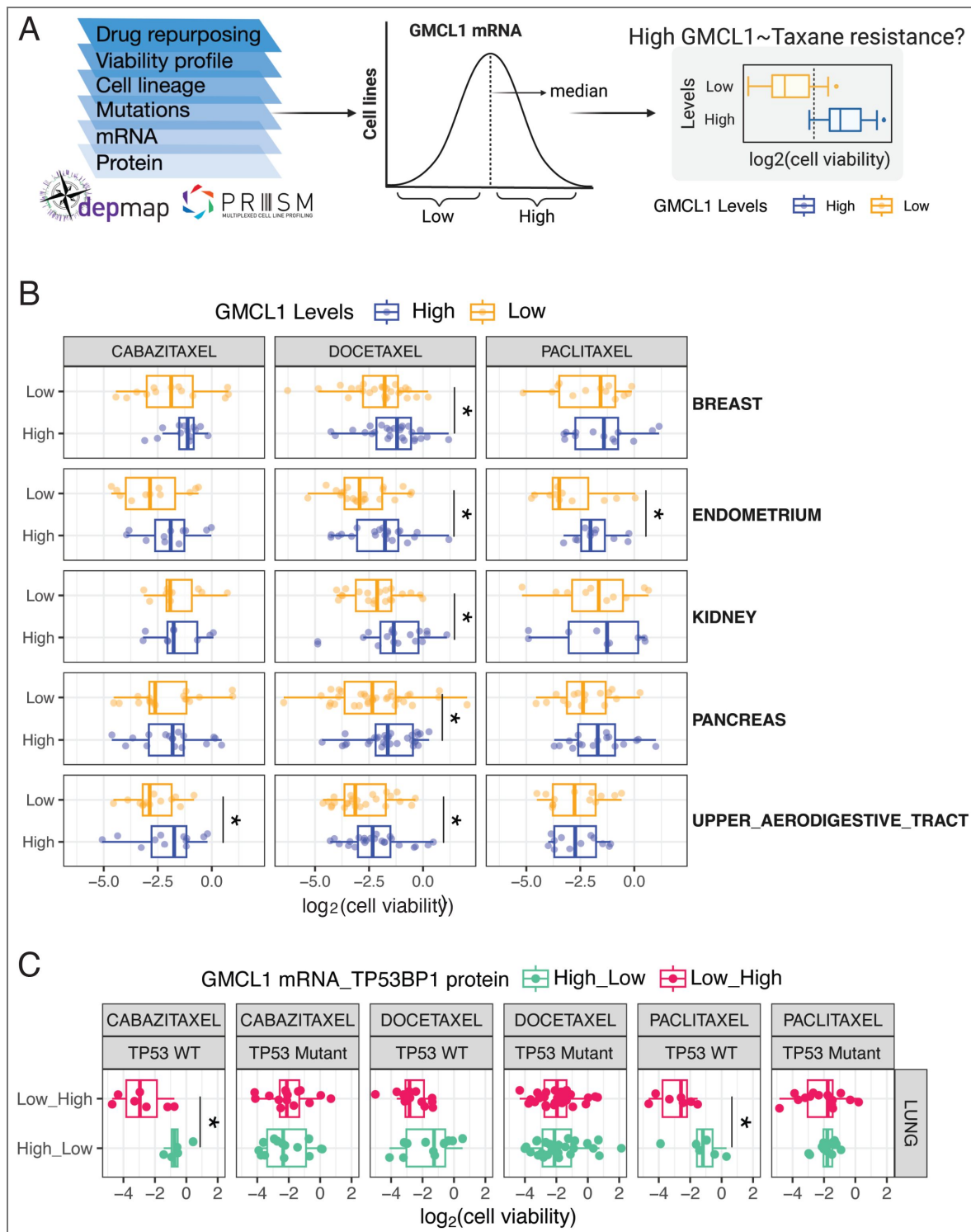


Figure 4. GMCL1 expression shows positive correlation with taxane resistance in cancer cell lines

A) Schematic overview of DepMap and PRISM data integration used in the analysis, including *GMCL1* mRNA (protein not available) and drug response for taxanes across DepMap cancer cell lines (left panel). Method for classification of cell lines into GMCL1-high and GMCL1-low groups based on its median mRNA expression levels within tissue types (middle panel). Schematic depicting comparison of taxane sensitivity between GMCL1-high and GMCL1-low groups (right panel). (B) Boxplots visualizing comparison of taxane sensitivity (i.e., cabazitaxel, docetaxel and paclitaxel; log-fold change in cell viability) between GMCL1-high and GMCL1-low groups. Statistical significance was assessed using a two-sided, unpaired Wilcoxon-rank sum test (* $p < 0.05$). (C) Boxplots visualizing comparison of taxane sensitivity (i.e., cabazitaxel, docetaxel and paclitaxel; log-fold change in cell viability) between *GMCL1* and *TP53BP1* High_Low and Low_High groups, respectively, further stratified by *TP53* mutation status. Statistical significance was assessed using a two-sided, unpaired Wilcoxon-rank sum test (* $p < 0.05$).

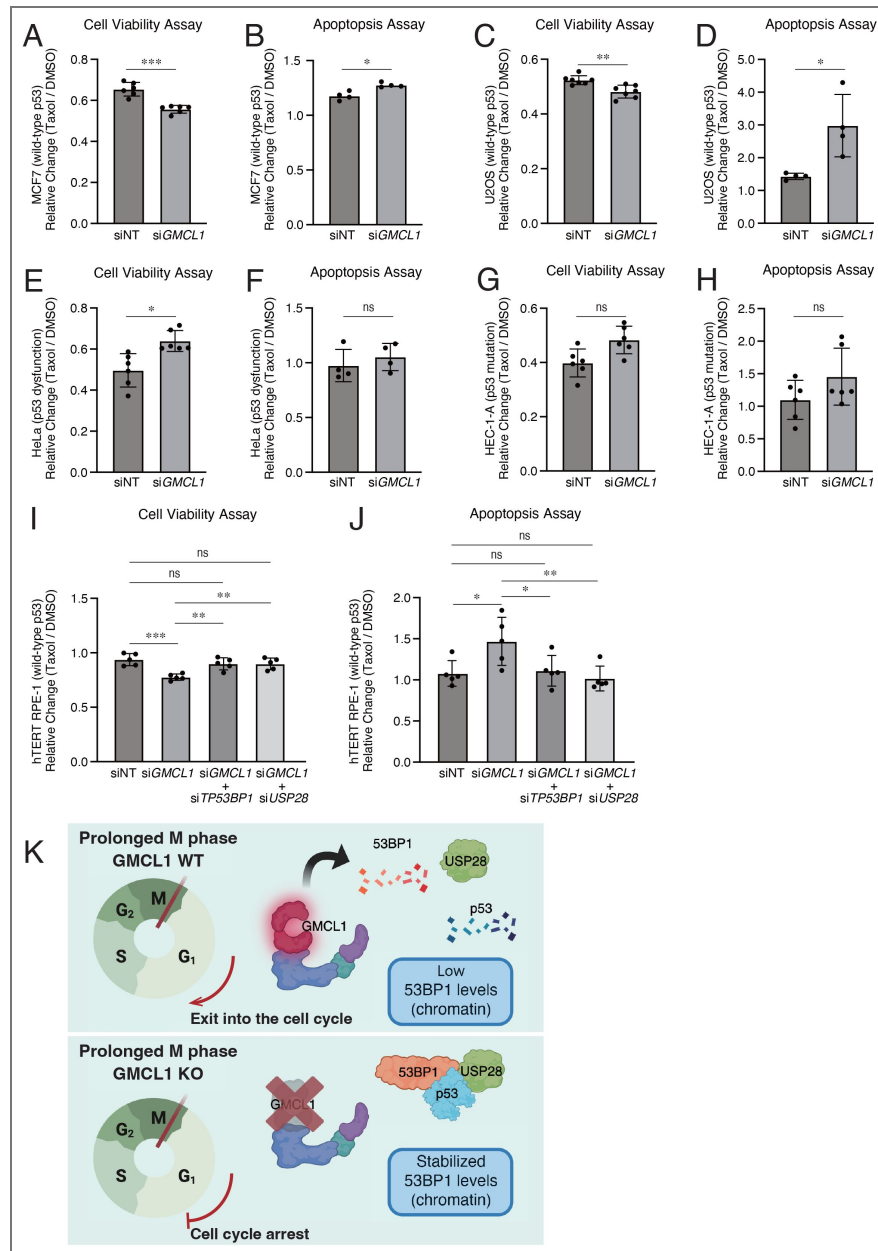


Figure 5. GMCL1 deficiency sensitizes cancers with wild-type p53 to paclitaxel-induced apoptosis

(A-G) MCF7 (A), U2OS (C), HeLa (E), HEC-1-A (G) cells were transfected with GMCL1-targeting siRNAs or non-targeting (NT) control for 72 h. Cells were treated with DMSO or 100 nM paclitaxel for 48 h, and cell viability was assessed using the CellTiter-Glo Cell Viability Assay from four or six independent measurements. (B-H) Apoptosis was measured in the same conditions, *i.e.*, MCF7 (B), U2OS (D), HeLa (F), HEC-1-A (H), using the RealTime-Glo Annexin V Apoptosis and Necrosis Assay from four or six independent measurements. For comparisons between two independent groups (A-H), a two-tailed Welch's test was applied (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$). (I) hTERT-RPE1 cells were transfected with a non-targeting (NT) control or GMCL1-targeting siRNAs alone or in combination with either TP53BP1 or USP28 targeting siRNAs for 72 h, followed by treatment with 100 nM paclitaxel for 48 h. Cell viability was assessed using the CellTiter-Glo Cell Viability Assay from five independent measurements. (J) Apoptosis was measured in the same conditions, *i.e.*, RPE1 using the RealTime-Glo Annexin V Apoptosis and Necrosis Assay from five independent measurements. Error bars represent standard deviation. For analysis involving four groups (I and J), one-way ANOVA followed by Tukey's multiple-comparisons test was applied (* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$). (K) Schematic model of this study. During prolonged mitosis, GMCL1 promotes degradation of 53BP1, thereby releasing p53 from the 53BP1-p53-USP28 ternary complex and leading to p53 degradation. As a result, daughter cells proceed through the cell cycle. In the absence of GMCL1, excessive accumulation of 53BP1 results in inheritance of the 53BP1-p53-USP28 ternary complex into daughter cells, where p21 expression is induced and cell cycle progression is arrested.

We found that GMCL1 primarily regulates the levels of chromatin-associated 53BP1. A PLK1-dependent 53BP1 re-localization from chromatin to the nucleoplasm has been reported in mitosis^{10,36,37}. However, multiple *GMCL1* KO clones display elevated chromatin-bound 53BP1 during M-phase arrest without a corresponding decrease in the soluble fraction, indicating the effect is not due to re-localization. The increased 53BP1 half-life in *GMCL1* KO daughter cells supports this conclusion. Discrepancies in the reported localization of 53BP1 (chromatin vs. nucleoplasm) during mitosis may reflect differences in biochemical fractionation methods (*e.g.*, differences in the concentrations of salt or detergent and/or sonication conditions).

Our findings suggest that GMCL1 functions as a regulator of mitotic stress response, with potential oncogenic properties in certain contexts. This underscores the role of GMCL1 in mitotic regulation and chromosome stability, which may vary based on tumor type, genetic background, and additional oncogenic mutations. Accordingly, we observed that a subset of cell lines exhibiting resistance to taxane-based agents, such as paclitaxel, cabazitaxel, and docetaxel, display elevated *GMCL1* mRNA expression. Clinical application of our findings is currently limited by tumor heterogeneity and by the variable efficacy and cellular availability of taxanes during treatment.

GMCL1 has been primarily studied in the germ cells of *D. melanogaster*, where we have shown that it forms an active CRL3 complex during mitosis²³. Our new findings suggest a critical role for GMCL1 in mammalian somatic cell fate decisions by regulating 53BP1 stability during prolonged mitosis. We further show that GMCL1 loss sensitizes p53-wild-type, but not p53-mutant, cancer cells to paclitaxel-induced death, suggesting that GMCL1 inhibition may offer a selective therapeutic strategy for tumors with intact p53 function.

Materials and methods

Cell culture

Cell lines were purchased from ATCC and were routinely checked for mycoplasma contamination with the MycoStrip Mycoplasma Detection Kit (Invivogen). HEK293T (ATCC CRL-3216), HeLa (ATCC CCL-2) and hTERT RPE-1 (ATCC CRL-4000) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco). U-2 OS (ATCC HTB-96), HCT-116 (ATCC CCL-247) and HEC-1-A (ATCC HTB-112.NM) cells were maintained in McCoy's 5A medium (Gibco). MCF7 (ATCC HTB-22) were maintained in Eagle's Minimum Essential Medium (EMEM) (ATCC). All media were supplemented with 10% fetal bovine serum (FBS) (Corning Life Sciences) and 1% penicillin/streptomycin/L-glutamine (Corning Life Sciences); however, MCF7 was further supplemented with human recombinant insulin (zinc solution; Gibco) to a final concentration of 11.2 µg/mL. All cell lines were maintained at 37°C and 5% CO₂ in a humidified atmosphere.

Plasmids, siRNA, and transfection

Homo sapiens cDNAs were amplified by PCR using KAPA HiFi DNA Polymerase (Kapa Biosystems) and sub-cloned into a variety of vector backbones, including modified pCDNA3.1 and pLVX-PURO lentiviral vectors containing C-terminal Flag, HA or V5 tags. Site-directed mutagenesis was performed using KAPA HiFi DNA Polymerase (Kapa Biosystems). All cell lines were transiently transfected using Lipofectamine 3000 (ThermoFisher Scientific) based on the manufacturer's recommendation. siRNA oligo transfections were performed using RNAiMax (ThermoFisher Scientific) according to the manufacturer's instructions.

Virus-mediated gene transfer

For the generation of lentivirus, HEK293T cells were transfected with pLVX constructs carrying the genes of interest, alongside the packaging plasmids pCMV-delta-R8.2 and pCMV-VSV-G. Viral supernatant was harvested 48 hours post-transfection, passed through a 0.45-µm sterile Millex-HV filter unit (Millipore Sigma), and supplemented with polybrene at a final concentration of 8 µg/ml (Sigma). Target cells were infected by replacing their culture medium with the virus-containing supernatant for an 8-hour incubation period. Selection of successfully transduced cells was performed using puromycin at a concentration of 1-2 µg/ml (Sigma).

