Reviewer 1

The authors study the regulation of centrosome disjunction, the result of intercentrosomal linker disassembly. This step of the centrosomal cycle facilitates the physical separation of the centrosomes and the correct formation of the mitotic spindle, and is tightly regulated by phosphorylation. Under the control of epidermal growth factor receptors, the G protein–coupled receptor kinase GRK2 has been shown to phosphorylates Mst2, in turn resulting in the activation of the NIMA kinase Nek2A, the major controller of intercentrosomal linker stability, through a convoluted mechanism that involves the phosphatase PP1 gamma. The authors describe the importance of GRK2 for the temporal regulation of centrosomal disjunction. Their findings suggest that GRK2 is degraded in G2 through the action of Mdm2, an E3 ligase previously described by the authors to be responsible for the ubiquitination of GRK2 upon stimulation of G protein coupled receptors. This degradation is proposed to restrain centrosome disjunction and thus subsequent centrosome separation. Phosphorylation of GRK2 at a specific site, Ser670, is necessary for ubiquitination and degradation of the kinase. This phosphorylation additionally modulates GRK2 activity and specificity, suggesting that GRK2 is tightly regulated in order to properly control centrosome disjunction in G2.

The experiments are well done and clearly presented, with adequate quantifications and statistics when needed. I would agree that they may suggest that the cellular amount of GRK2 has a role controlling centrosome separation and that Ser670 is important for this. Indeed the authors show that interference with GRK2 degradation (through the modulation of Mdm2 activity and the expression of GRK2 Ser670 mutants) results in abnormal centrosome separation. But in my opinion it is difficult to draw conclusions from some of the experiments, and additional data would be needed to grant the acceptance of the paper. *Specifically, a clearer proof that GRK2 is degraded and ubiquitinated specifically in G2 should be produced.*

We appreciate the comments of the reviewer, which have contributed to clarify and improve the manuscript. In particular, the revised version of the manuscript provides new results demonstrating higher endogenous ubiquitination of GRK2 protein in cells that are synchronized in G2. Moreover, the occurrence of such G2-context dependent ubiquitination of GRK2 depends on protein phosphorylation at Ser670. These data are shown in figure 2 as a new panel C.

Major points

● Etoposide is used to arrest cells in G2. As this drug obviously causes DNA damage, it would be desirable to see whether other forms of G2 arrest that do not affect DNA integrity (i.e. CDK1 inhibition by RO-3306) also induce GRK2 degradation (as indeed is suggested by synchronization experiments)

We agree with the reviewer that whenever possible is better to synchronize cells without triggering genotoxic damage, which might influence manifold proteins through activation of the DNA Damage Response pathway. However, we are confident that the decay of GRK2 in cells treated with etoposide is not caused by genotoxic damage *per se*. Using the double thymidine/aphidicolin synchronization method, GRK2 levels decrease by default in G2 (circa 22-44% of reduction in figure 2B, 30-50% reduction in figure 4D and 5A) as previously shown (doi: 10.1073/pnas.0905778107). In this synchronization method, thymidine interrupts the deoxynucleotide metabolism pathway through competitive inhibition, while aphidicolin reversibly inhibits DNA polymerase α without disrupting DNA repair. Thus, arrested DNA replication can be resumed in the absence of genotoxicity. In cells synchronized with etoposide in G2, GRK2 protein levels dropped in a similar way (figure 1A and 2A), pointing out that

