RIPK1 and Caspase-8 Ensure Chromosome Stability Independently of Their Role in Cell Death and Inflammation

Highlights
- A RIPK1/FADD/Caspase-8/cFLIP complex (rioptosome) forms during mitosis
- The mitotic kinase PLK1 binds to RIPK1 and is recruited into mitotic ripoptosomes
- PLK1 is cleaved by Caspase-8 in a RIPK1-dependent manner
- Deletion and inhibition of RIPK1 or Caspase-8 lead to chromosomal instability

In Brief
Liccardi et al. show that ripoptosome complexes assemble during physiological mitosis and regulate PLK1 activity via RIPK1-dependent recruitment and Caspase-8-mediated cleavage. These results reveal a direct role for RIPK1 and Caspase-8 in controlling chromosome segregation during mitosis.

Authors
Gianmaria Liccardi, Laura Ramos Garcia, Tencho Tenev, ..., Manolis Pasparakis, Katiuschia Bianchi, Pascal Meier

Correspondence
pmeier@icr.ac.uk

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RIPK1 and Caspase-8 Ensure Chromosome Stability Independently of Their Role in Cell Death and Inflammation

Gianmaria Liccardi, Laura Ramos Garcia, Tencho Tenev, Alessandro Annibaldi, Arnaud J. Legrand, David Robertson, Rebecca Feltham, Maurice Darding, Nieves Peltzer, Marius Dannappel, Hannah Schünke, Luca L. Fava, Manuel D. Haschka, Timo Glatter, Alexander Schmidt, Philip A. Harris, John Bertin, Peter J. Gough, Andreas Villunger, John Silke, Manolis Pasparakis, Katiuscia Bianchi, and Pascal Meier

1The Breast Cancer Now Toby Robins Research Centre, Institute of Cancer Research, Mary-Jean Mitchell Green Building, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, UK
2The Walter and Eliza Hall Institute, 1G Royal Parade, Parkville, Victoria 3052, Australia
3Centre for Cell Death, Cancer, and Inflammation (CCCI), UCL Cancer Institute, University College, London WC1E 6BT, UK
4Institute for Genetics, Centre for Molecular Medicine (CMMC) and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases (CECAD), University of Cologne, 50931 Cologne, Germany
5Division of Dev. Immunology, Biocenter, Medical University of Innsbruck, Innsbruck, A-6020, Austria
6Proteomics Core Facility, Biocentrum of the University of Basel, Basel, Switzerland
7Max Planck Institute for Terrestrial Microbiology, Karl-von-Frisch Str. 10, 35043 Marburg, Germany
8Department of Pathology, Department of Computational Medicine & Bioinformatics, University of Michigan, Ann Arbor, MI, USA
9Pattern Recognition Receptor Discovery Performance Unit, Immuno-Inflammation Therapeutic Area, GlaxoSmithKline, Collegeville, PA 19426, USA
10Tyrolean Cancer Research Institute, A-6020 Innsbruck, Austria
11Barts Cancer Institute, Queen Mary, John Vane Science Centre, University of London, Charterhouse Square, London EC1M 6BQ, UK
12Senior author
13Lead Contact
1Correspondence: pmeier@icr.ac.uk
https://doi.org/10.1016/j.molcel.2018.11.010

SUMMARY

Receptor-interacting protein kinase (RIPK) 1 functions as a key mediator of tissue homeostasis via formation of Caspase-8 activating ripoptosome complexes, positively and negatively regulating apoptosis, necroptosis, and inflammation. Here, we report an unanticipated cell-death- and inflammation-independent function of RIPK1 and Caspase-8, promoting faithful chromosome alignment in mitosis and thereby ensuring genome stability. We find that ripoptosome complexes progressively form as cells enter mitosis, peaking at metaphase and disassembling as cells exit mitosis. Genetic deletion and mitosis-specific inhibition of Ripk1 or Caspase-8 results in chromosome alignment defects independently of MLKL. We found that Polo-like kinase 1 (PLK1) is recruited into mitotic ripoptosomes, where PLK1’s activity is controlled via RIPK1-dependent recruitment and Caspase-8-mediated cleavage. A fine balance of ripoptosome assembly is required as deregulated ripoptosome activity modulates PLK1-dependent phosphorylation of downstream effectors, such as BUBR1. Our data suggest that ripoptosome-mediated regulation of PLK1 contributes to faithful chromosome segregation during mitosis.

INTRODUCTION

RIPK1 functions as a critical signaling node in various innate immune pathways (Rickard et al., 2014; Takahashi et al., 2014; Weinlich and Green, 2014). Its activity is subject to tight regulation, most notably by phosphorylation, ubiquitylation, and Caspase-8 (Casp8)-mediated cleavage. Proper regulation of the kinase activity and scaffolding function of RIPK1 is crucial, as deregulation of RIPK1 is associated with various human pathologies (Silke et al., 2015).

Following its activation, RIPK1 associates with the endopeptidase Casp8 and cellular FLICE-like inhibitor protein (cFLIP), through the adaptor molecule Fas-associated protein with Death Domain (FADD) to form a RIPK1-based Casp8 activating complex, variably referred to as complex-IIb (Petersen et al., 2007; Wang et al., 2008), ripoptosome (Tenev et al., 2011), or necrosome when the RIP kinase family member RIPK3 is also recruited to this complex (Pasparakis and Vandenabeele, 2015). For simplicity, we will subsequently refer to this RIPK1/FADD/Casp8/cFLIP multiprotein complex as “ripoptosome.” Formation of the ripoptosome frequently occurs under physiological conditions without killing the cell, generating a burst of sub-lethal caspase activity (Boatright et al., 2004; Micheau et al., 2002) that...
causes cleavage of RIPK1 and RIPK3 and destabilization of ripoptosome complexes (Feng et al., 2007; Oberst et al., 2011). IAP-mediated ubiquitylation of RIPK1 and Casp8 represents a further control point that restricts the lethal potential of RIPK1 and the ripoptosome (Tenev et al., 2011).

Beyond its role in regulating cell death in some cell types, Casp8 also fulfills non-cell-death functions (Maelaid and Beyaert, 2008). For example, evidence exists that indicates a possible tumor suppressor function of Casp8. Accordingly, deficiency of Casp8 facilitates cellular transformation in vitro (Krelin et al., 2008), acts as driver mutation in breast cancer (Stephens et al., 2012) and B cell lymphoma (Hakem et al., 2012), and is frequently found to be mutated in hepatocellular carcinomas (Soung et al., 2005b) and advanced gastric cancer (Soung et al., 2005a). Further, loss of Casp8 expression is associated with human neuroblastomas with N-Myc amplification (Teitz et al., 2000), small-cell lung carcinoma (Hopkins-Donaldson et al., 2003), and relapsed glioblastoma multiforme (Martinez et al., 2007). Moreover, Casp8 reportedly is essential for maintaining chromosomal stability (Hakem et al., 2012), independent of its role in cell death. Despite these data, compelling evidence is lacking to support a direct causal role for Casp8 inactivation in the generation of cancer chromosomal instability.

By studying why Casp8 is essential for maintaining chromosomal stability, we identified RIPK1 and Casp8 (rioptosome complexes) as negative regulators of polo-like kinase 1 (PLK1), a key kinase that regulates chromosomal segregation, spindle assembly checkpoint, and maintenance of genomic integrity (Medema et al., 2011; Zitouni et al., 2014). We noticed that ripoptosome complexes form physiologically during mitosis and that active PLK1 is recruited into these complexes by RIPK1. Upon its recruitment, PLK1 is cleaved at D457 by Casp8, similarly to other ripoptosome components. In the absence of Ripk1, active PLK1 accumulates, resulting in excessive phosphorylation of BUBR1 and mitotic defects. Conversely, enhancing ripoptosome formation during mitosis suppresses the interaction of PLK1 with its substrate BUBR1, resulting in hypo-phosphorylation of BUBR1 and defects in chromosome congression and segregation. Collectively, our data demonstrate that deregulation of key ripoptosome components causes chromosomal instability, independently of their role in cell death and inflammation. Our data provide a likely explanation as to why loss of Casp8 and low levels of Ripk1 can be driver mutations in certain types of cancer, leading to chromosome instability that may favor tumor evolution, heterogeneity, acquisition of drug resistance, and heightened risk for tumor relapse.