CRISPR-Cas9 genome editing

CRISPR-Cas9 genome editing techniques were carried out as previously described³⁸ with modifications. In brief, to generate *GMCL1*-knockout U-2 OS cells, optimal gRNA target sequences closest to the start codon of the genes were designed using the Benchling CRISPR Genome Engineering tool (<https://www.benchling.com>). For transient Cas9 expression, gRNAs specific for *GMCL1* gene was incorporated into the pRP [CRISPR]-Hygro-hCas9-U6 vector, which was obtained from VectorBuilder (<https://en.vectorbuilder.com/>). The following oligos were used to generate the proper gRNA in the vector: *GMCL1* (5'-CGTGCCCCACGTACCTTCG-3'). To generate *GMCL1* 2×Flag knock-in HCT116 cells, an optimal gRNA target sequence closest to the genomic target site and a ~2 kb homologous recombination (HR) donor template was designed using the Benchling CRISPR Genome Engineering tool. The HR donor template was designed to introduce a 2×Flag tag in frame with the C terminus of *GMCL1*, in the following order: *GMCL1*-linker-FLAG-linker-FLAG-Stop codon and was purchased from VectorBuilder (<https://en.vectorbuilder.com/>). The following single gRNA sequence was used for the transient hCas9 expression vector: *GMCL1* (5'-AAGTTACAGCAGATATATAA-3').

Genomic DNA was collected using QuickExtract (Epicentre). Genotyping PCRs were performed with MyTaq HS Red Mix (Bioline), using primers surrounding the genomic target sites. The following primers were used for genotyping: *GMCL1* (F: 5'-GCAGGCTTCTGATCTCCCT-3', R: 5'-ACTTGTCATCGTCGTCCTTGT-3'), and *GMCL1* (F: 5'-GGGTGGGAGTTTGGAGAGTG-3', R: 5'-TCTGGATTTTCTGGTGACGA-3'). The resulting PCR products were then purified and sequenced to determine the presence of insertion or deletion events. Clones positive for insertion or deletion events were then validated by western blot.

Antibodies

The following antibodies were used: β -actin (1:5,000, Sigma-Aldrich A5441), CUL3 (1:1,000, Bethyl Laboratories A301-109A), FLAG (1:2,000, Sigma-Aldrich F7425), *GMCL1* (1:1,000, Proteintech 15575-1-AP), HA (1:2,000, Bethyl Laboratories A190-108A), Histone H3 (1:1,000, Abcam, ab1791), p21 (1:1,000, Cell Signaling Technology 2947S), p53 (1:1,000, Proteintech 10442-1-AP), pHistone H3 (D2C8) (Ser10, 1:1,000, Cell Signaling Technology, #3377), Ubiquitin (p37) (1:1,000, Cell Signaling Technology, 58395S), USP28 (1:1,000, Proteintech 17707-1-AP), 53BP1 (1:2,000, Abcam ab36823), α -tubulin (1:5,000, Sigma-Aldrich T6074).

Drug treatment procedures

Where indicated, cells were treated with 400 ng/ml Nocodazole (Sigma-Aldrich M1404) for 16 hours, 100 nM paclitaxel for 48 hours, 10 μ M MG132 for 3 hours, 2.5 μ M MLN4924 for 3 hours, 100 μ g/ml cycloheximide (CHX) for the indicated time.

Cell synchronization

Cells were synchronized using nocodazole. Cells were treated with 100 ng/ml nocodazole for 14 hours. Mitotic cells were then collected by shake-off (M phase cells), or washed three times with PBS, and replated in normal medium to allow them to resume cell cycle (to analyze subsequent cell cycle progression in the daughter cells).

qRT-PCR

Total RNA was purified using RNeasy mini kits (Qiagen). cDNA was generated using Double Primed EcoDry kits (Takara). The qPCR reaction was carried out using PowerUp SYBR Green (Applied Biosystems) and the Applied Biosystems QuantStudio 3 Real-Time PCR system in a 96-well format. ROX was used as a reference dye for fluorescent signal normalization and for well-to-well optical variations correction. Bar graphs represent the relative ratios of target genes to β -actin housekeeping gene values. For each biological sample, triplicate reactions were analyzed using absolute relative quantification method alongside in-experiment standard curves for each primer set to control for primer efficiency. The oligos used for qRT-PCR analysis were: β -actin (F: 5'-

CATGTACGTTGCTATCCAGGC-3', R: 5'-CTCCTTAATGTCACGCACGAT-3'), GMCL1 (F: 5'-GGAGATTCTTGACCAGAACATTG-3', R: 5'-CGACTGGGCTTTATCAAGACAT-3'), GMCL2 (F: 5'-CCACGCAGCGGGTCTGT, R: 5'-TGGATTTTCTGGGTGACGATTATT), p21 (F: 5'-TGTCGTCAGAACCCATGC-3', R: 5'-AAAGTCGAAGTCCATCGCTC-3'), NOXA (F: 5'-CCAAGCCGTGACCAAGGAC-3', R: 5'-CGCCACATTGTGTAGCACCT-3'), PUMA delta (F: 5'-GCCAGATTTGTGAGACAAGAGG-3', R: 5'-CAGGCACCTAATTGGGCTC-3').

Fractionation and immunoprecipitation

For whole-cell lysates, cells were directly lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 2 mM MgCl₂, and 1 mM dithiothreitol (DTT). Lysates were incubated on ice for 20 min and subsequently clarified by centrifugation at 20,000 × g for 15 min at 4 °C. When cellular fractionation was performed, it followed a previously established method³⁹. Briefly, cells were lysed in CSK buffer (10 mM HEPES, pH 7.4, 100 mM NaCl, 300 mM sucrose, 0.1% Triton X-100, 3 mM MgCl₂, and 1 mM EGTA) for 5 minutes. The soluble fraction was collected by centrifugation at 1,300 × g for 3 minutes at 4°C. Cell pellets were subsequently washed in CSK buffer and then lysed in chromatin extraction buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 50mM NaF, 1 mM EGTA, 2 mM MgCl₂, and 250 U/mL Benzonase (Sigma-Aldrich)) for 30 minutes. Insoluble debris was removed by centrifugation at 20,000 × g for 15 minutes at 4°C. All buffers were supplemented with protease inhibitors (Complete ULTRA, Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 2, Sigma-Aldrich).

For immunoprecipitation and affinity purification, samples were incubated with FLAG-M2 magnetic beads (Sigma-Aldrich) or anti-HA magnetic beads (Thermo Fisher Scientific) at 4°C for 2 hours. Beads were thoroughly washed with wash buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% NP-40, 1 mM EDTA, 1 mM EGTA, 2 mM MgCl₂, and 1 mM dithiothreitol (DTT), and protein elution was performed using either 3xFLAG peptide (Sigma-Aldrich) for mass spectrometry analysis or 1x Laemmli sample buffer for Western Blot analysis.

Immunoblotting

Western blotting was carried out as described previously³⁸. Protein samples were resolved under denaturing and reducing conditions on 4%–12% Bis-Tris gels (NuPAGE) and transferred onto PVDF membranes (Immobilon-P, Millipore). Membranes were blocked with 5% nonfat dried milk, incubated overnight at 4°C with primary antibodies, followed by washes and incubation with HRP-conjugated secondary antibodies (Amersham GE). Immunoreactive bands were visualized using enhanced chemiluminescence reagents (Pierce) and detected with a ChemiDoc MP imaging system (Bio-Rad). Each Western blot experiment was conducted at least three times to ensure reproducibility, with representative blots shown in the figures.

Cell cycle analysis by flow cytometry

EdU incorporation and propidium iodide staining were performed either on asynchronous or synchronized cells. Visualization of EdU and propidium iodide staining was performed following the instructions of the manufacturer³⁸. In brief, cells were pulsed with EdU (10 μM), fixed and permeabilized, and EdU was detected by copper-free click chemistry using the Click-iT™ Plus EdU Alexa Fluor™ 488 Flow Cytometry Assay Kit (Thermo Fisher Scientific). Flow cytometry analysis of cell cycle distribution was conducted using a CytoFlex Analyzer (Beckman Coulter) and data were processed with FlowJo v10 software (Becton Dickinson).

Mass spectrometry analysis of GMCL1 immunoprecipitations

The eluted anti FLAG-tag antibody purified protein complexes were reduced with 2 μl of 0.2 M DTT for 1 h at 57°C and subsequently alkylated with 2 μl of 0.5 M iodoacetamide (Sigma) for 45 min at room temperature in the dark. 250 ng of SP3 beads (Cytiva) were added proteins precipitated onto the beads by adding ethanol. Samples were placed in a thermomixer at 25°C for 10 min. Beads were washed three times with 80% ethanol and then digested overnight with 400ng of sequencing

grade modified Trypsin (Promega) in 100 mM ammonium bicarbonate. Next, the samples were spun down 21,000 x g for 1 minute. The supernatant was transferred to a new tube while the beads were washed twice with 0.5% acetic acid. The washes were then combined with the supernatant collection. Samples were acidified with 10% TFA to pH1 and loaded onto a 0.1% TFA equilibrated Pierce C18 spin column using a microcentrifuge. The samples were rinsed twice with 0.1% TFA and twice more using 0.5% acetic acid. Peptides were eluted with 80% acetonitrile in 0.5% acetic acid. The organic solvent was removed using a SpeedVac concentrator and the sample reconstituted in 0.5% acetic acid.

An equal aliquot of each sample was loaded onto a trap column (Acclaim PepMap 100 pre-column, 75 $\mu\text{m} \times 2\text{cm}$, C18, 3 μm , 100 Å, Thermo Scientific) connected to an analytical column (EASY-Spray column, 50 m $\times$ 75 μm internal diameter, PepMap RSLC C18, 2 μm , 100Å, Thermo Scientific) using the autosampler of an Easy nLC 1200 (Thermo Fisher Scientific) with solvent A consisting of 2% acetonitrile in 0.5% acetic acid and solvent B consisting of 80% acetonitrile in 0.5% acetic acid. The peptide mixture was gradient eluted using the following gradient: 5% solvent B for 5 minutes, 5-35% solvent B in 60 min, 35-45% solvent B in 10 min, followed by 45-100% solvent B in 10 min. The samples were acquired on the Orbitrap Eclipse using the following parameters: full MS spectra resolution of 120,000, an AGC target of 4e5, maximum ion time of 50 ms, scan range from 400 to 1,500 m/z. The MS/MS spectra were collected with the following parameters: a resolution of 30,000, an AGC target of 2e5, maximum ion time of 30 ms, one microscan, 2 m/z isolation window, normalized collision energy (NCE) of 27 and a dynamic exclusion of 30 s. To identify binding partners, all acquired MS2 spectra were searched against a UniProt human database using Sequest HT within Proteome Discoverer 1.4 (Thermo Fisher Scientific). Fixed modifications were set on cysteine (carbamidomethyl), variable modifications of oxidation on methionine, and deamidation on glutamine and asparagine. The resulting peptide spectra matches and proteins are filtered to better than 1% false discovery rate (FDR) and only proteins with at least two different peptides are reported. Proteins differentially expressed between GMCL1 WT and EK, as determined by SAINT scores⁴⁰ with a 5% FDR, were considered significantly enriched interactions when comparing to GMCL1 BTB.

Taxane resistance analysis

Taxane sensitivity data were obtained from the PRISM Repurposing dataset (DepMap 24Q2, www.depmap.org), which reports log2-fold changes (LFC) in cell viability across 578 cancer cell lines treated with various compounds, including paclitaxel, cabazitaxel, and docetaxel^{3,4}. Data preprocessing and integration: We integrated the GMCL1 RSEM-normalized mRNA expression (DepMap filename:

OmicsExpressionProteinCodingGenesTPMLogp1BatchCorrected.csv), with TP53BP1 protein abundance⁴¹ and TP53 mutation status (DepMap filename:

OmicsSomaticMutationsMatrixHotspot.csv) across all cell lines catalogued in the PRISM dataset into a harmonized dataset using R version 4.2.1 (Supplemental Table 2, 4A raw data). Cell lines with missing GMCL1 or TP53BP1 expression, taxane LFC data, or tissue type annotation were excluded from the analysis.

Stratification by expression levels: For tissue-level comparisons (e.g., breast, endometrium, kidney, pancreas, etc.), cell lines were stratified in parallel based on the median within tissue type of GMCL1 mRNA expression or TP53BP1 protein abundance into “high” and “low” GMCL1 or TP53BP1 groups, respectively. This allowed us to assess the relationship between baseline GMCL1 or TP53BP1 levels, TP53 mutation status, and taxane resistance under standardized, non-physiological screening conditions.

Statistical analysis: Differences in taxane sensitivity (LFC values) between high and low expression groups were assessed using two-sided, and unpaired Wilcoxon rank-sum tests between “high” vs “low” groups (* $P < 0.05$). To account for multiple testing across different tissue types and drug

combinations, we applied the Benjamini Hochberg false discovery rate (FDR). Importantly, the dataset does not specifically isolate M-phase cells, but rather represents mixed populations, and the findings should be interpreted accordingly.