regulatory events related to G2 phase but not DNA damage are responsible for triggering GRK2 decay with etoposide. In fact, we have described previously that genotoxic damage upregulates GRK2 instead of decreasing its levels. The genotoxic drug doxorubicin prevents the decay of GRK2 in HeLa cells that are progressing in G2 after being released from a G1/S arrest (doi: 10.1073/pnas.0905778107). Moreover, in asynchronously growing HeLa cells doxorubicin promote accumulation of GRK2 protein in acute or chronic treatments (see Fig. R1-1A for the consideration of the reviewer). Akin to etoposide, doxorubicin inhibits topoisomerase II producing double strand breaks (DSB) but it displays additional mechanisms of action causing DSB and toxicity, thereby precluding its use in cellular synchronization. In this regard, it is reported that DNA lesions produced by doxorubicin persisted and even increased following drug removal, while a complete disappearance of etoposide-induced DNA breaks was noted after drug removal (DOI: 10.1002/ijc.2910450223). Regarding the suggestion of the reviewer of using CDK1 inhibitors for arresting cells in G2 phase, we have rule out this synchronization method because we are aware that Ser670 can be phosphorylated by CDK1 in *in vitro* assays (although less efficiently than cyclinA-Cdk2, the main kinase regulating GRK2 in G2), and the CDK inhibitor olomoucin (selective inhibitor of CDK1/cyclin B (IC_{50} = 7 µM) and related kinases, including CDK2/cyclin A (IC_{50} = 7 µM)) cause a stabilizing effect on GRK2 when added to cells released from a G1/S arrest (Fig R1-1B for the consideration of the reviewer). In short, our available evidence supports the preferential choice of etoposide for G2 synchronization and that effects on GRK2 protein stability are unrelated to genotoxicity. A sentence pointing out this issue is included in the text of the manuscript, line 240.

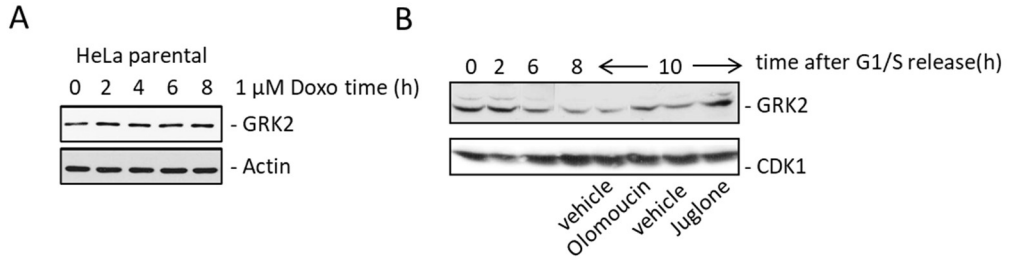


Figure R1-1. GRK2 levels of asynchronously growing HeLa cells in response to genotoxic damage (A) and effect of inhibitor olomoucin (B) on GRK2 downmodulation. Inhibition of CDK 1/2 activity by treatment of cells with olomoucin 2h after release from a G1/S arrest cause stabilization of GRK2 in G2/M, in a similar way as Pin1 inhibition by juglone.

● Figure 1 (and other figures): besides the geminin and H3-P blots, DNA content as assessed by FACS should be shown to demonstrate that cells are indeed in the assigned phases of the cell cycle (ideally combined with H3-P detection to quantify the percentage of mitotic cells in the G2/M peak)

The methods described in the manuscript for G2 and G1/S synchronization are very well documented and previously optimized for our cellular models (doi: 10.1073/pnas.0905778107). Routinely, we have detected upregulation of geminin to confirm cells in G2 phase. Geminin levels oscillate through the cell cycle, accumulating from S phase to G2 and being rapidly degraded during mitosis with very low levels at the end of this phase (doi: 10.1074/jbc.M105406200). Moreover, in prometaphase geminin is phosphorylated by Aurora A which results in a band shift (doi: 10.1038/ncomms2859) which provides additional information for assign the cell cycle phase. We acknowledge that flow cytometry could have been a valuable

approach to further confirm synchronization of cells in G2 by assessing 4N DNA content. Unfortunately, it is not possible to re-evaluate by flow cytometry the extent of G2 synchronization as cells were processed for obtaining cellular lysates. However, following the reviewer's indication, to corroborate that cells are indeed synchronized in G2, we have assessed additional synchronization markers where there was sufficient material left over for immunoblot detection. In figure 3D, detection of phosphorylated histone H3 (p-His3) was added to the panel. This marker accumulates from late G2 phase to mitosis. As expected, levels of p-His3 were low at G1/S transition and in S phase but they start to increase after 6h post-release from G1/S arrest with the concurrent geminin upregulation, which together allows to identify the cells in late G2 phase.

● GRK2 degradation seems to coincide with the peak of H3 phosphorylation, usually happening in mitosis, not in G2. Comment.