RESULTS

The Ripoptosome Assembles during Physiological Mitosis

Immunoprecipitation of Casp8 from cells in different stages of the cell cycle revealed that RIPK1, FADD, Casp8, and cFLIP associated during mitosis of HT1080, primary MEFs, and HT29 cells, suggesting that the ripoptosome can form during mitosis (Figures 1A–1C and S1A). To visualize ripoptosome complexes in their native state in intact cells, we utilized in situ proximity ligation assay (PLA) to detect RIPK1/Casp8 complexes (Orme et al., 2016). While ripoptosome formation was undetectable in G2, ripoptosome complexes steadily formed as cells entered mitosis (prophase), peaking at metaphase and declining as cells exited M-phase (Figure 1D). Although TRADD can also activate Casp8 (Anderton et al., 2018; Wang et al., 2008), we found no evidence for TRADD/Casp8 complexes during mitosis (Figure 1E). Additional PLA controls are provided in Figures S1B and S1C.

Ripoptosome complexes also formed in M-phases of unperturbed, non-synchronized cells (Figure S1D), indicating that this complex transiently forms during normal mitosis. PLA analysis detected mitosis-specific association of RIPK3/FADD, RIPK3/Ripk1, and RIPK3/Casp8 in MEFs (Figure 1F) and HT29 cells (Figure 1G) and confirmed cFLIP/Casp8 interactions in mitotic cells (Figures 1H and S1I). All antibodies were tested for PLA signal specificity (Figures S1E–S1I). Ripoptosome formation during mitosis also occurred in a panel of additional cell types, implying that ripoptosome formation during mitosis is a general phenomenon (Figure 1I). Using 3D reconstruction, RIPK1/Casp8 complexes appeared to be distributed throughout the nucleoplasm and not associated with the spindle, centromeres, or kinetochores of mitotic cells (Figure S1J), which was in agreement with RIPK1- and Casp8-staining during mitosis (Figure S1K).

Consistent with the formation of an active ripoptosome in mitosis, cells in M-phase showed significantly higher levels of caspase activity than cells in other cell cycle phases (Figures 1J and S1L). Mitotic caspase activity was Casp8- and RIPK1-dependent as RNAi-mediated depletion of Casp8, or treatment with the RIPK1 kinase inhibitor Nec-1, suppressed this caspase activity (Figures 1J and 1K). Similarly, expression of the viral Casp8 inhibitor CrmA, or treatment with the caspase inhibitor zVAD-FMK, also suppressed caspase activity in M-phase (Figures 1J and S1L). Despite measurable levels of caspase activity, mitotic cells remained fully viable (Figure S1M, and data not shown). Consistently, mitotic cells were “unprimed” to die by a
mitochondria-dependent form of apoptosis (Figure S1N). Further, RNAi-mediated depletion of cFLIP suppressed the caspase activity in M-phase, indicating the non-killing activity of this complex (Figure S1O). RNAi-mediated depletion of Tnfr1, Trail-r1/r2, and CD95/Fas did not prevent formation of Casp8/RIPK1 complexes (Figure S1P). Under the same condition, RNAi-mediated depletion of these death receptors suppressed cell death mediated by their respective ligands (Figure S1Q). Together, these data indicate that RIPK1/FADD/Casp8/cFLIP form a complex during mitosis (mitotic ripoptosome), generating a pulse of sub-lethal caspase-8 activity that is dependent on cFLIP and RIPK1 kinase activity.

**PLK1 Is Recruited to RIPK1 and the Ripoptosome**

To gain further insights into the mitotic ripoptosome, we undertook a proteomic-based approach using RIPK1 as affinity agent. In addition to identifying known interactors of RIPK1, the mass spectrometry experiment also identified PLK1 as a putative binding partner of RIPK1 (Figure S2A). Under the same conditions, PLK1 was not co-purified with GFP, LacZ, FADD, Casp8, and cFLIP (data not shown), implying that the identification of PLK1 is not due to nonspecific interaction.

Reciprocal immunoprecipitation of endogenous RIPK1 and PLK1 from cells in different stages of the cell cycle revealed that PLK1 and RIPK1 associated during mitosis (Figure 2A). Immunoprecipitation of endogenous Casp8, RIPK1, and FADD from mitotic cells also co-purified PLK1 along with other components of the ripoptosome (Figures 2B and 2C). No such interaction with PLK1 was observed outside of mitosis. While wild-type and the constitutively active form of PLK1 (PLK1-T210D) bound to RIPK1, the PBD domain of PLK1 in isolation failed to bind to RIPK1, suggesting that PLK1 binds to RIPK1 independently of PBD-mediated phospho-recognition (Figure 2D). Recruitment of PLK1 to ripoptosome complexes was strictly RIPK1-dependent, as PLK1 failed to be co-purified by Casp8 in the absence of RIPK1 (Figure 2E). Interestingly, both FADD and cFLIP associated with Casp8 independently of RIPK1, suggesting that RIPK1 does not nucleate or trigger this complex during mitosis. RIPK1 mutants, lacking the RHIM or DD domain, readily associated with PLK1 (Figure S2B), indicating that RIPK1 can bind to PLK1 independently of FADD, caspase-8, or RIPK3.

The interaction between RIPK1 and PLK1 in mitosis was also detectable using PLA with antibodies against RIPK1 and total PLK1 or phospho-specific PLK1 (anti-PLK1-pT210) (Figures S2C–S2F). Mitosis-specific interaction between RIPK1 and PLK1, as well as RIPK3 and PLK1, was also detected in HT29 and primary MEFs (Figures 2F and 2G), expanding this observation to additional cell types. RNAi-mediated depletion of Tnfr1, Trail-r1/r2, and CD95/Fas did not interfere with the formation of PLK1/RIPK1 complexes (Figure S2G). Together, our data demonstrate that PLK1, RIPK1, RIPK3, FADD, cFLIP, and Casp8 form ripoptosome complexes in mitosis.

**The Ripoptosome Restrains PLK1 in Mitosis**

Casp8 can cleave RIPK1, RIPK3, and cFLIP within ripoptosome complexes (Oberst et al., 2011). Since PLK1 is also recruited to such complexes during mitosis, we tested whether Casp8 cleaves PLK1 in a RIPK1-dependent manner (Figure 3A). Using CDK1i-synchronized and released primary Ripk1<sup>−/−</sup> intestinal organoids, we noticed that RIPK1 and PLK1 were readily cleaved (Figure 3B). PLK1 cleavage was RIPK1-dependent, as conditional ablation of Ripk1 caused accumulation of full-length PLK1 while diminishing the cleaved form. Ripk1 deletion also resulted in enrichment of active PLK1 (PLK1-pT210). Similar results were observed in synchronized Mouse Dermal-Fibroblasts (MDFs) where expression of RIPK1 was ablated by CRISPR/Cas9 (Figure S3A). Depletion of Casp8 resulted in a stronger PLK1/RIPK1 PLA signal (Figure 3C), suggesting that Casp8 negatively regulates PLK1/RIPK1 interactions.

To gain further insights on how Casp8 regulates PLK1, we mapped the Casp8 cleavage site of PLK1. To this end, we purified N- or C-terminally HA-tagged PLK1 from cells (Figure S3B) and offered it to recombinant Casp8 in presence or absence of zVAD-fmk. This revealed the presence of a Casp8 cleavage site (Figures 3D and 3E). Mutagenesis of putative cleavage sites revealed that Casp8 cleaves PLK1 at D457, which is surface exposed and evolutionarily conserved from flies to human (Figures 3D, 3E, and S3B). Together, these data suggest that recruitment of PLK1 to ripoptosome complexes induces Casp8-mediated PLK1 cleavage in a RIPK1-dependent manner and that active PLK1, similarly to RIPK1, is regulated via Casp8-mediated proteolysis.