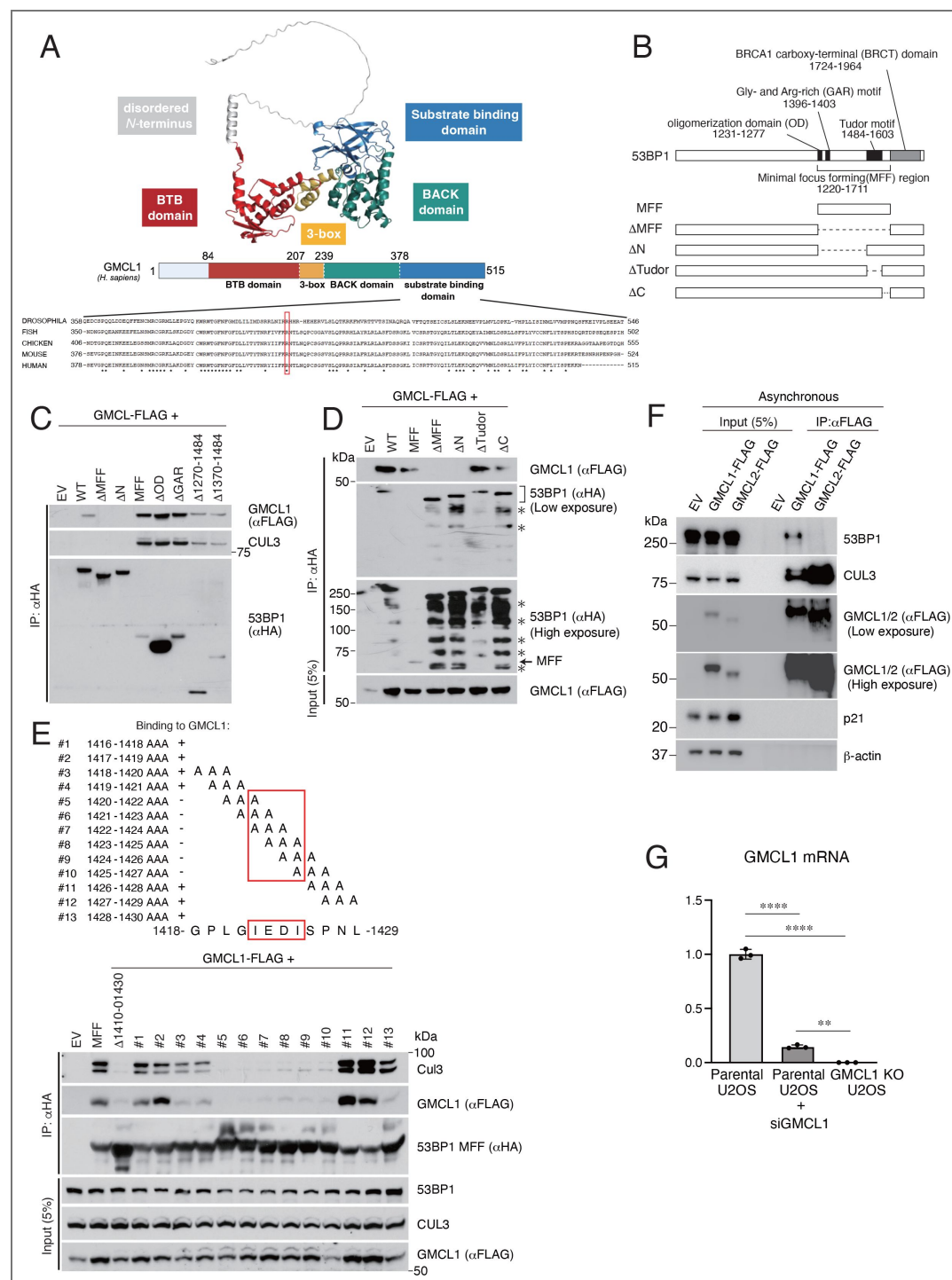
Cell viability and apoptosis assays

Cells (2500/well) were plated in a 96-well plate. The medium was replaced with 50 μ L of medium containing the target siRNA (at a final concentration of 20 nM). After 24 hours, an additional 50 μ L of medium containing either DMSO or paclitaxel (at a final concentration of 100 nM) was added. Luminescence was measured using BioTek Synergy Neo2 (Agilent) 48 hours post-treatment, using the CellTiter-Glo 2.0 Cell Viability Assay Kit (Promega) or RealTime-Glo Annexin V Apoptosis and Necrosis Assay Kit (Promega) following to the manufacturer's recommendations.

Quantification and statistical analysis

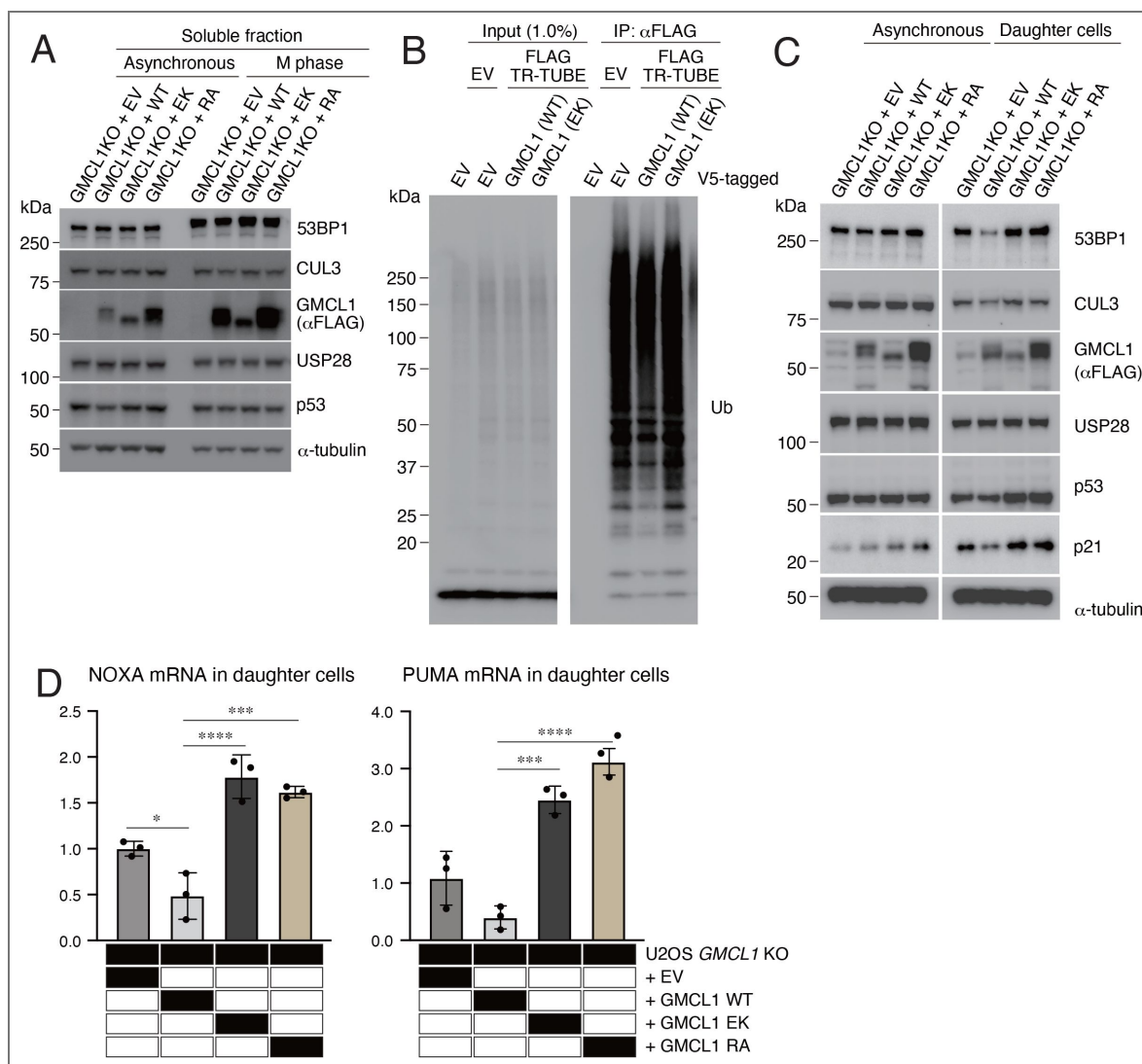
Data analysis was performed using GraphPad Prism version 10.2.1. For comparisons involving three or more groups, One-way ANOVA followed by Bonferroni's post hoc test or the Brown-Forsythe and Welch ANOVA test followed by Dunnett'S T3 multiple comparisons test was applied.

Supplemental figures



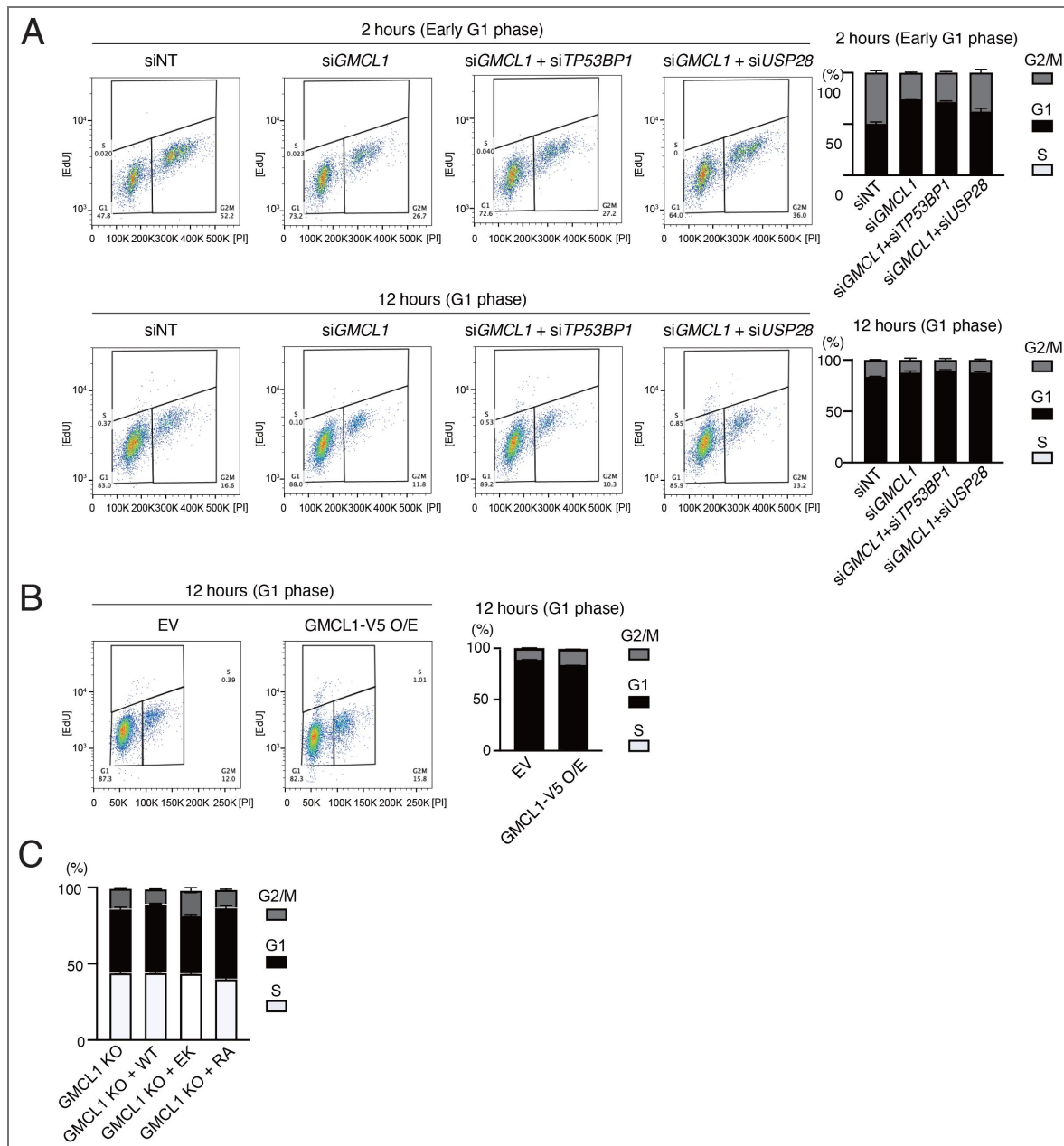
Supplemental Figure 1. Mapping 53BP1 binding sites on GMCL1 (A) Predicted structure of GMCL1, domain architecture overview, and comparative analysis of the substrate-binding domain across *Drosophila*, fish, chicken, mouse, and human. Conserved amino acids are indicated by asterisks, with the human R433 residue highlighted in red. (B) Schematic representation of 53BP1 domains. (C) HEK293T cells were co-transfected with FLAG-GMCL1 and either EV, HA-53BP1 WT, or deletion mutants: HA-53BP1 ΔMFF (minimal focus forming), HA-53BP1 ΔN (lacking the N-terminus of MFF), HA-MFF, HA-MFF ΔOD (oligomerization domain), HA-MFF ΔGAR (glycine-arginine-rich motif), HA-MFF Δ1270-1484, or HA-MFF Δ1370-1484. Immunoprecipitation of 53BP1 was performed using HA beads, followed by immunoblotting of co-purified proteins. (D) HEK293T cells were transfected with EV or FLAG-

GMCL1, together with HA-53BP1 WT or mutants: HA-MFF (minimal focus forming), HA-53BP1 Δ MFF, HA-53BP1 Δ N (lacking the N-terminus of MFF), HA-53BP1 Δ Tudor, and HA-53BP1 Δ C (lacking the C-terminus of MFF). 53BP1 was immunoprecipitated with HA-beads, followed by immunoblotting of co-purified proteins. Asterisk indicates non-specific bands. (E) To narrow down the GMCL1-binding region on 53BP1, sequential 20-amino-acid deletions within the MFF (minimal focus forming) domain were generated. HEK293T cells were co-transfected with EV or FLAG-GMCL1, along with HA-MFF, HA-MFF Δ 1410-1430, and site-specific mutants (every three amino acids mutated within 1410–1430 region of 53BP1). Immunoprecipitation of 53BP1 was conducted with HA-beads, followed by immunoblotting. (F) HEK293T cells were transfected with FLAG-GMCL1 or FLAG-GMCL2. GMCL1 and GMCL2 were immunoprecipitated with FLAG-beads and analyzed by immunoblotting. This experiment was performed two times, and a representative blot is shown. (G) RNA was extracted from asynchronous parental, *GMCL1* silenced, and *GMCL1* knockout U2OS cells (Clone 1). *GMCL1* Mrna levels were quantified by Qpcr from three independent experiments. Error bars represent standard deviation.