We appreciate the importance this reviewer gives to this observation. Indeed, GRK2 protein levels are actively down-modulated at the G2/M transition and during mitosis when levels of Mdm2 fall down (doi: 10.1016/s0014-5793(03)00502-7), pointing that other ligases involved in GRK2 modulation may be engaged during the cell cycle, a notion stressed by the fact that GRK2 is still degraded at the onset of mitosis in the absence of Mdm2 (Figure 1B) as mentioned in the manuscript text (lines 245-246). We are actively investigating the occurrence of additional ligases involved in the downmodulation of GRK2 in mitosis. These ongoing results reveal a multilayered degradation pathway with several ligases acting independently but sequentially to keep at bay GRK2 from G2 to mitosis for regulation of different processes. The description of such additional mechanisms are part of a different manuscript now in preparation.

● Figure 1C should be part of 1B and show an example of the western blots quantified. I fail to see them in the supplementary materials.

As indicated by the reviewer, we have put together panels B and C of the original Figure 1 as a new panel 1B in the revised manuscript with the incorporation of representative blots of pCDK1 quantification.

● Why is the western in Figure 2A cut? The data (Figure S2) seem to show almost no variation in the levels of GRK2 in parental cells.

In the representative blot of panel 2A, the asynchronous condition was performed in duplicate only for parental cells and one of the lanes was eliminated for homogeneity with the other cell types. For the sake of clarity, we have included a new representative blot comparing endogenous versus overexpressing wild-type GRK2 cells, which more clearly shows downregulation of GRK2 protein levels in the parental etoposide-treated condition.

● Figure 2B, the western blot does not seem to support the quantification

Co-immunoprecipitation of GRK2 with Pin1 is technically difficult because of the dynamic interactions of Pin1 and the high background signal resulting from the use of capture antibody (anti-Pin1 rabbit polyclonal antibody of Upstate Biotechnology) and detection antibody (anti-GRK2 rabbit polyclonal antibody of Santa Cruz) of the same species. We have probed other antibodies but without positive results. We have changed the blot for one over-exposed to improve detection of endogenous GRK2 protein bound to Pin1.

● I fail to see ubiquitinated GRK2 in figure 2C. Several strong bands and a smear at high MW are observed in the ubiquitin western in the lane that lacks GRK2. A more clear experiment should be presented

Specific poly-ubiquitination of GRK2 is marked with a dashed box around 250kDa in the blot of figure 2C (which correspond now to figure 2D in the revised version of the manuscript). The smear detected with an anti-ubiquitin antibody is sharp and intense in the presence of GRK2 plus Mdm2, wild-type ubiquitin and E2 enzyme, while in the presence of GRK2-S670A mutant the smear is markedly reduced to those levels found with only Mdm2, which is known to be auto-ubiquitinated. These results indicate that only wild-type GRK2 is modified by Mdm2 over the basal signal due to ligase auto-ubiquitination. We show for the consideration of the reviewer a similar in vitro ubiquitination assay immunoblotted with an anti-ubiquitin antibody and reblotted with a mouse monoclonal antibody c5/1.1 for GRK2 detection (Fig R1-2). Compared to Mdm2 alone the ubiquitin antibody reveals a polyubiquitination smear where Mdm2 is present and a more intense smear when GRK2 is added, while GRK2 antibody detects species of high molecular weight upon incubation of GRK2 with Mdm2, but not in the absence of Mdm2 or with the presence of Mdm2 alone. These results confirm the occurrence of a specific GRK2 polyubiquitination driven by Mdm2

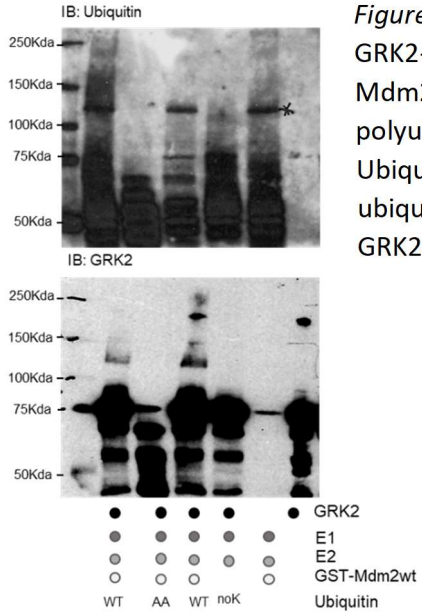


Figure R1-2. In vitro ubiquitination driven by Mdm2. Recombinant GRK2-wt was incubated in the presence of E1 and E2 protein, GST-Mdm2 and purified ubiquitin-wt, no-K unable to form polyubiquitin chains, and AA unable to be conjugated. Ubiquitination was detected by immunoblotting with an anti-ubiquitin antibody and by GRK2 band shift detected with anti-GRK2 antibody.