To further study how the activity and size of mitotic ripoptosome affect PLK1, we decided to utilize pharmacological compounds known to modulate the activity and formation of the ripoptosome (Figure S3C) (Silke and Meier, 2013; Tenev et al., 2011). Pharmacological inhibition of IAPs (via SMAC mimetics, SM), like treatment with the caspase inhibitor QVD, resulted in an increase in ripoptosome formation during mitosis, which was accompanied with enhanced PLK1 recruitment (Figures S3D–S3M).
Co-treatment with the RIPK1 inhibitor GSK'963 (Berger et al., 2015) (subsequently referred to as RIPK1i) normalized complex formation (Figures 3F and 3G). While treatment with SM or QVD diminished the recruitment of active PLK1 (pPLK1-T210) into ripoptosome complexes, co-treatment with RIPK1i normalized this interaction (Figures 3F and S3D–S3G). Interestingly, treatment with the RIPK1i alone significantly reduced the association of RIPK1 and PLK1 to the complex, albeit it did not completely block their recruitment to Casp8 (Figure 3F). While the immunoprecipitation in Figure 3F appears to contradict the PLA-based results (Figure 3G), it should be noted that PLA only assesses whether RIPK1 is in proximity to Casp8 but cannot assess how many molecules of RIPK1 are in proximity to Casp8. Given that the same number of PLA-speckles is formed following release into DMSO and RIPK1i, but significantly more RIPK1 co-purifies with caspase-8 under IP/Western conditions, we conclude that the RIPK1 inhibitor suppresses the “chaining” of RIPK1 within the mitotic ripoptosome but does not affect its nucleation. The significant reduction of PLK1 is in line with our observation that recruitment of PLK1 is dependent (and directly proportional) to the recruitment of RIPK1 (Figure 2E). Together, our data are consistent with the notion that enhanced ripoptosome formation negatively impacts PLK1.

The Ripoptosome Negatively Regulates PLK1-Mediated Phosphorylation of its Downstream Substrates

To test whether the ripoptosome negatively regulates PLK1-mediated phosphorylation of its downstream substrates, we monitored the phosphorylation status of BUBR1 (Elowe et al., 2010). PLK1-mediated phosphorylation of BUBR1 is required for chromosome congression and the bipolar spindle attachment that forms the metaphase plate (Suijkerbuijk et al., 2012). We noticed that treatment with SM and QVD caused a dramatic loss of BUBR1 phosphorylation at T680 (Figures 4A, S4A, and S4B). SM and QVD reduced phosphorylation at T680 of BUBR1 to a similar extent as treatment with PLK1i, or following Pkt1 siRNA (Figures S4A and S4B). Importantly, co-treatment with RIPK1i normalized BUBR1 phosphorylation at T680 (Figures 4A and S4B). Consistent with our observed reduction in BUBR1 phosphorylation, we found that treatment with SM caused reduced binding of PLK1 to BUBR1 (Figure 4B). While treatment with SM reduced PLK1-BUBR1 interactions, co-treatment with RIPK1i restored the binding of PLK1 to BUBR1 to control levels. Similar results were obtained by PLA analysis (Figures 4C, 4D, S4C, and S4D). Since the levels of BUBR1 were unchanged following treatment with the respective inhibitors (or inhibitor combinations) (Figures 4A and S4C), our data suggest that deregulation of the ripoptosome interferes with PLK1-mediated interaction and phosphorylation of BUBR1. Consistently, we found that the level of BUBR1 phosphorylation was dramatically increased upon RNAi-mediated depletion of RIPK1 (Figures 4E and 4F). Although ripoptosome modulation affects PLK1-mediated phosphorylation of BUBR1, BUBR1 is unlikely to be the only PLK1 substrate that is affected. This is because treatment with SM and QVD caused mis-localization of PLK1 and abnormal mitosis (Figure 4G). Together, our data demonstrate that enhanced ripoptosome formation negatively impacts PLK1’s ability to phosphorylate downstream substrates such as BUBR1.

Acute Pharmacological Modulation of RIPK1 or Casp8 Activity, Specifically during Mitosis, Results in Chromosome Alignment Defects

We next tested whether RIPK1i-mediated deregulation of PLK1 results in mitosis defects. First, we evaluated mitotic timing via live-cell imaging. While there was no significant difference in the length of mitosis of cells treated with DMSO, SM, or SM/ RIPK1i (Figure 5A; Videos S1, S2, and S3), careful analysis of individual frames revealed that SM-treated cells presented accumulation of chromosomal defects during both metaphase and/or anaphase (Figures 5B, 5C, and 5S; Videos S1, S2, and S3). Interestingly, these defects were completely rescued by co-treatment with RIPK1i, which is in agreement with our observation that RIPK1i restores defects in BUBR1-pT680 phosphorylation upon SM treatment (Figures 4A, 4B, and 4D–4G).

To gain further insights on the type of chromosomal abnormalities, we evaluated fixed mitotic cells released into media containing MG132, which allows accurate evaluation of chromosome segregation defects (Janssen et al., 2009) (Figure 5D). Microscopic images of mitotic HT1080 and RPE-1 cells, treated with SM, QVD, zVAD, or RIPK1i, revealed a significantly higher number of chromosome alignment defects at the metaphase plate and abnormalities at anaphase (Figures 5E, 5F, and 5S; SSB–SSF). Importantly, the SM- and QVD/zVAD-mediated chromosomal abnormalities were fully rescued upon co-treatment with two different RIPK1 kinase inhibitors, RIPK1i and Nec-1s (Figures 5E, 5F, and 5S; SSB–SSF). In agreement with our data from HT1080 cells (Figure 5G), analysis of synchronized RPE-1 cells also showed that ripoptosome modulation caused the appearance of multipolar spindles (Figure 5H). This is consistent with a role of PLK1 in regulating Kizuna, TPX2, chTOG, TACC3, and clathrin (Maiato and Logarinho, 2014). Despite the abundance of such abnormalities, cells seemed to be able to complete

Figure 3. RIPK1 Negatively Regulates PLK1
(A) Schematic representation of RIPK1- and Casp8-mediated regulation of PLK1.
(B) Immunoblots of primary intestinal organoids from two Ripk1fl/fl,IEC-creERTM animals. Organoids were treated with ETOH (vehicle control) or 4-OHT, synchronized with CDK1i, and released into media. Cells were lysed and analyzed by immunoblotting with the indicated antibodies.
(C) In situ PLA detection of PLK1 and RIPK1 in HT1080 cells, following Casp8 or Pkt1 siRNA. Green dots indicate PLA signals between PLK1 and RIPK1. Graph shows quantifications of RIPK1/PLK1 PLA speckles (mean ± SD). 15 cells were counted for each condition. Scale bars: 10 μm.
(D and E) In vitro cleavage assay. Purified HA-tagged PLK1 construct and recombinant Casp8 were incubated for 1 h. Immuno blot analysis using the indicated antibodies is shown.
(F) HT1080 cells were synchronized with CDK1i and released into media containing the indicated drugs. Lysates from asynchronous or synchronized HT1080 cells were immunoprecipitated with anti-Casp8 antibody. Immunoblot analysis using the indicated antibodies is shown.
(G) In situ PLA detection of Casp8/RIPK1 in CDK1i-synchronized and released HT1080 cells treated with the indicated agents. Green dots indicate PLA speckles. Scale bars: 10 μm.
mitosis (on average at the same time), though in some cases without achieving daughter cell separation. Nevertheless, this had significant consequences as treatment with SM, QVD, or RIPK1i led to a significant drop in clonogenic potential, which was restored upon co-treatment with the RIPK1 inhibitors RIPK1i and Nec-1s (Figures 5G and S5I). No such effect was observed when cells were acutely (2 hr) treated with these compounds outside of M-phase (Figure S5J). Together, these data strongly suggest that RIPK1 and Casp8 contribute to proper chromosome dynamics during mitosis and that their deregulation can result in chromosome alignment defects.