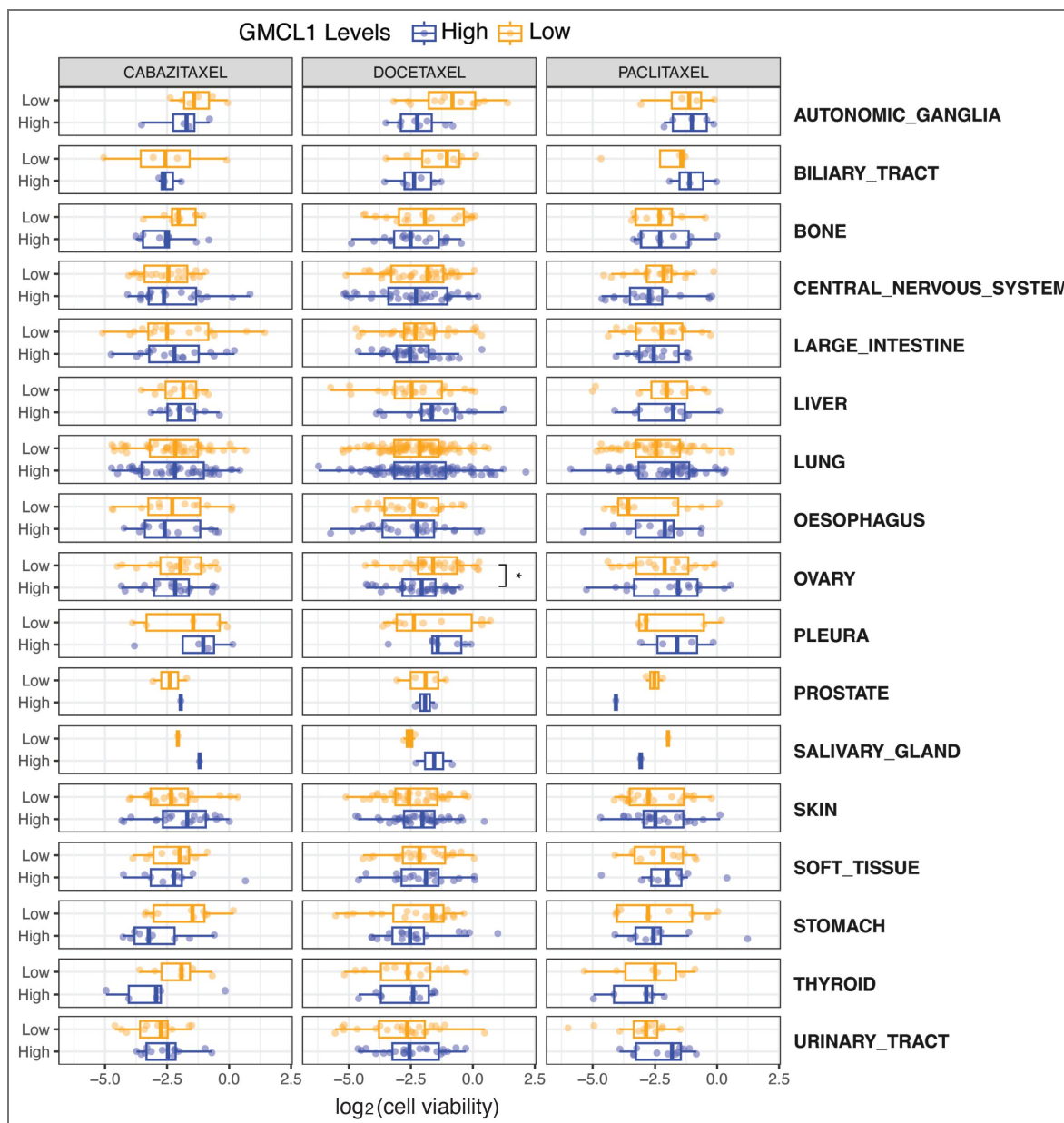
Supplemental Figure 2. Mitotic stress imprints apoptotic memory in daughter cells

(A) Stable U2OS cell lines expressing EV, FLAG-GMCL1 WT, FLAG-GMCL1 EK, or FLAG-GMCL1 RA in a *GMCL1* KO background were synchronized into M phase and fractionated and analyzed by immunoblotting; this panel presents the soluble fraction corresponding to Figure 2B. (B) HEK293T cells were transfected with EV, V5-GMCL1 WT, or V5-GMCL1 EK, together with FLAG-TR-TUBE where indicated. FLAG immunoprecipitates were probed for ubiquitin. (C) Stable U2OS cell lines expressing EV, FLAG-GMCL1 WT, FLAG-GMCL1 EK, or FLAG-GMCL1 RA in a *GMCL1* KO background were synchronized into M phase by mitotic shake-off following and subsequently release into fresh FBS-containing medium for 20 hours. Daughter cells were fractionated into chromatin-bound fractions and analyzed by immunoblotting. (D) RNA was extracted from the same cells as in (C), and *NOXA* and *PUMA* mRNA levels were quantified by Qpcr from three independent experiments. Error bars represent standard deviation from three independent experiments. Differences among four groups were tested by one-way ANOVA followed by Tukey's multiple comparisons test (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$).



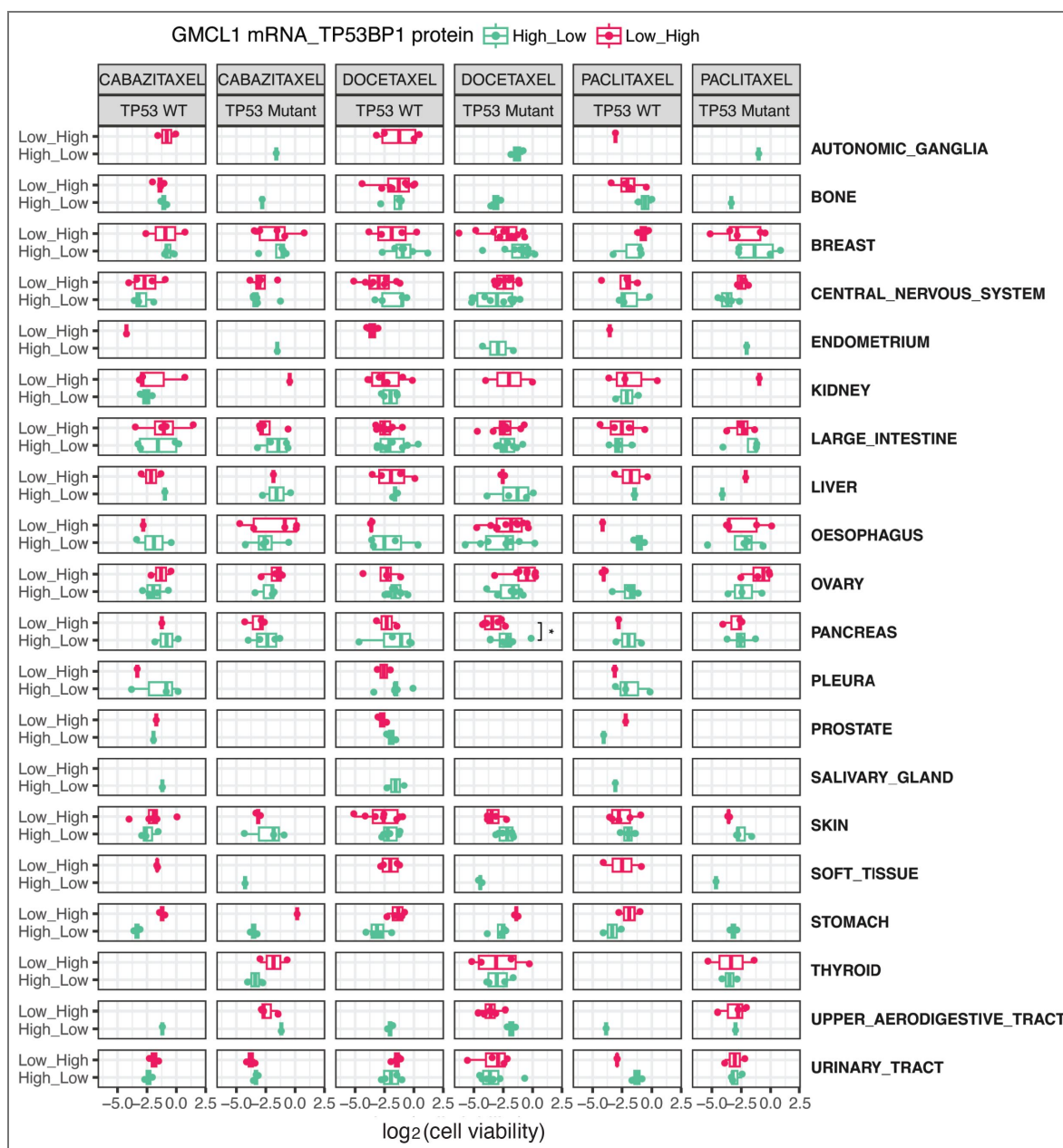
Supplemental Figure 3. Cell Cycle Fate Determination of Daughter Cells Following Prolonged Mitosis in GMCL1 Knockdown Cells with 53BP1 or USP28.

(A) Dot plots and graphs show RPE1 cell cycle distribution 2 hours and 12 hours following nocodazole release, corresponding to the data in Figure 3A. The y-axis indicates EdU incorporation, and the x-axis represents DNA content measured by PI staining. (B) Dot plots and graphs show RPE1 cell cycle distribution 12 hours following nocodazole release, corresponding to the data in Figure 3C. The y-axis indicates EdU incorporation, and the x-axis represents DNA content measured by PI staining. (C) Cell cycle distribution of asynchronous parental or the indicated FLAG-GMCL1 over-expressing U2OS cells were determined using EdU pulse and PI. Error bars represent standard deviation from three independent experiments.



Supplemental Figure 4.

(A) Boxplots visualizing comparison of taxane sensitivity (i.e., cabazitaxel, docetaxel and paclitaxel; log-fold change in cell viability) between GMCL1-high and GMCL1-low groups. Statistical significance was assessed using a two-sided, unpaired Wilcoxon-rank sum test (* $p < 0.05$). (B) Boxplots visualizing comparison of taxane sensitivity (i.e., cabazitaxel, docetaxel and paclitaxel; log-fold change in cell viability) between GMCL1 and TP53BP1 High_Low and Low_High groups, respectively, further stratified by TP53 mutation status. Statistical significance was assessed using a two-sided, unpaired Wilcoxon-rank sum test (* $p < 0.05$).



Supplemental Figure 4. (continued)

Data availability

We used taxane sensitivity from PRISM Repurposing (DepMap 24Q2) and merged it with DepMap 24Q2 transcriptome (GMCL1), TP53 mutation status, and TP53BP1 protein abundance. Original western blot images have been deposited at Mendeley at DOI:10.17632/gj3x6r263d.1 and are publicly available as of the date of publication. <https://data.mendeley.com/preview/gj3x6r263d?alpha=1e452dfc-bf85-472c-83ce-0e997ba6fa40>. The mass spectrometric raw files are accessible at <https://massive.ucsd.edu> under accession MassIVE MSV000097235 and at www.proteomexchange.org under accession PXD061458.

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Additional information

Author contributions

Y.K., G.R. and M.P. conceived, supervised, and coordinated the study. T.G.R. performed all analyses from PRISM and DepMap data. Y.K. performed the mass spectrometric sample preparation, generated CRISPR KO and KI cell lines, designed plasmid constructs, and performed the cell-based immunoprecipitations, fractionation and protein stability assays. A.M. and J.P. performed the initial mass spectrometric sample preparation and immunoprecipitations. B.M.U and J.O.P performed mass spectrometry analysis. S.F.G. performed structural predictions using AlphaFold3. The manuscript was written with inputs from all authors. Dr. Ruth Lehmann was included as a co-author in the original submission due to her intent to contribute to the manuscript's preparation, but her name was later removed when she was unable to participate.

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Competing interests: MP is or has been an advisor for SEED Therapeutics, CullGen, Deargen, Kymera Therapeutics, Lumanity, Serinus Biosciences, Sibylla Biotech, Triana Biomedicines, and Umbra Therapeutics. He also has financial interests in CullGen, Kymera Therapeutics, SEED Therapeutics, Thermo Fisher Scientific, and Triana Biomedicines. The other authors declare no conflict of interest.

Additional files

[Supplementary Table 1](#) 

[Supplementary Table 2](#) 

References

1. Hosea R., Hillary S., Naqvi S., Wu S., Kasim V. (2024) The two sides of chromosomal instability: drivers and brakes in cancer. *Signal Transduct Target Ther* **9**:75 <https://doi.org/10.1038/s41392-024-01767-7>
2. Lens S.M.A., Medema R.H. (2019) Cytokinesis defects and cancer. *Nat Rev Cancer* **19**:32-45 <https://doi.org/10.1038/s41568-018-0084-6>
3. Lambrus B.G., Holland A.J. (2017) A New Mode of Mitotic Surveillance. *Trends Cell Biol* **27**:314-321 <https://doi.org/10.1016/j.tcb.2017.01.004>
4. Uetake Y., Sluder G. (2010) Prolonged prometaphase blocks daughter cell proliferation despite normal completion of mitosis. *Curr Biol* **20**:1666-1671 <https://doi.org/10.1016/j.cub.2010.08.018>
5. Lambrus B.G., Daggubati V., Uetake Y., Scott P.M., Clutario K.M., Sluder G., Holland A.J. (2016) A USP28-53BP1-p53-p21 signaling axis arrests growth after centrosome loss or prolonged mitosis. *J Cell Biol* **214**:143-153 <https://doi.org/10.1083/jcb.201604054>
6. Meitinger F., Anzola J.V., Kaulich M., Richardson A., Stender J.D., Benner C., Glass C.K., Dowdy S.F., Desai A., Shiau A.K., et al. (2016) 53BP1 and USP28 mediate p53 activation and G1 arrest after centrosome loss or extended mitotic duration. *J Cell Biol* **214**:155-166 <https://doi.org/10.1083/jcb.201604081>
7. Fong C.S., Mazo G., Das T., Goodman J., Kim M., O'Rourke B.P., Izquierdo D., Tsou M.F. (2016) 53BP1 and USP28 mediate p53-dependent cell cycle arrest in response to centrosome loss and prolonged mitosis. *eLife* **5** <https://doi.org/10.7554/eLife.16270>
8. Stracker T.H. (2024) Regulation of p53 by the mitotic surveillance/stopwatch pathway: implications in neurodevelopment and cancer. *Front Cell Dev Biol* **12**:1451274 <https://doi.org/10.3389/fcell.2024.1451274>
9. Sparr C., Meitinger F. (2025) Prolonged mitosis: A key indicator for detecting stressed and damaged cells. *Curr Opin Cell Biol* **92**:102449 <https://doi.org/10.1016/jceb.2024.102449>
10. Meitinger F., Belal H., Davis R.L., Martinez M.B., Shiau A.K., Oegema K., Desai A. (2024) Control of cell proliferation by memories of mitosis. *Science* **383**:1441-1448 <https://doi.org/10.1126/science.add9528>
11. Chan K.S., Koh C.G., Li H.Y. (2012) Mitosis-targeted anti-cancer therapies: where they stand. *Cell Death Dis* **3**:e411 <https://doi.org/10.1038/cddis.2012.148>
12. Giannakakou P., Robey R., Fojo T., Blagosklonny M.V. (2001) Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. *Oncogene* **20**:3806-3813 <https://doi.org/10.1038/sj.onc.1204487>
13. Maloney S.M., Hoover C.A., Morejon-Lasso L.V., Prosperi J.R. (2020) Mechanisms of Taxane Resistance. *Cancers (Basel)* **12** <https://doi.org/10.3390/cancers12113323>
14. Gupta N., Gupta P., Srivastava S.K. (2019) Penfluridol overcomes paclitaxel resistance in metastatic breast cancer. *Sci Rep* **9**:5066 <https://doi.org/10.1038/s41598-019-41632-0>
15. Baird R.D., Tan D.S., Kaye S.B. (2010) Weekly paclitaxel in the treatment of recurrent ovarian cancer. *Nat Rev Clin Oncol* **7**:575-582 <https://doi.org/10.1038/nrclinonc.2010.120>
16. Sosa Iglesias V., Giuranno L., Dubois L.J., Theys J., Vooijs M. (2018) Drug Resistance in Non-Small Cell Lung Cancer: A Potential for NOTCH Targeting? *Front Oncol.* **10**:3389 <https://doi.org/10.3389/fonc.2018.00267>
17. Jongens T.A., Ackerman L.D., Swedlow J.R., Jan L.Y., Jan Y.N. (1994) Germ cellless encodes a cell type-specific nuclear pore-associated protein and functions early in the germ-cell specification pathway of *Drosophila*. *Genes Dev* **8**:2123-2136 <https://doi.org/10.1101/gad.8.18.2123>
18. Jongens T.A., Hay B., Jan L.Y., Jan Y.N. (1992) The germ cell-less gene product: a posteriorly localized component necessary for germ cell development in *Drosophila*. *Cell* **70**:569-584 [https://doi.org/10.1016/0092-8674\(92\)90427-e](https://doi.org/10.1016/0092-8674(92)90427-e)
19. Liebe B., Petukhova G., Barchi M., Bellani M., Braselmann H., Nakano T., Pandita T.K., Jasin M., Fornace A., Meistrich M.L., et al. (2006) Mutations that affect meiosis in male mice influence the dynamics of the mid-preleptotene and bouquet stages. *Exp Cell Res* **312**:3768-3781