● Is proteasome inhibition recovering GRK2 amounts in G2? does it result in an accumulation of high molecular weight ubiquitinated GRK2 forms?

We provide solid evidence that GRK2 protein decay in G2 depends on Mdm2, a well-known ligase that ubiquitinates and enables proteasome-dependent degradation of GRK2 in physiological settings (doi: 10.1172/jci.insight.95998; doi: 10.1007/s00395-019-0730-5) and in response to GPCR activation in different cellular contexts (doi: 10.1038/sj.emboj.7601351). As suggested by the reviewer, proteasome inhibitors are expected to stabilize proteins targeted to this protease in a ubiquitin-dependent manner, leading to increased levels of protein ubiquitination. However, these inhibitors prevent degradation of the bulk of cellular proteasome substrates, including many cell cycle regulators that orchestrate G2 progression, thereby making difficult to discriminate whether lack of GRK2 decay might be due to protection from proteasome in G2 or disruption of the G2-specific regulatory events that instruct GRK2

degradation. In addition, ubiquitinated protein accumulation in cells as result of proteasome inhibition might be hardly detected as cellular deubiquitinases are very abundant and active. Therefore, we have performed an alternative approach to trap endogenous poly-ubiquitinated proteins in G2 with TUBEs, a molecular entity that not only display high affinity for ubiquitination but also protect ubiquitinated proteins from both de-ubiquitination and proteasome-mediated degradation. As shown in the new panel C of figure 2, poly-ubiquitinated GRK2 was markedly increased in G2-synchronized HeLa cells that express extra wild-type GRK2 compared to asynchronous cells. Conversely, polyubiquitination of GRK2-S670A mutant was barely detected in G2 despite similar levels of bound polyubiquitinated proteins in both Hela-WT5 and Hela-A1 cells, confirming the pivotal role of S670 phosphorylation in GRK2 ubiquitination by Mdm2 noted in *in vitro* ubiquitination assays and pointing out that Mdm2 fails to ubiquitinate phosphorylation defective-S670 GRK2 protein in G2 phase.

● Figure 3C, 4D, if geminin "specifically accumulates in G2" (page 5), why does the figure show higher levels of the protein 0.5 hours post-G1/S release?

Our apologies for this sentence and the misunderstanding of the message we wanted to transmit that upregulation of geminin taking from S phase to mitosis is used as a marker for the confirmation of cells in G2 phase when synchronization protocols for G2 arrest or G2 enrichment are performed, and geminin protein does not undergo mobility shifts. It has been reported that in prometaphase geminin is phosphorylated by Aurora A which results in a band shift compared to the electrophoretic position in asynchronous or G2 conditions (doi: 10.1038/ncomms2859). We have more clearly mentioned this aspect in the text, line 217-219. The main function of geminin is to block pre-replication complex by means of binding to Cdt1 for the prevention of endoreplication, and geminin begins to appear in the nucleus at the initial phase of the S phase (doi: 10.1126/science.290.5500.2309). In the thymidine/aphidicolin method cells arrested at G1/S transition have already initiated the S phase, so a higher level of geminin is expected compared to asynchronous cells as reported by others (doi: 10.1074/jbc.M105406200).

● Besides FACS data an additional manner to show convincingly that cells are in G2 in Figure 3 (and 4) would be to co-stain cells with anti-cyclin B

Following the suggestion of the reviewer, we have detected the expression level of phosphorylated Histone 3 in cellular lysates of Figure 3. Geminin upregulation is present from the beginning of S phase until early M phase, while phosphorylated Histone 3 rise from late G2 to the end of mitosis. Dual detection of geminin and phosphorylated Histone 3 allows to assign cells more precisely to late G2 phase during progression after G1/S release as shown in figure 3C.

Figure 4: I fail to see how Mdm2 inhibition could fail to interfere with centrosome separation in an asynchronously growing culture (where only cells in G2 would separate the centrosomes) but do so in cells synchronously going through G2.