**Cells from Ripk1 and Casp8 Knockout Animals Exhibit Chromosome Alignment Defects**

Next, we wished to characterize cells from available knockout animals to determine whether genetic deletion of ripoptosome components results in chromosome alignment defects. To this end, we assessed chromosomal segregation defects in synchronized primary MEFs derived from WT, Casp8fl/fl RosaCreER (Casp8−/− MEFs were obtained following treatment with Tamoxifen), Mlkl−/−, and Casp8/Mlkl double-knockout (DKO) mice using a blinded-experimental design (Figures 6A–6C). Knockout cell lines were validated functionally and via western blot (Figures S6A–S6E). Our data showed that 63% of Casp8−/− mitotic cells had chromosome segregation defects (Figure 6B). Importantly, co-deletion of Mlkl did not rescue these defects, as 64% of Casp8/Mlkl DKO MEFS exhibited chromosome alignment defects (Figure 6B). This demonstrates that the alignment defects of primary Casp8−/− MEFS occur independently of the necrotic program. Intriguingly, similar to Casp8−/− MEFS, 66% of primary Ripk1−/− MEFS displayed chromosome alignment defects (Figure 6C). Since Ripk1−/− and Casp8−/− cells exhibited comparable levels of chromosome alignment defects, these data suggest that RIPK1 and Casp8 together contribute to proper chromosome alignment. The observed chromosome alignment defects were also evident in conditional Ripk1tamEIC-KO intestinal organoids (Figures 6D and S6F–S6J) but were absent in ETOH-treated WT organoids. Importantly, 4-OHT-treated Cre control organoids also did not present any abnormalities, indicating that the observed chromosomal defects in Ripk1tamEIC-KO were due to the conditional deletion of Ripk1 (Figures 6D and S6G–S6J). Like genetic deletion, RNAi-mediated knockdown of Ripk1 or Casp8 also caused chromosome misalignment in primary MEFs and human HT1080 cells (Figures 6E, S6K, S6L, and data not shown). Knockdown efficiency was determined functionally (Figure S6K) and biochemically (Figure S6L). Interestingly, co-knockdown of Casp8 and Ripk1 rescued chromosome alignment defects in primary MEFs (Figure 6E), which is in agreement with our pharmacological study using QVD and RIPK1i. Primary MEFs derived from Ripk1K45A knockin mice showed modest chromosome alignment defects compared to control. However, these were numerically lower than those observed in Ripk1−/− (Figure 6F).

Chromosome alignment defects were also apparent in tissue sections derived from Casp8−/− (64%) (E10.5), Casp8/Mlkl DKO (50%) (E13.5), Ripk1−/− (large intestine 78%, liver 49%, and skin 55%) (E19.5), and Ripk1/Tnfr1 DKO (large intestine 64%, liver 49%, and skin 55%) (E19.5) but were absent in tissue sections from Ripk1/Casp8 DKO (E19.5) embryos or Mlkl−/− (E13.5) embryos (Figures 6G, 6H, and 6I). Together, this demonstrates that RIPK1 and Casp8 are involved in regulating chromosome segregation, independently of their role in inhibiting necroptosis or inflammation. While 78% of mitotic cells in the large intestine of Ripk1−/− embryos had chromosome segregation defects, co-deletion of Casp8 completely rescued the Ripk1−/− segregation defects at E19.5 (Figure 6I). In contrast, co-deletion of Tnfr1 did not rescue the alignment defects of Ripk1−/− animals, even though it did rescue caspase-3 activation and apoptosis in the large intestine and liver (Figure S6M; Rickard et al., 2014). This demonstrates that cell death and chromosome alignment defects can be uncoupled genetically and provides further evidence for a non-cell death function of RIPK1 and the ripoptosome in mitosis.

**Ripk1 mRNA Levels Correlate with Aneuploidy in Human Cancers**

Chromosomal mis-segregation defects are known to induce aneuploidy, which in turn has been shown to correlate with tumor onset and progression, but also therapeutic treatment (Bakhroun and Swanton, 2014). Recently, a score of aneuploidy was calculated for most tumor samples, accessible by The Cancer Genome Atlas (TCGA) (Taylor et al., 2018), allowing us to correlate gene expression to aneuploidy. Our results clearly indicate that low expression of RIPK1 mRNA is significantly associated with higher aneuploidy in breast, lung, and colorectal carcinomas (Figures 7A, 7B, and 7A). Our analysis excluded samples bearing TP53 mutations (Taylor et al., 2018) and copy number variations of the RIPK1 gene. Also, we utilized CENP-A

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Figure 4. RIPK1 Negatively Regulates PLK1-Mediated Phosphorylation of BUBR1

(A) Scheme illustrating how mitotic ripoptosome interacts and modulates PLK1 and how pharmacological inhibition regulates such interaction and downstream substrate activation. Immunofluorescence analysis using anti-BUBR1 or anti-BUBR1-pT680 antibodies. HT1080 cells were synchronized with CDK1i and released into media containing the indicated agents. Scale bars: 10 μm.

(B) HT1080 cells were synchronized with CDK1i and released. Lysates from asynchronous or synchronized HT1080 cells were immunoprecipitated with anti-PLK1 antibody. Immunoblot analysis using the indicated antibodies is shown.

(C and D) In situ PLA detection of PLK1/BUBR1 (C) or PLK1/BUBR1-pT680 (D) in synchronized HT1080 cells, treated with the indicated agents. Scale bars: 10 μm.

(E) Immunofluorescence analysis using anti-BUBR1-pT680 antibodies (under extraction conditions) in CDK1i-synchronized HT1080 (left) and HT29 (right) cells under the indicated RNAi conditions. Cells were released for 30 min, after which MG132 was added for 90 min to arrest cells in metaphase. N.T. indicates non-targeting RNAi Control oligos. Scale bars: 10 μm.

(F) Western blot analysis of phosphorylated BUBR1 following knockdown of Control (Ctrl), Ripk1, or Plk1 in CDK1i-synchronized and released HT1080 cells.

(G) CDK1i-synchronized HT1080 cells were released into media containing the indicated agents. Cells were released for 30 min, after which MG132 was added for 90 min to arrest cells in metaphase. Only cells presenting mitotic abnormalities were scored for PLK1 localization. Images show representative examples of PLK1 mis-localization. Scale bars: 10 μm.
Experimental setting for colony formation assay:

- HT1080
- Release into media containing DMSO or compound
- Synchronization: CDK1i
- Miotic shake and drug washout
- Seeding of 1000 mitotic cells
- 10 days

Colony formation assay:

- % of Control (Fold change, M.U.)
- N.S.

(legend on next page)
(centromeric histone) and PLK1 as positive controls (Taylor et al., 2018). As expected, tumors with high levels of CENP-A or PLK1 displayed higher aneuploidy (Figures 7C, S7B, and S7C). Interestingly, a low ratio between normalized expression of RIPK1 and PLK1 mRNA levels showed an association with higher aneuploidy (Figure 7D). Together, these data suggest that a combination of low RIPK1 and high PLK1 might be associated with chromosomal mis-segregation and long-term aneuploidy in human cancers.

**DISCUSSION**

RIPK1 and Caspase-8 are key players in inflammation, cell death, and the regulation of tissue homeostasis, positively and negatively regulating these processes depending on the cellular context (Pasparakis and Vandenabeele, 2015). Beyond their role in inflammation and cell death, we find that RIPK1 and Casp8 also play critical roles in mitosis.