- <https://doi.org/10.1016/j.yexcr.2006.07.019>
20. Liu X.X., Cai L., Liu F.J. (2018) An in silico analysis of human sperm genes associated with asthenozoospermia and its implication in male infertility. *Medicine (Baltimore)* **97**:e13338 <https://doi.org/10.1097/MD.00000000000013338>
21. Consortium G. (2020) The GTEx Consortium atlas of genetic regulatory effects across human tissues. *Science* **369**:1318-1330 <https://doi.org/10.1126/science.aaz1776>
22. Park S.J., Yoon B.H., Kim S.K., Kim S.Y. (2019) GENT2: an updated gene expression database for normal and tumor tissues. *BMC Med Genomics* **12**:101 <https://doi.org/10.1186/s12920-019-0514-7>
23. Pae J., Cinalli R.M., Marzio A., Pagano M., Lehmann R. (2017) GCL and CUL3 Control the Switch between Cell Lineages by Mediating Localized Degradation of an RTK. *Dev Cell* **42**:130-142.e137. <https://doi.org/10.1016/j.devcel.2017.06.022>
24. Bonchuk A., Balagurov K., Georgiev P. (2023) BTB domains: A structural view of evolution, multimerization, and protein-protein interactions. *Bioessays* **45**:e2200179 <https://doi.org/10.1002/bies.202200179>
25. Holm L. (2022) Dali server: structural unification of protein families. *Nucleic Acids Res* **50**:W210-W215 <https://doi.org/10.1093/nar/gkac387>
26. Usher E.T., Sabri N., Rohac R., Boal A.K., Mittag T., Showalter S.A. (2021) Intrinsically disordered substrates dictate SPOP subnuclear localization and ubiquitination activity. *J Biol Chem* **296**:100693 <https://doi.org/10.1016/j.jbc.2021.100693>
27. Abramson J., Adler J., Dunger J., Evans R., Green T., Pritzel A., Ronneberger O., Willmore L., Ballard A.J., Bambrick J., et al. (2024) Accurate structure prediction of biomolecular interactions with AlphaFold 3. *Nature* **630**:493-500 <https://doi.org/10.1038/s41586-024-07487-w>
28. Panier S., Boulton S.J. (2014) Double-strand break repair: 53BP1 comes into focus. *Nat Rev Mol Cell Biol* **15**:7-18 <https://doi.org/10.1038/nrm3719>
29. Cong L., Ran F.A., Cox D., Lin S., Barretto R., Habib N., Hsu P.D., Wu X., Jiang W., Marraffini L.A., et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**:819-823 <https://doi.org/10.1126/science.1231143>
30. Yukiko Yoshida Y.S., Murakami Arisa, Kawawaki Junko, Tsuchiya Hikaru, Yoshihara Hidehito, Shindo Mayumi, Tanaka Keiji (2015) A comprehensive method for detecting ubiquitinated substrates using TR-TUBE. *PNAS* **112**:4630-4635 <https://doi.org/10.1073/pnas.1422313112>
31. Schiff P.B., Fant J., Horwitz S.B. (1979) Promotion of microtubule assembly in vitro by taxol. *Nature* **277**:665-667 <https://doi.org/10.1038/277665a0>
32. Xu A.P., Xu L.B., Smith E.R., Fleishman J.S., Chen Z.S., Xu X.X. (2023) Cell death in cancer chemotherapy using taxanes. *Front Pharmacol* **14**:1338633 <https://doi.org/10.3389/fphar.2023.1338633>
33. Mosca L., Ilari A., Fazi F., Assaraf Y.G., Colotti G. (2021) Taxanes in cancer treatment: Activity, chemoresistance and its overcoming. *Drug Resist Updat* **54**:100742 <https://doi.org/10.1016/j.drup.2020.100742>
34. Corsello S.M., Nagari R.T., Spangler R.D., Rossen J., Kocak M., Bryan J.G., Humeidi R., Peck D., Wu X., Tang A.A., et al. (2020) Discovering the anti-cancer potential of non-oncology drugs by systematic viability profiling. *Nat Cancer* **1**:235-248 <https://doi.org/10.1038/s43018-019-0018-6>
35. Zhao B., Rothenberg E., Ramsden D.A., Lieber M.R. (2020) The molecular basis and disease relevance of non-homologous DNA end joining. *Nat Rev Mol Cell Biol* **21**:765-781 <https://doi.org/10.1038/s41580-020-00297-8>
36. Burigotto M., Vigorito V., Gliech C., Mattivi A., Ghetti S., Bisio A., Lolli G., Holland A.J., Fava L.L. (2023) PLK1 promotes the mitotic surveillance pathway by controlling cytosolic 53BP1 availability. *EMBO Rep* **24**:e57234 <https://doi.org/10.15252/embr.202357234>
37. Lee D.H., Acharya S.S., Kwon M., Drane P., Guan Y., Adelmant G., Kalev P., Shah J., Pellman D., Marto J.A., et al. (2014) Dephosphorylation enables the recruitment of 53BP1 to double-strand DNA breaks. *Mol Cell* **54**:512-525 <https://doi.org/10.1016/j.molcel.2014.03.020>

38. Rona G., Miwatani-Minter B., Zhang Q., Goldberg H.V., Kerzhnerman M.A., Howard J.B., Simoneschi D., Lane E., Hobbs J.W., Sassani E., *et al.* (2024) CDK-independent role of D-type cyclins in regulating DNA mismatch repair. *Mol Cell* <https://doi.org/10.1016/j.molcel.2024.02.010>
39. Rona G., Roberti D., Yin Y., Pagan J.K., Homer H., Sassani E., Zeke A., Busino L., Rothenberg E., Pagano M. (2018) PARP1-dependent recruitment of the FBXL10-RNF68-RNF2 ubiquitin ligase to sites of DNA damage controls H2A.Z loading. *eLife* **7** <https://doi.org/10.7554/eLife.38771>
40. Choi H., Larsen B., Lin Z.Y., Breitkreutz A., Mellacheruvu D., Fermin D., Qin Z.S., Tyers M., Gingras A.C., Nesvizhskii A.I. (2011) SAINT: probabilistic scoring of affinity purification-mass spectrometry data. *Nat Methods* **8**:70-73 <https://doi.org/10.1038/nmeth.1541>
41. Gonçalves E., Poulos R.C., Cai Z., Barthorpe S., Manda S.S., Lucas N., Beck A., Bucio-Noble D., Dausmann M., Hall C., *et al.* (2022) Pan-cancer proteomic map of 949 human cell lines. *Cancer Cell* **40**:835-849.e838. <https://doi.org/10.1016/j.ccell.2022.06.010>
 Corsello, S.M., Nagari, R.T., Spangler, R.D., Rossen, J., Kocak, M., *et al.* (2024) Repurposing Public 24Q2 (PRISM Repurposing). depmap. ID 24Q2
 Gonçalves, E., Poulos, R.C., Cai, Z., Barthorpe, S., Manda, S.S., *et al.* (2022) Pan-cancer proteomic map of 949 human cell lines. PRIDE. ID PXD030304
<https://www.ebi.ac.uk/pride/archive/projects/PXD030304>

Peer reviews

Reviewer #2 (Public review):

Summary

This study investigates the role of GMCL1 in regulating the mitotic surveillance pathway (MSP), a protective mechanism that activates p53 following prolonged mitosis. The authors identify a physical interaction between 53BP1 and GMCL1, but not with GMCL2. They propose that the ubiquitin ligase complex CRL3-GMCL1 targets 53BP1 for degradation during mitosis, thereby preventing the formation of the "mitotic stopwatch" complex (53BP1-USP28-p53) and subsequent p53 activation. The authors show that high GMCL1 expression correlates with resistance to paclitaxel in cancer cell lines that express wild-type p53. Importantly, loss of GMCL1 restores paclitaxel sensitivity in these cells, but not in p53-deficient lines. They propose that GMCL1 overexpression enables cancer cells to bypass MSP-mediated p53 activation, promoting survival despite mitotic stress. Targeting GMCL1 may thus represent a therapeutic strategy to re-sensitize resistant tumors to taxane-based chemotherapy.

Strengths

This manuscript presents potentially interesting observations. The major strength of this article is the identification of GMCL1 as 53BP1 interaction partner. The authors identified relevant domains and show that GMCL1 controls 53BP1 stability. The authors further show a potentially interesting link between GMCL1 status and sensitivity to Taxol.

Weaknesses

A major limitation of the original manuscript was that the functional relevance of GMCL1 in regulating 53BP1 within an appropriate model system was not clearly demonstrated. In the revised version, the authors attempt to address this point. However, the new experiment is insufficiently controlled, making it difficult to interpret the results. State-of-the-art approaches would typically rely on single-cell tracking to monitor cell fate following release from a moderately prolonged mitosis.

In contrast, the authors use a population-based assay, but the reported rescue from arrest is minimal. If the assay were functioning robustly, one would expect that nearly all cells

depleted of USP28 or 53BP1 should have entered S-phase at a defined time after release. Thus, the very small rescue effect of siTP53BP1 suggests that the current assay is not suitable. It is also likely that release from a 16-hour mitotic arrest induces defects independent of the 53BP1-dependent p53 response.

Furthermore, the cell-cycle duration of RPE1 cells is less than 20 hours. It is therefore unclear why cells are released for 30 hours before analysis. At this time point, many cells are likely to have progressed into the next cell cycle, making it impossible to draw conclusions regarding the immediate consequences of prolonged mitosis. As a result, the experiment cannot be evaluated due to inadequate controls.

To strengthen this part of the study, I recommend that the authors first establish an assay that reliably rescues the mitotic-arrest-induced G1 block upon depletion of p53, 53BP1, or USP28. Once this baseline is validated, GMCL1 knockout can then be introduced to quantify its contribution to the response.

A broader conceptual issue is that the evidence presented does not form a continuous line of reasoning. For example, it is not demonstrated that GMCL1 interacts with or regulates 53BP1 in RPE1 cells-the system in which the limited functional experiments are conducted.

There are also a number of inconsistencies and issues with data presentation that need to be addressed:

- (1) Figure 2C: p21 levels appear identical between GMCL1 KO and WT rescue. If GMCL1 regulates p53 through 53BP1, p21 should be upregulated in the KO.
- (2) Figure 2A vs. 2C: GMCL1 KO affects chromatin-bound 53BP1 in Figure 2A, yet in Figure 2C it affects 53BP1 levels specifically in G1-phase cells. This discrepancy requires clarification.
- (3) Figure 2C quantification: The three biological repeats show an unusual pattern, with one repeat's data points lying exactly between the other two. It is unclear what the line represents; please clarify.
- (4) Figure nomenclature: Some abbreviations (e.g., FLAG-KI in Fig. 1F, WKE in Fig. 1C-D, Δ MFF in Fig. 1E) are not defined in the figure legends. All abbreviations must be explained.
- (5) Figure 2D: Please indicate how many times the experiment was reproduced. Quantification with statistical testing would strengthen the result. Pull-downs of 53BP1 with calculation of the ubiquitinated/total ratio could also support the conclusion.
- (6) Figures 3A and 3C: The G1 bars share the same color as the error bars, making the graphs difficult to interpret. Please adjust the color scheme.