Respectfully, maybe the reviewer has misunderstood cellular experimental conditions for determining centrosome separation in figure 4. As detailed in figure legend, HeLa cells from panel A to panel D were synchronized at G1/S transition and released for G2 progression in the presence of EGF with or without the Mdm2 inhibitor HLI373. In panel A, cells were sorted according to separation (≥2µm) or non-separation (<2µm) of centrosomes, while in panel B distances of cells with separated centrosomes were plotted for each cell condition. An explicit mention to cell synchronization was added in the text introducing results of figure 4, line 387-388.

●Figures 4B and 4C should use the same type of graphic

We would like to draw your attention to the different category of data displayed in panel B and panel C of Figure 4. The former shows inter-centrosomal distances of individual cells in a column scatter plot with median values and standard deviations. Panel C illustrate numerical proportion of whole cells (100%) represented in panels A and B sorted by ranges of centrosome separation. Respectfully, we believe that graphs types in panel A and B are adequate for the nature of data plotted in each case.

● Figure 4 should show that the cells are indeed in G2 (or any other phase of the cell cycle, asynchronously growing in 4A, if I understood correctly). This is specially important here to discard the possibility that HLI373 affects cell cycle progression and distribution

We agree with the reviewer that it is important to rule out that HLI373 alters cell cycle progression in G2, which *per se* could be enough to distort centrosome separation independently of its effect on GRK2 degradation by Mdm2. So far, the only effect of Mdm2 on cell cycle progression has to do with G1 arrest in wild type p53 contexts and stimulation of multiple rounds of DNA replication when Mdm2 is overexpressed (Deb SW. Mol Cancer Res. 2003 Dec;1(14):1009-16). Levels are cell-cycle regulated and MDM2 protein is accumulated from S phase to G2 with a pattern similar to that of cyclin B1 but MDM2 protein decays before (doi: 10.1016/s0014-5793(03)00502-7), which may play a role in cell cycle progression. The ring domain of the ligase but not the ligase activity is required for cell cycle-dependent regulation of Mdm2 protein and S phase entry (doi: 10.1016/s0014-5793(03)00502-7). Therefore, it is not expected that HLI373 alters Mdm2 dynamics or cell cycle progression in S phase/G2 in our cell models that are functionally deficient in p53 (p53 knock-out MEFs and HeLa cells).

Minor comments

● line 43: What is the "S-M linker"? Do the authors mean the Daughter-Mother linker?

The *S-M* linker refers to the connection between the procentriole formed (daughter) adjacent to a pre-existing centriole (mother) during centriole duplication and is maintained from S phase (*S*) to Mitosis (*M*).

● line 216: "Levels of Geminin, a protein that specifically accumulates in G2", a reference should be provided.

Sentence has been modified to read "Levels of Geminin, a protein that accumulates from S phase to G2 and maintained during mitosis in which it shows a phosphorylation-dependent band shift" and proper references have been incorporated in line 219 of the revised version: [36] Wohlschlegel JA, Dwyer BT, Dhar SK, Cvetic C, Walter JC, et al. (2000) Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science 290: 2309; [37] Aurora-A controls pre-replicative complex assembly and DNA replication by stabilizing geminin in mitosis. Tsunematsu T, Takihara Y, Ishimaru N, Pagano M, Takata T, Kudo Y. Nat Commun. 2013;4:1885.

● The observed degradation of GRK2 in G2 is not total (possibly ~25% diminution in total amounts). The authors could comment on how such a limited change in the amount of an enzyme that by definition acts catalytically could result in a physiological response (i.e. is GRK2 specifically degraded in G2 at the centrosome?).

We thank the reviewer for raising this interesting point. We agree that catalytic functions of kinases might be only modestly affected by variations of ~25% in their overall protein levels.

However, the “cellular and molecular context” in which protein fluctuations take place might strongly amplify their consequences on kinase activity. As suggested by the reviewer, it is possible that most of the GRK2 protein targeted by Mdm2 resides at the centrosome. Such local degradation would allow higher reductions of GRK2 protein, which would readily affect the phosphorylation of the pool of Mst2 localized in centrosomes. A comment about this point has been incorporated in the Discussion section, text lines 576-586.

- Figure 4C would benefit from arrows indicating where the centrosomes are.

Thanks for this suggestion for the sake of clarity. Arrows have been added to point out representative centrosomes.