Several lines of evidence demonstrate that RIPK1 and Casp8 contribute to normal mitosis. First, in mouse and human, the ripoptosome naturally forms during mitosis, peaking at metaphases and disassembling as cells exit M-phase. Consistently, mitotic cells harbor sub-lethal levels of caspase activity that is dependent on RIPK1, Casp8, and cFLIP. Second, PLK1 is incorporated into the ripoptosome specifically in mitosis, an observation that is consistent with previous reports demonstrating an interaction between PLK1 and Casp8 (Matthess et al., 2014) and FADD (Jang et al., 2011). Like for RIPK1 and RIPK3, recruitment of PLK1 to the ripoptosome results in Casp8-mediated cleavage. Third, ripoptosome-mediated regulation of PLK1 fine-tunes the localization and activity of PLK1. Downregulation and hyper-activation of ripoptosome cause defects in PLK1 localization, PLK1-BUBR1 interaction, and loss of PLK1-mediated phosphorylation of BUBR1 (and most likely other PLK1 substrates), resulting in mitotic defects. Fourth, genetic deficiency or mitosis-specific pharmacological inhibition of ripoptosome components causes severe chromosomal alignment defects. These defects occur independently of cell death or inflammation. This is evident as Casp8/Plk1 DKO mice still present chromosomal instability, despite rescuing necroptotic cell death. Likewise, Ripk1/Tnfr1 DKO animals, which are fully viable at E19.5, harbor significant levels of chromosome alignment defects in various tissues in the absence of caspase-3 activation. Hyperformation of mitotic ripoptosomes, following SM treatment, may possibly inhibit PLK1 function by a two-pronged mechanism: (1) via proteolytic cleavage of PLK1 within ripoptosome complexes and (2) by recruitment of PLK1 into ripoptosome complexes and sequestration away from its substrates. Together, these reduce the availability of active PLK1 to phosphorylate important downstream substrates required for chromosome alignment and congression. Consistent with a role of sequestration, Casp8 knockdown enhances the recruitment of PLK1 to RIPK1. Conversely, loss of RIPK1 de-represses PLK1, leading to substrate hyper-phosphorylation. Our current model proposes that PLK1 is recruited into the ripoptosome complexes via RIPK1 and that RIPK1 negatively regulates PLK1. This interaction is further regulated by Casp8, which cleaves not only RIPK1 but also PLK1 at D457. The cleavage of RIPK1 is required to regulate the recruitment of PLK1 and keep its activity under tight regulation. Upon loss of Casp8 or IAP depletion, the RIPK1-PLK1 association is enhanced leading to sequestration and inactivation of PLK1, ultimately interfering with PLK1-mediated phosphorylation of BUBR1. Under this condition, deregulated RIPK1 might bind to PLK1 and sequester it away from the spindle or chromosomes. On the other hand, when RIPK1 is missing, Casp8-mediated cleavage of PLK1 is lost (Figures 3B and S3A), leading to accumulation of active PLK1. However, this alone would not explain why co-deletion of Ripk1/Casp8 normalizes the chromosomal instability observed in absence of either Casp8 or RIPK1. It is possible that, in the absence of Ripk1, Casp8 might (indirectly) lead to PLK1 over-activation, potentially by cleaving and inactivating a protein involved in PLK1 regulation at the spindle. Hence, concomitant loss of both RIPK1 and Casp8 would not only prevent RIPK1-induced inhibition of PLK1 but also Casp8-mediated over-activation of PLK1, leaving levels of PLK1 intact. Mice with a Ripk1 kinase-dead knock-in-allele (Ripk1<sup>*K45A</sup>) develop normally (Berger et al., 2014), even though primary MEFs of such animals do have some chromosome alignment defects, although such defects are very modest when compared to Ripk1<sup>−/−</sup>. Similar chromosome alignment defects, as well as aneuploidy, are also seen in Balb/cJ<sup>Bubr1<sup>−/−</sub></sup> mice, yet such mice are fertile and die of old age and present only splenomegaly and abnormal megakaryopoiesis (Baker et al., 2004; Kapanidou et al., 2015). The tumorigenic phenotype due to this lingering chromosomal instability is manifested only upon challenge with...
Statistical analysis was performed via the two-way ANOVA multiple comparison analysis with *p < 0.05, **p < 0.01, ***p < 0.001. Total amount of abnormalities was considered in determining statistical significance. Scale bars: 10 µm.

Intriguingly, the mitotic ripoptosome seems to differ from other situations where ripoptosome/complex-II/necrosome complexes are formed. While depletion of cIAPs, activation of cytokine, or pattern recognition receptors results in RIPK1-driven formation of RIPK1/FADD/Casp8/cFLIP complexes, during mitosis the formation of this complex seems to be driven by either FADD or Casp8. This is evident as FADD/Casp8 complexes form independently of RIPK1. Further, while RIPK1 kinase inhibition blocks “canonical” ripoptosome formation, treatment with RIPK1i only prevents chaining of RIPK1 onto the mitotic ripoptosome without affecting the nucleation of the complex. This suggests an important difference between kinase activity of RIPK1 and scaffolding function of RIPK1, both equally important for the regulation of PLK1. This is especially highlighted by the fact that loss of RIPK1 kinase activity in animals only induces low levels of chromosome instability, a phenotype somewhat intermediate to the loss of RIPK1.

Our findings that the ripoptosome contributes to mitosis are consistent with earlier reports demonstrating that Casp8 is essential for maintaining chromosome stability, suppressing consistent with earlier reports demonstrating that Casp8 is essential for maintaining chromosome stability, suppressing
B cell lymphomagenesis (Hakem et al., 2012), restraining oncogene-induced transformation (Krelin et al., 2008), and acting as driver mutation in breast cancer (Stephens et al., 2012); also, it is frequently found to be mutated or lost in different cancer types (Hopkins-Donaldson et al., 2003; Martinez et al., 2007; Soung et al., 2005b). This, together with our analysis that mRNA levels of RIPK1 are associated with aneuploidy in breast, lung, and colon cancer, strongly suggests that Casp8- and RIPK1-mediated regulation of PLK1 is required for the maintenance of chromosome stability, consequent aneuploidy, and suppression of cancer (at least in the B cell lineage for Casp8).

Our observations have important ramifications, as they may help to explain why Casp8 is frequently lost in several kinds of human tumors, including small-cell lung carcinoma, neuroblastoma, hepatocellular carcinoma, and others. Interestingly, Casp8 deficiency occurs by multiple mechanisms, including hyper-methylation of regulatory regions in the Casp8 gene or (less frequently) gene mutation, suggesting that it is a causal factor in the oncogenic transformation rather than a consequence of it. Consistently, our data suggest that loss of Casp8 and subsequent deregulation of RIPK1 would provide cells with evolvability (chromosomal instability), leading to establishment of cellular transformation and tumorigenesis.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and three videos and can be found with this article online at https://doi.org/10.1016/j.molcel.2018.11.010.

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AUTHOR CONTRIBUTIONS

G.L. planned, performed and analyzed experiments shown in Figures 1, 2, 3, 4, 5, 6, S1, S2B–S2F, S3B–S3F, S4, S5, and S6. L.R.G. performed experiments shown in Figures S1E–S1I, S1P–S1Q, and S2F. K.B. and M.D. planned and performed preliminary experiments that led to the initial observations of this study. T.T. created tools to conduct this study and performed experiments shown in Figures 3D, 3E, and S3B. A.J.L. performed experiments shown in Figure 7 and S7. L.L.F. and A.V. provided important insights during the duration of this study. A.A. isolated primary WT MEFs for the knockdown studies. M.D. and M.P. designed and supervised Figure S6F and together with H.S. the isolation and synchronization of Ripk1flox/flox intestinal organoids. D.R. helped with imaging setup of organoids and of the histology slides. R.F. prepared the histology slides for Figure 6L. R.F. and H.A. performed the experiment in Figure S6M. J.S. provided the KO animals for Figure 6L. N.P. prepared the embryo blocks for Figure 6G and provided stained slides for Figure 6H. J.B., P.A.H., P.J.G. provided the Ripk1i GSK’963. T.G., A.N., and A.S. performed the mass spec analysis of Figure S2A. G.L. and P.M. designed and supervised the study and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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**STAR METHODS**

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-RIPK1 (C-terminal) – for IP western and PLA | BD Bioscience | 610459 |
| Anti-PLK1 for Human WB, PLA, IF | Bethyl Laboratories | A300-251A |
| Anti-PLK1 for Mouse WB | Abcam | Ab178666 |
| Anti-pPLK1-T210 for IF | Abcam | A8150095 |
| Anti-Actin for WB | Santa Cruz Biotechnology | sc-1615 |
| Anti-BUBR1 for WB, IF, PLA | BD bioscience | 8242 |
| Anti-BUBR1-pT680 for IF | Gert Kops | N/A |
| Anti-BUBR1-pS676 WB | Erich A. Nigg | sc-371 |
| Anti-p-H-H3 for WB | Millipore | 06-570 |
| Anti-cFLIP WB PLA | Adipogene | AG-20B-0056 |
| Anti-MYC for IP and WB | SIGMA | M5546 |
| Anti-RIPK3 – Human WB PLA | Novus Biological | NBP2-24588 |
| Anti-RIPK3 – Mouse WB PLA | ProSci Inc | 2283 |
| Anti-FADD – for IP and PLA | Santa Cruz Biotechnology | sc-6036 |
| Anti-FADD – for WB | BD | 610400 |
| Anti-RIPK1 (N-terminal) WB PLA | Cell Signaling | 3493 |
| Anti-phalloidin633 IF | Invitrogen | A22284 |
| Anti-alpha-Tubulin IF | Serotec | mca78g |
| Anti-CASPASE-8 - for WB - post IP Human | MBL | M032-3 |
| Anti-CASPASE-8 - for IP [C-20] Human | Santa Cruz Biotechnology | sc-6136 |
| Anti-HA For IP | Roche | 11867423001 |
| Anti-HSP90 WB | Santa Cruz Biotechnology | sc-7947 |
| Anti-PLK1 for Mouse WB and PLA | AB178666 |
| Anti-Casp8 for Mouse PLA | Santa Cruz Biotechnology | SC7890 |
| Anti-Crest for IF | Kind Gift of Stephan Geley | N/A |
| Donkey anti-rat 488 | Invitrogen | A21208 |
| Donkey anti-rat 633 | Biotium | 20137 |
| Goat anti-human 555 | Invitrogen | A21433 |
| Goat anti-rabbit 488 | Invitrogen | A11034 |
| Donkey anti-mouse 488 | Invitrogen | A21202 |
| Goat anti-rabbit 633 | Invitrogen | A21071 |
| Anti-alpha–tubulin 488 | Invitrogen | 322588 |
| Anti-goat plus | SIGMA | DUO 82003 |
| Anti-mouse minus | SIGMA | DUO 82004 |
| Anti-mouse plus | SIGMA | DUO 92004 |
| Anti-rabbit plus | SIGMA | DUO 82002 |
| Anti-Rabbit Minus | SIGMA | DUO 82005 |