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Reviewer #3 (Public review):

Summary:

In this study, Kito et al follow up on previous work that identified *Drosophila* GCL as a mitotic substrate recognition subunit of a CUL3-RING ubiquitin ligase (CRL3) complex. Here they identified mutants of the human ortholog of GCL, GMCL1, that disrupt the interaction with CUL3 (GMCL1E142K) and that lack the substrate interaction domain (GMCL1 BBO). Immunoprecipitation followed by mass spectrometry identified 9 proteins that interacted with wild type FLAG-GMCL1 but not GMCL1 EK or GMCL1 BBO. These proteins included 53BP1, which plays a well characterized role in double strand break repair but also functions in a USP28-p53-53BP1 "mitotic stopwatch" complex that arrests the cell cycle after a substantially prolonged mitosis. Consistent with the IP-MS results, FLAG-GMCL1

immunoprecipitated 53BP1. Depletion of GMCL1 during mitotic arrest increased protein levels of 53BP1, and this could be rescued by wild type GMCL1 but not the E142K mutant or a R433A mutant that failed to immunoprecipitate 53BP1. Using a publicly available dataset, the authors identified a relatively small subset of cell lines with high levels of GMCL1 mRNA that were resistant to the taxanes paclitaxel, cabazitaxel, and/or docetaxel. This type of analysis is confounded by the fact that paclitaxel and other microtubule poisons accumulate to substantially different levels in various cell lines (PMID: 8105478, PMID: 10198049) so careful follow up experiments are required to validate results. The correlation between increased GMCL1 mRNA and taxane resistance was not observed in lung cancer cell lines. The authors propose this was because nearly half of lung cancers harbor p53 mutations, and lung cancer cell lines with wild type but not mutant p53 showed the correlation between increased GMCL1 mRNA and taxane resistance. However, the other cancer cell types in which they report increased GMCL1 expression correlates with taxane sensitivity also have high rates of p53 mutation. Furthermore, p53 status does not predict taxane response in patients (PMID: 10951339, PMID: 8826941, PMID: 10955790). The authors then depleted GMCL1 and reported that it increased apoptosis in two cell lines with wild type p53 (MCF7 and U2OS) due to activation of the mitotic stopwatch. This is surprising because the mitotic stopwatch paper cited (PMID: 38547292) reported that U2OS cells have an inactive stopwatch. Though it can be partially restored by treatment with an inhibitor of WIP1, the stopwatch was reported to be substantially impaired in U2OS cells, in contrast to what is reported here. Additionally, activation of the stopwatch results in cell cycle arrest rather than apoptosis in most cell types, including MCF7. Beyond this, it has recently been shown that the level of taxanes and other microtubule poisons achieved in patient tumors is too low to induce mitotic arrest (PMID: 24670687, PMID: 34516829, PMID: 37883329). Physiologically relevant concentrations are achieved with approximately 5-10 nM paclitaxel, rather than the 100 nM used here. The findings here demonstrating that GMCL1 mediates chromatin localization of 53BP1 during mitotic arrest are solid and of interest to cell biologists, but it is unlikely that these findings are relevant to paclitaxel response in patients.

Strengths:

This study identified 53BP1 as a target of CRL3GMCL1-mediated degradation during mitotic arrest. AlphaFold3 predictions of the binding interface followed by mutational analysis identified mutants of each protein (GMCL1 R433A and 53BP1 IED1422-1425AAAA) that disrupted their interaction. Knock-in of a FLAG tag into the C-terminus of GMCL1 in HCT116 cells followed by FLAG immunoprecipitation confirmed that endogenous GMCL1 interacts with endogenous CUL3 and 53BP1 during mitotic arrest.

Weaknesses:

The clinical relevance of the study is overinterpreted. The authors have not taken relevant data about the clinical mechanism of taxanes into account. Supraphysiologic doses of microtubule poisons cause mitotic arrest and can activate the mitotic stopwatch. However, in physiologic concentrations of clinically useful microtubule poisons, cells proceed through mitosis and divide their chromosomes on mitotic spindles that are at least transiently multipolar. Though these low concentrations may result in a brief mitotic delay, it is substantially shorter than the arrest caused by high concentrations of microtubule poisons, and the one mimicked here by 16 hours of 0.4 mg/mL nocodazole or 48 hours of 100 nM paclitaxel. Resistance to mitotic arrest occurs through different mechanisms than resistance to multipolar spindles, raising concerns about the relevance of prolonged mitosis to paclitaxel response in cancer. Nocodazole is a microtubule poison that is not used clinically and does not induce multipolar spindles, so a similar apoptotic response to both drugs increases concern about a lack of physiological relevance. Moreover, clinical response to paclitaxel does not correlate with p53 status (PMID: 10951339, PMID: 8826941, PMID:

10955790). No evidence is presented that GMCL1 affects cellular response to clinically relevant doses of paclitaxel.

Comments on revisions:

(1) The claim that GMCL1 modulates paclitaxel sensitivity in cancer should be toned down. Inaccurate statements based on an outdated understanding of the anti-cancer mechanism of paclitaxel should be removed (eg lines 42-44: "In cancers that are resistant to paclitaxel, a microtubule-targeting agent, cells bypass mitotic surveillance activation, allowing unchecked proliferation...", lines 73-75: "Proper mitotic arrest is critical for the efficacy of microtubule-targeting therapies...", lines 78-79: "This resistance is frequently associated with loss of MSP activity, for example due to defective p53 signaling". As cited in the public review, p53 status does not correlate with paclitaxel response in cancer.)

(2) Perform timelapse experiments +/- GMCL1 siRNA in the absence of drug and in the presence of low, physiologically relevant concentrations of paclitaxel (5-10 nM), as well as supraphysiologic concentrations (100 nM) and correlate mitotic duration with cell cycle arrest. Test if co-depletion of 53BP1 with GMCL1 rescues cell cycle arrest after a substantially prolonged mitosis. Perform these experiments in a cell line with an intact mitotic stopwatch.

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Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1(Public review):

In this manuscript, Pagano and colleagues test the idea that the protein GMCL1 functions as a substrate receptor for a Cullin RING 3 E3 ubiquitin ligase (CUL3) complex. Using a pulldown approach, they identify GMCL1 binding proteins, including the DNA damage scaffolding protein 53BP1. They then focus on the idea that GMCL1 recruits 53BP1 for CUL3-dependent ubiquitination, triggering subsequent proteasomal degradation of ubiquitinated 53BP1.

In addition to its DNA damage signalling function, in mitosis, 53BP1 is reported to form a stopwatch complex with the deubiquitinating enzyme USP28 and the transcription factor p53 (PMID: 38547292). These 53BP1-stopwatch complexes generated in mitosis are inherited by G1 daughter cells and help promote p53-dependent cell cycle arrest independent from DNA damage (PMID: 38547292). Several studies show that knockout of 53BP1 overcomes G1 cell cycle arrest after mitotic delays caused by anti-mitotic drugs or centrosome ablation (PMID: 27432897, 27432896). In this model, it is crucial that 53BP1 remains stable in mitosis and more stopwatch complex is formed after delayed mitosis.

Major concerns:

Pagano and coworkers suggest that 53BP1 levels can sometimes be suppressed in mitosis if the cells overexpress GMCL1. They carry out a bioinformatic analysis of available public data for p53 wild-type cancer cell lines resistant to the anti-mitotic drug paclitaxel and related compounds. Stratifying GMCL1 into low and high expression groups reveals a weak ($p = 0.05$ or ns) correlation with sensitivity to taxanes. It is unclear on what basis the authors claim paclitaxel-resistant and p53 wild-type cancer cell lines bypass the mitotic surveillance/timer pathway. They have not tested this. Figure 3 is a correlation assembled from public databases but has no experimental tests. Figure 4 looks at proliferation but not cell cycle progression or the length of mitosis. The main

conclusions relating to cell cycle progression and specifically the link to mitotic delays are therefore not supported by experimental data. There is no imaging of the cell cycle or cell fate after mitotic delays, or analysis of where the cells arrest in the cell cycle. Most of the cell lines used have been reported to lack a functional mitotic surveillance pathway in the recent work by Meitinger. To support these conclusions, the stability of endogenous 53BP1 under different conditions in cells known to have a functional mitotic surveillance pathway needs to be examined. A key suggestion in the work is that the level of GMCL1 expression correlates with resistance to taxanes. For the mitotic surveillance pathway, the type of drug (nocodazole, taxol, etc) used to induce a delay isn't thought to be relevant, only the length of the delay. Do GMCL1-overexpressing cells show resistance to anti-mitotics in general?

We thank the reviewer for this insightful comment. We propose that GMCL1 promotes CUL3-dependent ubiquitination of 53BP1 during prolonged mitotic arrest, thereby facilitating its proteasome-dependent degradation. To evaluate the potential clinical relevance of this mechanism, we stratified cancer cell lines based on GMCL1 mRNA expression using publicly available datasets from DepMap (PMID: 39468210). We observed correlations between GMCL1 expression levels and taxane sensitivity that appear to reflect specific cancer type-drug combinations. To experimentally evaluate this correlation and obtain mechanistic insights, we performed knockdown experiments in hTERT-RPE1 cells, which are known to possess an intact mitotic surveillance pathway. Silencing of GMCL1 alone inhibited cell proliferation and induced apoptosis, while co-depletion of either TP53BP1 or USP28 significantly rescued these effects. These results suggest that GMCL1 modulates the stability of 53BP1 and therefore the availability of the 53BP1-USP28-p53 ternary complex in cells with a functional mitotic surveillance pathway (MSP) (new Figure 5I,J) directly linking GMCL1 to the regulation of the MSP complex. Moreover, to further support our mechanism, we assessed the effect of GMCL1 levels on cell cycle progression. Briefly, following nocodazole synchronization and release, we treated cells with EdU and performed FACS analyses at different times. Knockdown of GMCL1 alone led to a delayed cell cycle progression, but co-depletion of either TP53BP1 or USP28 restored this phenotype (new Figure 3A and new Supplementary Figure 3A-C). These results are consistent with our proliferation data and suggest that the observed effects of GMCL1 are specific to mitotic exit. Finally, overexpression of GMCL1 accelerates cell cycle progression (as assessed by FACS analyses) upon release from prolonged mitotic arrest (new Figure 3B and new Supplementary Figure 3D-E).

Importantly, if GMCL1 specifically degrades 53BP1 during prolonged mitotic arrests, the authors should show what happens during normal cell divisions without any delays or drug treatments. How much 53BP1 is destroyed in mitosis under those conditions? Does 53BP1 destruction depend on the length of mitosis, drug treatment, or does 53BP1 get degraded every mitosis regardless of length? Testing the contribution of key mitotic E3 ligase activities on mitotic 53BP1 stability, such as the anaphase-promoting complex/cyclosome (APC/C) is important in this regard. One previous study reported an analysis of putative APC/C KEN-box degron motifs in 53BP1 and concluded these play a role in 53BP1 stability in anaphase (PMID: 28228263).

Physiological mitosis under unperturbed conditions is typically brief (approximately 30 minutes), making protein quantification during this window challenging. Despite this, we tried by synchronizing cells using RO-3306 and releasing them into drug-free medium to assess GMCL1 dynamics during normal mitosis. Under these conditions, GMCL1 expression was similar to that in asynchronous cells and higher than the levels upon extended mitosis. However, when we attempted to measure the half-life of proteins using cycloheximide, most cells died, likely due to the toxic effect of cycloheximide in cells subjected to co-treatment with RO-3306 or nocodazole. This is the same reasons why in Figure 2C, we assessed 53BP1 in daughter cells rather than mitotic cells.

There is no direct test of the proposed mechanism, and it is therefore unclear if 53BP1 is ubiquitinated by a GMCL1-CUL3 ligase in cells, and how efficient this process would be at different cell cycle stages. A key issue is the lack of experimental data explaining why the proposed mechanism would be restricted to mitosis. Indirect effects, such as loss of 53BP1 from the chromatin fraction during M phase upon GMCL1 overexpression, do not necessarily mean that 53BP1 is degraded. PLK1-dependent chromatin-cytoplasmic shuttling of 53BP1 during mitotic delays has been described previously (PMID: 38547292, 37888778). These papers are cited in the text, but the main conclusions of those papers on 53BP1 incorporation into a stopwatch complex during mitotic delays have been ignored. Are the authors sure that 53BP1 is destroyed in mitosis and not simply re-localised between chromatin and non-chromatin fractions? At the very least, these reported findings should be discussed in the text.

To examine whether GMCL1 promotes 53BP1 ubiquitination in cells, we expressed in cells Trypsin-Resistant Tandem Ubiquitin-Binding Entity (TR-TUBE), a protein that binds polyubiquitin chains. Abundant, endogenous ubiquitinated 53BP1 co-precipitated with TR-TUBE constructs only when wild-type GMCL1 but not the E142K GMCL1 mutant, was expressed (new Figure 2D). The PLK1-dependent incorporation of 53BP1 into the stopwatch complex and the chromatin-cytoplasmic shuttling of 53BP1 during mitotic delays is now discussed in the text. That said, compared to parental cells, 53BP1 levels in the chromatin fraction are high in two different GMCL1 KO clones in M phase arrested cells (Figure 2A-B). This increase does not correspond to a decrease in the 53BP1 soluble fraction (Figure 2A and new Supplementary Figure 2D), suggesting decreased 53BP1 is not due to re-localization. The increased half-life of 53BP1 in daughter cells (Figure 2C), also supports this hypothesis.

The authors use a variety of cancer cell line models throughout their study, most of which have been reported to lack a functional mitotic surveillance pathway. U2OS and HCT116 cells do not respond normally to mitotic delays, despite being annotated as p53 WT. Other studies have used p53 wild-type hTERT RPE-1 cells to study the mitotic surveillance pathway. If the model is correct, then over-expressing GMCL1 in hTERT-RPE1 cells should suppress cell cycle arrest after mitotic delays, and GMCL1 KO should make the cells more sensitive to delays. These experiments are needed to provide an adequate test of the proposed model.

We greatly appreciate the reviewer's suggestion regarding overexpression of GMCL1 in hTERT-RPE1 cells. To address this, we generated stable RPE1 cells expressing V5-tagged GMCL1 and conducted EdU incorporation assays following nocodazole synchronization and release. Overexpression of GMCL1 enhanced cell cycle progression compared to control cells (new Figure 3B and new Supplementary Figure 3D-E) after mitotic arrest, consistent with our model. We, therefore, propose that GMCL1 controls 53BP1 stability to suppress p53-dependent cell cycle arrest.

We also want to point out that while some papers suggest that HCT116 and U2OS cells do not have an intact mitotic surveillance pathway, others have shown that the MSP is indeed functioning in HCT116 cells and can be triggered with variable efficiency in U2OS cells (PMID: 38547292). This is likely due to high heterogeneity and extensive clonal diversity of cancer cell lines grown in different labs. Please see examples in PMIDs: 3620713, 30089904, and 30778230. In particular, PMID: 30089904 shows that this heterogeneity correlates with considerably different drug responses.

To conclude, while the authors propose a potentially interesting model on how GMCL1 overexpression could regulate 53BP1 stability to limit p53-dependent cell cycle arrest, it is unclear what triggers this pathway or when it is relevant. 53BP1 is known to function in DNA damage signalling, and GMCL1 might be relevant in that context. The manuscript

contains the initial description of GMCL1-53BP1 interaction but lacks a proper analysis of the function of this interaction and is therefore a preliminary report.