**Chemicals, Peptides, and Recombinant Proteins**

| Mouse recombinant TNF | Enzo Life Sciences | ALX-522-009-C050 |
| GSK’963 (RIPK1 inhibitor) | Gift from X. Wang | N/A |
| Complete EDTA-free protease inhibitor tablets | Roche | 11873580001 |
| Halt Protease and phosphatase inhibitor | Thermo Scientific | 78443 |

(Continued on next page)
Continued

| REAGENT or RESOURCE SOURCE IDENTIFIER | RESOURCE SOURCE | IDENTIFIER |
|--------------------------------------|-----------------|------------|
| QVD Apex Bio A1901                  |                 |            |
| zVAD-FMK Apex Bio A1902              |                 |            |
| Protein G Sepharose Sigma            |                 | P3296      |
| Dapi Invitrogen DB571                 |                 |            |
| Propidium iodide solution Sigma      |                 | P4864      |
| Ac-DEVD-AMC Sigma A1086              |                 |            |
| MG132 Sigma C2211                    |                 |            |
| CDK1i (RO3306) Tocris 4181           |                 |            |
| Bim Peptide Kind Gift of Tony Letai  |                 | N/A        |
| Nec1 Merck CAS-4311-880              |                 |            |
| Nec-1s Biovision 2263                 |                 |            |
| Recombinant Casp-8 Enzo AxAI-201-062-0100 |             |            |
| Prolong Gold Invitrogen P36934       |                 |            |
| SirDNA Spirochrome SC007             |                 |            |
| h-TNF Enzo Life Sciences ALX-804-034-C050 |           |            |

Critical Commercial Assays

| Critical Commercial Assays | Source |
|---------------------------|--------|
| Duolink In Situ Detection Reagents Green | Sigma DUO92014 |

Deposited Data

| Deposited Data | Source |
|---------------|--------|
| Raw data http://doi:10.17632/x8tvmp6rfb.1 | N/A |

Experimental Models: Cell lines

| Experimental Models: Cell lines | Source |
|-------------------------------|--------|
| Kym1 Gift from John Silke (Melbourne, Australia) | N/A |
| HT1080 ATCC CCL-121            |        |
| MDA-MB-231 In house           | N/A    |
| HEK293T In house              | N/A    |
| Flp-InTM-T-RExTM-HEK293        | Termo Scientific R78007 |
| HT29 In house                 | N/A    |
| RPE-1 In house                | N/A    |
| U2OS In house                 | N/A    |
| Primary MEFs In house         | N/A    |
| T47D In house                 | N/A    |
| Primary MEFs Ripk1+/+         | Gift from Manolis Pasparakis N/A |
| Primary MEFs Ripk1-/-         | Gift from Manolis Pasparakis N/A |
| Primary MEFs Casp8+/+         | Gift From Andrew Oberst N/A |
| Primary MEFs Casp8-/- Mirk-/- | Gift From Andrew Oberst N/A |
| MDFs In house                 | N/A    |
| MDFs Ripk3-/-                 | In house N/A |
| Intestinal organoids Ripk1+/+Tnfr1+/+IEC-creERTM | Gift from Manolis Pasparakis N/A |
| MEFs Ripk1+/-iK144A           | In house N/A |

Experimental Models: Organisms/Strains

| Experimental Models: Organisms/Strains | Source |
|----------------------------------------|--------|
| Mouse: C57BL/6 Ripk1-/-                | Gift from John Silke N/A |
| Mouse: C57BL/6 Ripk1+/+ Tnfr1+/+       | Gift from John Silke N/A |
| Mouse: C57BL/6 Mirk+/+                 | Gift from Henning Walczak N/A |
| Mouse: C57BL/6 Ripk1+/+ Casp8-/-       | Gift from Henning Walczak N/A |
| Mouse: C57BL/6 Ripk1-/+ Tnfr1--        | Gift from John Silke N/A |

Recombinant DNA

| Recombinant DNA | Source |
|-----------------|--------|
| pcDNA3           | Thermo Scientific V79020 |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Pascal Meier (p.meier@icr.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Experimental Model

All animal procedures were conducted in accordance with the guidelines of The Walter and Eliza Hall Institute Animal Ethics Committee, UK home office in accordance with the revised (2013) Animals (Scientific Procedures) Act (ASPA) and the institutional guidelines of the UCL Cancer Institute, or European, national and institutional guidelines approved by the local government authorities of the Landesamt fuer Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, Germany.

Cell lines

Cell lines were obtained from ATCC (American Type Culture Collection). HT1080, RPE-1, KYM1, MDA-MB-231, T47D, ZR75, primary MEFs (passage number 2), HEK293T, U20S were cultured in DMEM. Culture media were supplemented with 10% fetal bovine serum (GIBCO), penicillin, and streptomycin. Cells were cultured under 10% CO2.

METHOD DETAILS

Isolation of Primary cells

Primary Mouse Embryonic Fibroblasts (MEFs) were generated from E13.5 embryos. After removing the placenta, yolk sac, head and the dark red organs, embryos were finely minced and digested for 20 min in 0.25% trypsin. Single cell suspension was then obtained by pipetting up and down the digested embryos. Mouse Dermal Fibroblasts (MDFs) were isolated as described in (Etemadi et al., 2015). To generate Bone Marrow Derived Macrophages (BMDMs), bone marrow cells from tibia and femur of 2 month old mice were seeded in non-coated Petri dishes and cultured for 6 days in Dulbecco’s modified Eagle medium + 10% fetal bovine serum + 20% (v/v) L929 mouse fibroblast conditioned medium. Keratinocytes were isolated as described in (Lichti et al., 2008). Splenocytes were isolated from 2 month old mice. Mouse spleens were mashed through a cell strainer into the Petri dish using the plunger end of a syringe. Cells were then washed once in cold PBS and treated with 1X Red Blood Cell Lysis Buffer (BioLegend, Cat N 420301) for 5 min on ice. Cells were then washed again in PBS and counted.

Isolation of primary intestinal organoids

Small intestine crypts were isolated and grown as described previously (Dannappel et al., 2014). Deletion of Ripk1 in organoid cultures from Ripk1fl/fl,IEC-creERTM (RIPK1tamIEC-KO) was induced with 100 nM 4-OHT for 24 hr. Ripk1fl/fl,IEC-creERTM treated with ETOH for the duration of 24 hr were used as controls. To synchronize organoid cultures, cells were treated with 9 mM CDK1i for 20 hr, after which cells were released using 3x washes with media. 10 min after release, organoid cultures were supplemented with 20 μM MG132 for 90 min. Chromosome misalignment was assessed 48 hr and 72 hr after 4-OHT treatment. For western blot analysis: intestinal organoids were harvested, and passed through a 23 G syringe. Cells were centrifuged at 10000 rpm for 5 min. Pelleted organoids were resuspended in 80 μl RIPA buffer (20 mM HEPES, pH 7.6, 350 mM NaCl, 1 mM MgCl2, 0.5 mM EDTA, 20% glycerol) supplemented with protease and phosphatase inhibitor tablets (Roche).