We hope that the new experiments, along with the clarifications provided in this response letter and revised manuscript, offer the reviewer increased confidence in the robustness and validity of our proposed model.

Reviewer #2 (Public review):

This study investigates the role of GMCL1 in regulating the mitotic surveillance pathway (MSP), a protective mechanism that activates p53 following prolonged mitosis. The authors identify a physical interaction between 53BP1 and GMCL1, but not with GMCL2. They propose that the ubiquitin ligase complex CRL3-GMCL1 targets 53BP1 for degradation during mitosis, thereby preventing the formation of the "mitotic stopwatch" complex (53BP1-USP28-p53) and subsequent p53 activation. The authors show that high GMCL1 expression correlates with resistance to paclitaxel in cancer cell lines that express wild-type p53. Importantly, loss of GMCL1 restores paclitaxel sensitivity in these cells, but not in p53-deficient lines. They propose that GMCL1 overexpression enables cancer cells to bypass MSP-mediated p53 activation, promoting survival despite mitotic stress. Targeting GMCL1 may thus represent a therapeutic strategy to re-sensitize resistant tumors to taxane-based chemotherapy.

Strengths:

This manuscript presents potentially interesting observations. The major strength of this article is the identification of GMCL1 as a 53BP1 interaction partner. The authors identified relevant domains and showed that GMCL1 controls 53BP1 stability. The authors further show a potentially interesting link between GMCL1 status and sensitivity to Taxol.

Weaknesses:

However, the manuscript is significantly weakened by unsubstantiated mechanistic claims, overreliance on a non-functional model system (U2OS), and overinterpretation of correlative data. To support the conclusions of the manuscript, the authors must show that the GMCL1-dependent sensitivity to Taxol depends on the mitotic surveillance pathway.

To demonstrate that GMCL1-dependent taxane sensitivity is mediated through the mitotic surveillance pathway (MSP), we now performed experiments using hTERT-RPE1 (RPE1) cells, a widely used, non-transformed cell line known to possess a functional MSP. We compared RPE1 cells with knockdown of GMCL1 alone to those with simultaneous knockdown of GMCL1 and either TP53BP1 or USP28. Upon paclitaxel (Taxol) treatment, cells with GMCL1 knockdown exhibited suppressed proliferation and increased apoptosis. Notably, these phenotypes were rescued by co-depletion of TP53BP1 or USP28 (new Figure 5I,J). These results support the notion that GMCL1 contributes to MSP activity, at least in part, through its regulation of 53BP1.

To further strengthen our mechanistic experiments, we assessed the effect of GMCL1 levels on cell cycle progression. Following nocodazole synchronization and release, we treated cells with EdU and performed FACS analyses at different times. Knockdown of GMCL1 alone led to a delay in cell cycle progression, but co-depletion of either TP53BP1 or USP28 alleviate this phenotype (new Figure 3A and new Supplementary Figure 3A, B). These results are consistent with our proliferation data.

Reviewer #3 (Public review):

Summary:

In this study, Kito et al follow up on previous work that identified Drosophila GCL as a mitotic substrate recognition subunit of a CUL3-RING ubiquitin ligase (CRL3) complex.

Here they characterize mutants of the human ortholog of GCL, GMCL1, that disrupt the interaction with CUL3 (GMCL1E142K) and that lack the substrate interaction domain (GMCL1 BBO). Immunoprecipitation followed by mass spectrometry identified 9 proteins that interacted with wild-type FLAG-GMCL1 and GMCL1 EK but not GMCL1 BBO. These proteins included 53BP1, which plays a well-characterized role in double-strand break repair but also functions in a USP28-p53-53BP1 "mitotic stopwatch" complex that arrests the cell cycle after a substantially prolonged mitosis. Consistent with the IP-MS results, FLAG-GMCL1 immunoprecipitated 53BP1. Depletion of GMCL1 during mitotic arrest increased protein levels of 53BP1, and this could be rescued by wild-type GMCL1 but not the E142K mutant or a R433A mutant that failed to immunoprecipitate 53BP1.

Using a publicly available dataset, the authors identified a relatively small subset of cell lines with high levels of GMCL1 mRNA that were resistant to the taxanes paclitaxel, cabazitaxel, and docetaxel. This type of analysis is confounded by the fact that paclitaxel and other microtubule poisons accumulate to substantially different levels in various cell lines (DOI: 10.1073/pnas.90.20.9552 , DOI: 10.1091/mbc.10.4.947), so careful follow-up experiments are required to validate results. The correlation between increased GMCL1 mRNA and taxane resistance was not observed in lung cancer cell lines. The authors propose this was because nearly half of lung cancers harbor p53 mutations, and lung cancer cell lines with wild-type but not mutant p53 showed the correlation between increased GMCL1 mRNA and taxane resistance. However, the other cancer cell types in which they report increased GMCL1 expression correlates with taxane sensitivity also have high rates of p53 mutation. Furthermore, p53 status does not predict taxane response in patients (DOI: 10.1002/1097-0142(20000815)89:4<769::aid-cncr8>3.0.co;2-6 , DOI: 10.1002/(SICI)1097-0142(19960915)78:6<1203::AID-CNCR6>3.0.CO;2-A , PMID: 10955790).

The authors then depleted GMCL1 and reported that it increased apoptosis in two cell lines with wild-type p53 (MCF7 and U2OS) due to activation of the mitotic stopwatch. This is surprising because the mitotic stopwatch paper they cite (DOI:

10.1126/science.add9528) reported that U2OS cells have an inactive stopwatch and that activation of the stopwatch results in cell cycle arrest rather than apoptosis in most cell types, including MCF7. Beyond this, it has recently been shown that the level of taxanes and other microtubule poisons achieved in patient tumors is too low to induce mitotic arrest (DOI: 10.1126/scitranslmed.3007965 , DOI: 10.1126/scitranslmed.abd4811 , DOI: 10.1371/journal.pbio.3002339), raising concerns about the relevance of prolonged mitosis to paclitaxel response in cancer. The findings here demonstrating that GMCL1 mediates degradation of 53BP1 during mitotic arrest are solid and of interest to cell biologists, but it is unclear that these findings are relevant to paclitaxel response in patients.

Strengths:

This study identified 53BP1 as a target of CRL3GMCL1-mediated degradation during mitotic arrest. AlphaFold3 predictions of the binding interface, followed by mutational analysis, identified mutants of each protein (GMCL1 R433A and 53BP1 IED11422-1425AAAA) that disrupted their interaction. Knock-in of a FLAG tag into the C-terminus of GMCL1 in HCT116 cells, followed by FLAG immunoprecipitation, confirmed that endogenous GMCL1 interacts with endogenous CUL3 and 53BP1 during mitotic arrest.

Weaknesses:

The clinical relevance of the study is overinterpreted. The authors have not taken relevant data about the clinical mechanism of taxanes into account. Supraphysiologic doses of microtubule poisons cause mitotic arrest and can activate the mitotic stopwatch. However, in physiologic concentrations of clinically useful microtubule poisons, cells proceed through mitosis and divide their chromosomes on mitotic spindles that are at least transiently multipolar. Though these low concentrations may result in a brief mitotic delay, it is substantially shorter than the arrest caused by high concentrations of microtubule poisons, and the one mimicked here by 16 hours of 0.4 mg/mL nocodazole, which is not used clinically and does not induce multipolar spindles. Resistance to mitotic arrest occurs through different mechanisms than resistance to multipolar spindles. No evidence is presented in the current version of the manuscript that GMCL1 affects cellular response to clinically relevant doses of paclitaxel.

We agree that it would be an overstatement to claim that GMCL1 and p53 regulates paclitaxel sensitivity in cancer patients in a clinical context. The correlations we observed were based on publicly available cancer cell lines from datasets catalogued in CCLE and DepMap, which do not fully account for clinical heterogeneity and patient-specific factors. In response to this important point, we have revised the text accordingly.

In the experiments shown in former Figure 4A-H (now Figure 5A-H) and in those shown in the new Figure 5I-J, we used 100 nM paclitaxel to test the hypothesis that low GMCL1 levels sensitizes cancer cells in a p53-dependent manner. Here, paclitaxel was chosen to mimic the conditions reported in the PRISM dataset (PMID: 32613204), which compiles the proliferation inhibitory activity of 4,518 compounds tested across 578 cancer cell lines. Consistent with our cell cycle findings, the paclitaxel sensitivity caused by GMCL1 depletion was reverted by silencing 53BP1 or USP28 (new Figure 5I-J), again supporting the involvement of the stopwatch complex. We are unsure about how to model the “physiologic concentrations of clinically useful microtubule poisons” in cell-based studies. A recent review notes that “The time above a threshold paclitaxel plasma concentration (0.05 mmol/L) is important for the efficacy and toxicity of the drug” (PMID: 28612269). Two other reviews mention that the clinically relevant concentration of paclitaxel is considered to be plasma levels between 0.05–0.1 $\mu\text{mol/L}$ (approximately 50–100 nM) and that in clinical dosing, typical patient plasma concentrations after paclitaxel infusion range from 80–280 nM, with corresponding intratumoral concentrations between 1.1–9.0 μM , due to drug accumulation in tumor tissue (PMIDs: 24670687 and 29703818). We have now emphasized in the revised text the rationale for using 100 nM paclitaxel in our experiments.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

General comments on the Figures:

(1) Western blots lack molecular weight markers on most panels and are often over-exposed and over-contrasted, rendering them hard to interpret.

We have now included molecular weight markers in all Western blot panels. We have also reprocessed the images to avoid overexposure and excessive contrast, ensuring that the bands are clearly visible and interpretable.

(2) Input and IP samples do not show percentage loading, so it is hard to interpret relative enrichments.

In the revised figures, we have indicated what % of the input was loaded.

(3) The authors change between cell line models for their experiments, and this is not clear in the figures. These are important details for interpreting the data, as many of the cell lines used are not functional for the mitotic surveillance pathway.

In the revised manuscript, we have clearly indicated the specific cell lines used in each experiment in the figure legends. Additionally, to address concerns regarding the mitotic surveillance pathway, we have included new experiments using hTERT-RPE1 cells, which have been reported to possess a functional mitotic surveillance pathway (MSP) (Figure 4I-J).

(4) No n-numbers are provided in the figure legends. Are the Western blots provided done once, or are they reproducible? Many of the blots would benefit from quantification and presentation via graphs to test for reproducible changes to 53BP1 levels under the different conditions.

As now indicated in the methods section, we have conducted each Western blot no less than three times, yielding results that exhibit a high degree of reproducibility. A representative Western blot has been selected for each figure. We did not include densitometric quantification of immunoblots, given that the semi-quantitative nature of this technique would lead to an overinterpretation of our data; unfortunately, this is a limitation of the technique. In fact, eLife and other similar scientific journals do not adhere to the practice of quantifying Western blots. One exception to this norm is for protein half-life studies, which is done to measure the kinetics of decay rates and their internal comparisons. Accordingly, the experiments in Figure 2C were quantified.

(5) Graphs displayed in the supplementary figures are blacked out, and individual data points cannot be visualised. All graphs should have individual data points clearly visible.

We revised the quantified graphs and replaced them with scatter plots to clearly display individual data points, showing sample distribution.

Additional experiments with specific comments on Figures:

(1) Figure 1C-D: the relative amount of 53BP1 co-precipitating with FLAG-tagged GMCL1 WT appears very different between the two experiments. If the idea is that MLN4924 (Cullin neddylation inhibitor) makes the interaction easier to capture, then this should be explained in the text, and ideally shown on the same gel/blot +/- MLN4924.

We now present the samples treated with and without MLN4924 on the same gel/blot to allow direct comparison (new Figure 1D) and clarified this point in the text.

(2) Figure 1E: The figure legend states that GMCL1 was immunoprecipitated, but the Figure looks as though FLAG-tagged 53BP1 was the bait protein being immunoprecipitated? Can the authors clarify?

We thank the reviewer for pointing out the discrepancy between the figure and the figure legend in Figure 1E. The immunoprecipitation was indeed performed using FLAG-tagged 53BP1, and we have now rectified the figure legend accordingly.

(3) Figure 1F: Rather than parental cell lysate, the better control would be to IP FLAG from another FLAG-tagged expressing cell line, to rule out non-specific binding with the FLAG tag at the non-overexpressed level.

Figure 1F shows interaction at the endogenous level. The specificity of binding with overexpressed proteins is shown in Figures 1C and 1D.

The USP28 blot is over-exposed and makes it hard to see any changes in electrophoretic mobility - it looks as though there is a change between the parental and the KI cell line?

It is surprising that USP28 would co-IP with GMCL1 (presumably because USP28 is bound to 53BP1) if the function of GMCL1-53BP1 interaction is to promote 53BP1 degradation. Can the authors reconcile this? Crucially, if the authors claim that the 53BP1-GMCL1 interaction is specific to prolonged mitosis, then this experiment should be repeated and performed with asynchronous, normal-length mitosis, and prolonged mitosis conditions. This is vital for supporting the claim that this interaction only occurs during prolonged mitoses and does not occur in every mitosis regardless of length.

This is a good point. Unfortunately, many of the protein-protein interactions occur post lysis. Therefore, we could not observe differences in asynchronous vs. mitotic cells.

(4) Figure S1F: Label on blot should be CUL3 not CUI3.

We thank the reviewer for pointing this out and we have corrected the typo.

(5) Figure 2A: The authors suggest an increase in chromatin-bound 53BP1 in GMCL1 KO U2OS cells, specifically in M phase. Again, is this time in mitosis dependent, or would this be evident in every mitosis, regardless of length? Such an experiment would benefit from repetition and quantification to test whether the observed effect is reproducibly consistent. If the authors' model is correct, simply treating U2OS WT mitotic cells with MG132 during the mitotic arrest and performing the same fractionation should bring 53BP1 levels up to that seen in GMCL1 KO cells under the same conditions.

The reviewer's suggestion to assess 53BP1 accumulation in wild-type U2OS cells treated with MG132 during mitotic arrest is indeed highly relevant. However, treatment with MG132 during prolonged mitosis consistently led to significant cell death, making it technically challenging to evaluate 53BP1 levels under these conditions.

(6) Figure 2B: The authors restore GMCL1 expression in the KO U2OS cells using WT and 2 distinct mutant cDNAs. However, the expression of these constructs is not equivalent, and thus their effects cannot be directly compared. It is also surprising that GMCL1 is much higher in M phase samples in this experiment (shouldn't it be destroyed?), when no such behaviour has been observed in the other figures.