Reagents and Antibodies

The following reagents were used: zVAD-FMK (10 μM, Apex Bio), QVD (10 μM, Apex Bio), SM-164 (100 nM, gift from Shaomeng Wang), GSK’963 (referred as RIPK1i, 100 nM, GlaxoSmithKline), Nec-1 (10 μM Merck), Nec-1s (10 μM Merck), RO-3306 (9 μM, Merck), Thymidine (Sigma), BIM peptide (Kind gift of Tony Letai), FLAG-hTNF (10 ng/ml, Enzo), MG132 (1-20 μM, as indicated per cell line, see below, SIGMA), Recombinant Casp8 (1U Enzo). The following antibodies were used for western blotting: α-RIPK1 (1:1000, BD Biosciences), α-HA (1:1000, Roche), α-PLK1 (1:1000, Bethyl Laboratories), α-PLK1-pT210 (1:2000, AbCam), α-Cyclin
**RNA Interference, Transfections**

siRNAs were purchased from QIAGEN and Dharmacon. siRNA sequence information can be obtained upon request. All siRNA assays were performed using a total of 50 nM – 100 nM of siRNA. Upon multiple siRNAs combination, each siRNA was used at 25 nM and control siRNA was utilized to balance siRNA concentrations. All siRNA transfections were performed using DharmaFECT4 transfection reagent (GE Healthcare) and Opti-MEM (Life Technologies) according to manufacturer’s protocol. All siRNA transfections were performed using retro transfection and left for 40 hr from the time of transfection to induce knockdown. For overexpression studies, 5 × 10⁵ cells were plated, and the indicated constructs were transfected a day after according to the Genejuice transfection protocol (Merck Millipore). Cells were harvested 24 hr after transfection.

For the generation of CRISPR cells. Guide RNAs (gRNAs) were designed utilizing the website tool: www.crispr.tefor.net. Guide RNAs were cloned in pLC-mCherry vector, expressing Cas9. MDFs were seeded in a 6-well plate and transfected with 2 ug of CRISPR vector using Viromer yellow transfection reagent (Cambridge Bioscience) according to the manufacturer’s instructions. Three days later, cells were sorted using fluorescence-activated cell sorting (FACS), and single clones were isolated and screened for the deletion of RIPK1. Positive clones were characterized. Guide sequence utilized to delete RIPK1 is available upon request.

**Cell synchronization**

Cells were treated with 9 μM of CDK1i (RO-3306) for 20 hr. Inhibitor was washed out by 3 washes using full media, and cells were released in media containing DMSO (Ctrl) or media containing the indicated drugs. Asynchronized cells: cells not treated with CDK1i; G2 population: cells collected following 20 hr treatment with CDK1i; mitotic population: cells collected by shake off after a 2 hr release; non-M population: cells left at the bottom of the plate following mitotic shake off; G1 cells: cells collected 16 hr after release. For the scoring of chromosome misalignment, cells were released into media containing either DMSO or the indicated drugs for 30 minutes. After 30 min, cells were treated with 1 μM MG132 (HT1080), 10 μM MG132 (RPE-1 and primary MEFs) for 90 min. The addition of MG132 was added to assess the misalignments at metaphase plate (Janssen et al., 2009; Potapova et al., 2006) Cells were then fixed and processed as indicated above. Organoids were plated and treated with 4-OHT for 24 hr to allow deletion of Ripk1. 4-OHT was removed and cells were treated with CDK1i for 20 hr. Following 3x washes with media, organoids were released into media for 10 min. After 10 min, cells were treated with 20 μM MG132 for 90 min. Organoids were fixed for IF or lysed for western blotting. For the clonogenic assay, cells were synchronized with CDK1i for 20 hr, and released into media containing either DMSO or the indicated drugs. 30 min following release, cells were treated with MG132 for 90 minutes. Mitotic cells were collected by shake off, counted, and re-plated in media containing no drugs. For the clonogenic assay of non-M populations, cells were synchronized with CDK1i for 20 hr, then released into media for 16 hr. Subsequently, cells were treated for 2 hr with the indicated drugs. 30 min following treatment, media containing drugs was supplemented with MG132 for an additional 90 min. Cells were then trypsinised, counted, and re-plated for clonogenic growth in media containing no drugs.

**Histology**

E19.5 embryos were fixed in 10% neutral buffered formalin, paraffin embedded. E10.5 embryos were fixed in methanol, and agarose embedded. Embryos were then sectioned for routine histology staining (H&E). Slides were visualized and scored with a X100 objective. For immunofluorescent staining paraffin sections were dewaxed, subjected to heat-induced epitope retrieval with citrate buffer then blocked and permeabilised with 1% BSA and 0.3% Triton X-100 (for anti-CC3) or blocke in IFF buffer and permeabilised in 0.5% Triton X-100 (for anti-alpha Tubulin and DAPI). Images were taken using a DP72 microscope and cellSens Standard software (Olympus) for CC3 or with Zeiss LSM 710 for alpha Tubulin and DAPI.

**Immunofluorescence**

1×10⁵ cells were plated on 13 mm glass coverslips (VWR) and retro-transfected with siRNA or treated as indicated. Cells were then fixed in 4% PFA for 10 min. Following 10 min permeabilisation with 1x PBS, 0.5% Triton X-100, cells were blocked for 1 hr in 1x PBS, 5% BSA. Respective primary antibodies were diluted in 1x PBS, 1% BSA overnight at 4°C. These were: -PLK1 (1:200 for PLA; 1:1000 for IF, for mouse PLA-Abcam 1:50), -PLK1-pT210 (1:200 for DuoLink; 1:2000 for IF), -BUBR1 (1:50 PLA, 1:1000 IF), -BUBR1-pT680 (1:1000), -BUBR1-pT676 (1:1000), -FADD (1:50 for PLA), -RIPK3 (1:50 PLA 1:1000 for IF), -Casp8 (1:1000, for organoids), DAPI (Invitrogen), -human CREST serum (1:2000, kind gift from Stephan Geley), -Phallodin-633 (Invitrogen). Primary antibodies were washed 3x with 1x PBS, 0.1% Triton X-100 for 10 min. Secondary Alexa fluor-conjugated antibodies were diluted in 1x PBS, 1% BSA, and incubated for 1 hr at room temperature. These were: donkey -rat-488 (1:1000, Invitrogen), donkey -rat-633 (1:1000, Biotium), goat -human-555 (1:1000, Invitrogen), goat -rabbit-488 (1:1000, Invitrogen), goat -mouse-488 (1:1000, Biotium), goat -rabbit-633 (1:1000, Invitrogen). Secondary antibodies were washed 3x...
with 1x PBS, 0.1% Triton X-100 for 10 min. DAPI was added to one of the three washes. Each slide was then washed with sterile water and mounted using Prolong Gold anti-fade fluorescent mounting media (Invitrogen). Organoids were fixed with 4% PFA for 1 hr at 37°C. These were then washed 3x in 1x PBS, and permeabilised for 1 hr using 1x PBS, 0.5% Triton X-100. Cells were blocked for 2 hr using IFF blocking buffer (1x PBS, 1% BSA, 2% FCS). Primary antibodies were diluted in IFF and left incubating overnight at 4°C, and then washed 3x with 1x PBS. 0.1% Triton X-100 for 30 min. Secondary fluorescent Alexa Fluor-conjugated antibodies were diluted in IFF buffer, left for 4 hr at room temperature, and washed 3x with 1x PBS, 0.1% Triton X-100 for 20 min. DAPI was added to each of these washes. The organoids chambers were further washed once in 1x PBS and once in H2O. Stained slides and organoids were visualized using the LSM710 Zeiss microscope, objective 40x or 63x Zeiss. Images were acquired by sequential scanning.