There is no evidence in our study or others that GMCL1 should be destroyed in M phase. We show that the R433A mutant is expressed at a level very similar to the WT protein, yet it doesn't promote the degradation of 53BP1. It is true that the E142K is expressed less in mitotic cells whereas is the most expressed in asynchronous cells. For some reason, this mutant has an inverse behavior compared to the WT, limiting the interpretation of this result. We now mention this in the text.

(7) Figure 2C: The CHX experiment would benefit from inclusion of a control protein known to have a short half-life (e.g. c-myc, p53). Is GMCL1 known to have a relatively short half-life? It looks as though GMCL1 disappears after 1 h CHX treatment (although hard to definitively tell in the absence of molecular weight markers). 53BP1 appears to continue declining in the absence of GMCL1, which is surprising if p53BP1 degradation requires GMCL1. How can the authors reconcile this?

As a control for the CHX chase experiments, we included p21, whose protein levels decreased in a CHX-dependent. GMCL1 itself also appeared to undergo degradation upon CHX treatment, but it doesn't disappear completely.

(8) Supplemental Figure 2:

Transcription is largely inhibited in M phase, so the p53 target gene transcripts present in M phase are inherited from the preceding G2 phase. The qPCR's thus need a reference sample to compare against. I.e., was p21/PUMA/NOXA mRNA already low in G2 in the

GMCL1 KO + WT cells before they entered mitosis? Or is the mRNA stability affected during M phase specifically? Is this effect on the mRNA dependent on the time in mitosis?

It is well established that transcription is not entirely shut down during mitosis, particularly for a subset of genes involved in cell cycle regulation. For example, p21, PUMA, NOXA, and p53 mRNAs have been shown to remain actively transcribed during mitosis (see Table S5 in PMID: 28912132). However, we currently lack direct evidence that p53 activation during mitosis, specifically through the mitotic surveillance pathway, drives the transcription of p21, PUMA, or NOXA mRNAs during M phase. In the absence of such mechanistic data, we opted to exclude these analyses from the final figures.

Panel B: blots are too over-exposed to see differences in p53 stability under the different conditions. Mitotic samples should be included to show how these differ from the G1 samples.

The background of all blot images has been adjusted to ensure clarity and consistency.

Panel D: The authors show no significant difference in the cell cycle profiles of the GMCL1 KO and reconstituted cells compared to parental U2OS cells. This should also be performed in the G1 daughter cells following a prolonged mitosis, to test the effect of the different GMCL1 constructs on G1 cell cycle arrest. U2OS cells have been reported not to have a functional mitotic surveillance pathway (Meitinger et al, Science, 2024), so U2OS cells are perhaps not a good model for testing this.

We performed cell cycle profiling using EdU incorporation in hTERT-RPE1 cells, which possess a functional MSP, to evaluate cell cycle progression in daughter cells following prolonged mitosis. We observed that GMCL1 knockdown alone leads to G1-phase arrest. In contrast, co-depletion of GMCL1 with either 53BP1 or USP28 bypasses this arrest, indicating that GMCL1 regulates cell cycle progression in an MSP-dependent manner. Please see also the answer to the public review above.

(9) Figure 3:

The authors show expression data for GMCL1 in the different cancer cell lines. This should be validated for a subset of cancer cell lines at the GMCL1 protein level, and cross-correlated to their MSP/mitotic timer status. Does GMCL1 depletion or knockout in p53 wild-type cancer cell lines overexpressing GMCL1 protein restore mitotic surveillance function?

We were unable to assess GMCL1 protein levels using publicly available proteomics datasets, as GMCL1 expression was not detected. In p53 wild-type hTERT-RPE1 cells, GMCL1 knockdown impaired the mitotic surveillance pathway, as evidenced by G1-phase arrest following prolonged mitosis (new Figure 3A and new Supplementary Figure 3A, B). This arrest was rescued by co-depletion of either TP53BP1 or USP28, indicating that GMCL1 acts upstream of the MSP.

(10) Figure 4:

The authors show siRNA experiments depleting GMCL1 and testing the effects of GMCL1 loss on cell viability and apoptosis induction. This is performed in different cell line backgrounds. However, there is no demonstration that any of the observed effects are due to a lack of GMCL1 activity on 53BP1. These experiments need to be repeated in 53BP1 co-depleted cells to test for rescue. Without this, the interpretation is purely correlative.

We assessed the effects of GMCL1 knockdown, alone or in combination with TP53BP1 or USP28 knockdown, on cell viability and apoptosis in hTERT-RPE1 cells using siRNA.

Knockdown of GMCL1 alone led to a significant reduction in cell viability and an increase in apoptosis. However, co-depletion of GMCL1 with either TP53BP1 or USP28 restored both cell viability and apoptosis levels to those observed in control cells (new Figure 5I,J).

(11) Text comments:

Line 257: HeLa cells suppress p53 through the E6 viral protein and are not "mutant" for p53.

The authors should cite early work by Uetake and Sluder describing the effects of spindle poisons on the mitotic surveillance pathway.

We appreciate the reviewer's comments – We have now made the necessary corrections.

Reviewer #2 (Recommendations for the authors):

Major Points:

(1) Unsubstantiated Mechanistic Claims:

In Figures 3 and 4, the authors show correlations between GMCL1 expression and sensitivity to Taxol. However, they fail to demonstrate that the mitotic stopwatch is mechanistically involved. To support this conclusion, the authors must test whether deletion of 53BP1, USP28, or disruption of their interaction rescues Taxol sensitivity in GMCL1-depleted cells. Since 53BP1 also plays a role in DNA damage response, such rescue experiments are necessary to distinguish between mitotic surveillance-specific and broader stress-response effects. Deletion of USP28 would be particularly informative.

We sought to experimentally determine whether GMCL1 is involved in regulating the mitotic stopwatch. Knockdown of GMCL1 alone resulted in reduced cell proliferation and increased apoptosis. In contrast, co-depletion of GMCL1 with either TP53BP1 or USP28 restored both proliferation and apoptosis levels to those observed in control cells (new Figure 5I, J). To further strengthen our mechanistic experiments, we assessed the effect of GMCL1 levels on cell cycle progression. We conducted EdU incorporation assays following nocodazole synchronization and release. Knockdown of GMCL1 alone led to a delay in G1 progression, whereas co-depletion of either TP53BP1 or USP28 rescued normal cell cycle progression (new Figure 3A and new Supplementary Figure 3A, B). These results are consistent with our proliferation data and suggest that GMCL1 functions upstream of the ternary complex, likely by regulating 53BP1 protein levels.

(2) Model System Limitations (U2OS Cells):

The use of U2OS cells is highly problematic for investigating the mitotic surveillance pathway. U2OS cells lack a functional mitotic stopwatch and do not arrest following prolonged mitosis in a 53BP1/USP28-dependent manner (PMID: 38547292). Therefore, conclusions drawn from this model system about the function of the mitotic surveillance pathway are not substantiated. Key experiments should be repeated in a cell line with an intact pathway, such as RPE1.

We now performed all key experiments also hTERT-RPE1 cells (see above). We also would like to point out that while some papers suggest that HCT116 and U2OS cells do not have an intact mitotic surveillance pathway, others have showed that the MSP is indeed functioning in HCT116 cells and can be triggered with variable efficiency in U2OS cells (PMID: 38547292). This is likely due to high heterogeneity and extensive clonal diversity of cancer cell lines grown in different labs. Please see examples in PMIDs: 3620713, 30089904, and 30778230. In particular, PMID: 30089904 shows that this heterogeneity correlates with considerably different drug responses.

(3) Misinterpretation of p53 Activity Timing:

The manuscript states that "GMCL1 KO cells led to decreased mRNA levels of p21 and NOXA during mitosis" (line 194). However, it is well established that the mitotic surveillance pathway activates p53 in the G1 phase following prolonged mitosis-not during mitosis itself (PMID: 38547292). Therefore, the observed changes in mRNA levels during mitosis are unlikely to be relevant to this pathway.

We currently lack direct evidence that p53 activated during mitosis through the mitotic surveillance pathway directly influences the transcription of p21, PUMA, or NOXA mRNAs during M phase. Therefore, we have chosen to exclude these data from the final figures.

(4) Incorrect Interpretation of 53BP1 Chromatin Binding:

The authors claim that 53BP1 remains associated with chromatin during mitosis, which contradicts established literature. It is known that 53BP1 is released from chromatin during mitosis via mitosis-specific phosphorylation (PMID: 24703952), and this is supported by more recent findings (PMID: 38547292). A likely explanation for the discrepancy may be contamination of mitotic fractions with interphase cells. The chromatin fraction data in Figure 2C must be interpreted with caution.

Our method to synchronize in M phase is rather stringent (see Supplementary Figure 3D as an example). The literature indicates that the bulk of 53BP1 is released from chromatin during mitosis. Yet, even in the two publications mentioned by the reviewer, there is a difference in the observable amount of 53BP1 bound to chromatin (compare Figure 2B in PMID: 38547292 and Figure 5A in PMID: 24703952). The difference is likely due to the different biochemical approaches used to purify chromatin bound proteins (salt and detergent concentrations, sonication, etc.). Using our fractionation approach, we can reliably separate the soluble fraction (containing also the nucleoplasmic fraction) and chromatin associated proteins as indicated by the controls such as α -Tubulin and Histon H3. We have now mentioned these limitations when comparing different fractionation methods in our discussion section.

(5) Inadequate Citation of Foundational Literature:

The literature on the mitotic surveillance pathway is relatively limited, and it is essential that the authors provide a comprehensive and accurate account of its development. The foundational work by the Sluder lab (PMID: 20832310), demonstrating a p53-dependent arrest following prolonged mitosis, must be cited. Furthermore, the three key 2016 papers (PMID: 27432896, 27432897, 27432896) that identified the involvement of USP28 and 53BP1 in this pathway are critical and should be cited as the basis of the mitotic surveillance pathway.

In contrast, the manuscript currently emphasizes publications that either contribute minimally or have been contradicted by prior and subsequent work. For example: PMID: 31699974, which proposes Ser15 phosphorylation of p53 as critical, has been contradicted by multiple groups (e.g., Holland, Oegema, and Tsou labs).

PMID: 37888778, which suggests that 53BP1 must be released from kinetochores, is inconsistent with findings that indicate kinetochore localization is not relevant.

The authors should thoroughly revise the Introduction to reflect what this reviewer would describe as a more accurate and scholarly approach to the literature.

We have substantially revised both the Introduction and Discussion sections to incorporate important references kindly suggested by the reviewer.

Minor Points:

(1) Overexposed Western Blots:

The Western blots throughout the manuscript are heavily overexposed and saturated, obscuring differences in protein levels and hindering data interpretation. The authors should provide properly exposed blots with quantification where appropriate.

We have provided Western blot images with appropriate exposure levels and included quantification where appropriate (i.e., to measure the kinetics of decay rates as in Figure 2C). For all the other immunoblots, we did not include densitometric quantification, given that the semi-quantitative nature of this technique would lead to overinterpretation of our data. This is, unfortunately, a limitation of the technique. In fact, eLife and other similar scientific journals do not adhere to the practice of quantifying Western blot analyses.

(2) Missing information in the graphs in Figure 2C and 4; S2? How many repeats? What are the asterisks?

Panels referenced above have been repeated several times, and further details are now provided in the figure legends.

Reviewer #3 (Recommendations for the authors):

(1) The claim that GMCL1 modulates paclitaxel sensitivity in cancer should be toned down

We agree that it would be an overstatement to claim that GMCL1 regulates paclitaxel sensitivity in cancer patients in a clinical context. The correlations we observed were based on publicly available, cell line-based datasets, which do not fully account for clinical heterogeneity and patient-specific factors. In response to this important point, we have revised our statements and corresponding text accordingly. We now placed greater emphasis on our molecular and cell biology studies.

(2) Additional experiments in low, physiologically relevant concentrations of paclitaxel would be interesting. It is possible that these concentrations activate the mitotic stopwatch in a portion of cells, in addition to inducing cell death due to chromosome loss, activation of an immune response, and chromothripsis. Results should be interpreted in the context of this complexity.

Please see the response to the public review.

(3) It would be helpful to show that CUL3 interacts with 53BP1 only in the presence of GMCL1.

We show that the binding of 53BP1 to GMCL1 is independent of the ability of GMCL1 to bind CUL3 (Figure 1C, D). The binding between 53BP1 and CUL3 is difficult to detect (Figure 1F) likely because it's not direct but mediated by GMCL1.

(4) The GMCL1 "KO" lines appear to still express a low level of GMCL1 (Figure 2A), which should be acknowledged

We have included the GMCL1 mRNA expression data, as measured by RT-PCR, in Supplementary Figure 1G, demonstrating that GMCL1 expression was undetectable under the tested conditions.

(5) Additional description of the methods is warranted. This is particularly true for the database analysis that forms the basis for the claim that GMCL1 overexpression causes

resistance to paclitaxel and other taxanes presented in Figure 3, the methodology used to obtain M-phase cells, and the concentration and duration of taxol treatment.

We have now extensively revised the Methods section.

(6) "Taxol" and "paclitaxel" are used interchangeably throughout the manuscript. Consistency would be preferable.

We have revised the manuscript to maintain consistency in the use of the terms "Taxol" and "paclitaxel" and now refer to "paclitaxel" when discussing that individual compound; "taxanes" when referring collectively to cabazitaxel, docetaxel and paclitaxel; and "Taxol" has been removed entirely to avoid redundancy or confusion.

(7) It is unclear why it is claimed that GMCL1 interacts "specifically" with 53BP1 (line 176) since multiple interactors were identified in the IP-MS study

We meant that the GMCL1 R433A mutant loses its ability to bind 53BP1, suggesting that the GMCL1-53BP1 interaction is not an artifact. We have now clarified the text.

(8) The bottom row in Figure S3 is misleading. Paclitaxel is not uniformly effective in every tumor of any given type, and so resistance occurs in every cancer type.

We fully agree that cancer is highly heterogeneous and that paclitaxel efficacy varies across tumors, even within the same histological subtype. Our intention was not to suggest uniform sensitivity/resistance, but rather to provide a high-level overview using aggregated data. We acknowledge that this coarse-grained representation may unintentionally imply overly generalized conclusions. To avoid potential misinterpretation, we have removed the corresponding panel in the revised paper.

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