**Live cell imaging**

1x10^5 HT1080 cells were seeded into 12-well plates (Porvoir), equilibrated into Leibovitz 15 (Life Technologies) medium supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin. Cells were incubated with 10 nM SIR-DNA and after treatments with the indicated drugs, cells were imaged every 6 min by advanced spinning disc confocal microscopy (Zeiss Axio Observer Z1, CSU-W1 T2 Spinning Disc Confocal, 50 μm Disk, Yokogama, Incubation from Oko laboratories with Temperature and Manual CO_2 Control, Hamamatsu ORCA-Flash4.0 V2+ sCMOS Camera - USB3.0/30fps 1 with 82% peak QE, cooled to 37°C and mounted using Prolong Gold anti-fade fluorescent mounting media (Invitrogen). Organoids were fixed with 4% PFA for 1 hr at 37°C and mounted using Prolong Gold anti-fade fluorescent mounting media (Invitrogen). DNA Plasmids

PLK1 cDNA constructs were purchased from ADDgene. All constructs used for transient transfection experiments were cloned into the pcDNA3 expression vector (Invitrogen) and sequence verified.

**Caspase activity assays (DEVDase)**

1x10^4 cells were plated in 96-well plates and retro siRNA transfection was performed for 40 hr. For the caspase activity assays during prolonged mitosis, cells were plated in 96-well plates and treated as indicated for the indicated time points. After treatment, medium was removed and 20 μL of DISC lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol) was added to each well. Pellets were washed in 1x PBS and lysed in DISC lysis buffer. Plates were placed at –80°C to aid cell lysis. Plates were thawed at room temperature for 15 min, after which 180 μL DEVDase assay mix (20 μM Ac-DEVD-AMC (Sigma), 1 mM DTT, 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 5% glycerol) was added to each well (NB: cell lysates were not cleared). The plates were wrapped in foil and the reaction was incubated at room temperature for up to 24 hr. DEVDase activity was read at 380 nm excitation/460 nm emission. For Mitotic population, cells were collected by mitotic shake off, then centrifuged for 5 min at 2000 rpm. These were then lysed in DISC lysis buffer. All the other samples were trypsinised and cells were collected by centrifugation for 5 min at 2000 rpm as for the mitotic population. Following addition of lysis buffer, samples were incubated at –80°C to aid cell lysis. 50 μL of each samples was added to a 24-well plate and 450 μL of DEVDase assay mix was added to each well. Caspase activity was then measured as indicated above. The remaining of these samples was boiled and protein amounts were quantified. These were then utilized to normalize the individual caspase activity of each sample.

**In vitro caspase cleavage assay**

Recombinant Casp8 (2 U) and/or PLK1 (1 μg) were incubated in caspase cleavage buffer (50 mM HEPES, pH 7.2, 50 mM sodium chloride, 0.1% CHAPS, 10 mM EDTA, 5% Glycerol, 10 mM DTT) for 1 and 2 hr at 37°C. Samples were then diluted in 2X sample buffer and analyzed by western blotting.
Cell Death assay (FACS)
Cell mixtures were incubated with medium containing 1 µg/ml propidium iodide and analyzed by FACS using a plate reader. Data shown are from 5000–10000 cells per condition. For cell viability of parental and Bcl-2 overexpressing HT1080 cells were assessed using a high-content imaging system (ImageXpress Micro-XL, Molecular Devices). Cells were plated in 96-well thin bottom plates in triplicate wells, and treated as indicated. A 20x long distance objective was used to image 16 sites in each well. Values were averaged for the 16 sites for each well, and shown are the mean ± SEM of 3 wells for each condition. Before imaging cells were loaded with 40 nM SYTOX Green nucleic acid stain (ThermoFisher Scientific), 1 µg/mL Hoechst 33342 (Sigma) for 30 min. The dyes were present during imaging. Images were analyzed using the Multiwavelength Cell Scoring module of the MetaXpress software (Molecular Devices). Cells were identified based on Hoechst staining, then nuclear SYTOX Green intensity was scored to identify cells with permeabilised plasma-membrane (SYTOX positive cells). Scoring was based on the size of area above threshold, determined by intact, non-treated cells.

Clonogenic Assays
1000 cells were left for approximately 10 days in medium. Cells were fixed in 4% PFA/PBS for 10 min and stained with crystal violet/ PBS for 20 min. Colonies were counted and recorded using ImageJ.

Immuno-Precipitation Assays
Cells were lysed in DISC lysis buffer (20 mM Tris-HCL pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10% glycerol) supplemented with protease and phosphatases inhibitors. Cell lysates were left in the −80°C to aid cell lysis for 20 min, then rotated at 4°C until thawed. Cell lysates were collected by centrifugation at 4°C at 14000 rpm for 15 min. 20 µL of G agarose (SIGMA); 7.5 µL of Casp8 antibody/mg protein lysis (Santa Cruz) or 5 µL of Myc antibody/mg of protein lysis, were rotated with cleared protein lysates overnight at 4°C. 5x washes in wash buffer (1x PBS, 1% Triton X-100, 1 mM EDTA, 5% glycerol) were performed, and samples eluted by boiling for 5 min in 60 µL of 2x SDS loading dye.

Proximity Ligation Assay
1x10⁵ cells were plated on a 13 mm glass coverslip (VWR) and retro-transfected with siRNA as indicated. Cells were then synchronized as described above and fixed in 4% PFA for 10 min. Following 10 min permeabilisation with 1x PBS, 0.5% Triton X-100, cells were blocked for 1 hr in 1x PBS, 5% BSA. Respective pair of primary antibodies were then added as indicated. Proximity ligation was performed according to the manufacturer’s instructions using the Duolink Detection Kit (Cambridge BioScience Ltd, Cambridge UK). Ligation and amplification reactions were carried out according to manufacturer’s instructions. Cy3 signal amplification was utilized for the assay. Following PLA, cells were immune-blocked with x-rat α-tubulin (Serotec) diluted in PBS 1% BSA for 1 hr. Following three washes with 1x PBS, 0.1% Triton X-100, slides were incubated with donkey x-rat alexa fluor-633 diluted in 1x PBS, 1% BSA for 1 hr. Secondary antibody was washed three time with 1x PBS, 0.1% Triton X-100, and stained with DAPI for 10 min. Each slide was then washed with sterile water and mounted using Prolong Gold anti-fade fluorescent mounting media (Invitrogen) and left to air dry overnight. Cells were examined with a confocal microscope (objective 40x, Zeiss LSM 710).

Mass Spectrometry
Prior to mass spectrometry analysis of RIPK1 interactors, eluted protein complexes were digested with Trypsin and peptides were purified using C18 Microspin columns (Harvard Apparatus) according to the manufactures instruction. LC-MS/MS analysis was performed on a dual pressure LTQ-Orbitrap mass spectrometer (Thermo Scientific), which was connected to an electrospray ion source (Thermo Scientific). Peptide separation was carried out using an easy nano-LC systems (Proxeon Biosystems) equipped with an RP-HPLC column packed with C18 resin (Magic C18 AQ 3 μm; Michrom BioResources). A 0.3 μl/min linear gradient from 96% solvent A (0.15% formic acid, 2% acetonitrile) and 4% solvent B (98% acetonitrile, 0.15% formic acid) to 40% solvent B over 40 min. The data acquisition mode was set to obtain one high-resolution MS scan in the FT part of the mass spectrometer at a resolution of 60,000 FWHM followed by MS/MS scans in the linear ion trap of the 20 most intense ions. Raw files were converted to the mzXML format, and searched against the human swissprot protein database. Further data processing including SAINT was carried out as described previously (Choi et al., 2011).

Cell cycle profile
5x10⁵ cells where plated in a six well plate. Cells were synchronized as described above. Following synchronization and release in media containing the indicated drugs, cells were fixed in 70% ETOH for 24 hr, and stained with rabbit alexa fluor 488 (pH-H3) and propidium iodide (PI). 5000 Cells were then analyzed via FACS, and percentage of different cell cycle stages were obtained.

QUANTIFICATION AND STATISTICAL ANALYSIS

Experimental procedure
Each experiment was conducted at least three times to generate three biological repeats. For western blots, immunoprecipitations and IF experiment the most representative experiment is shown. Bar graphs for quantifications show the average of three
independent experiments the SE and P value generated following two-way ANOVA multiple comparison test. No adjustments for multiple comparison was utilized. Number (n) of cell counted per experiment is noted for each experiment either in the legend or on the figure.

Statistics
Data shown represent the mean ± SD or SEM, as indicated in the figure legends. Two-way ANOVA multiple comparison analysis was performed for all the data unless otherwise indicated using Prism6. *p < 0.05 **p < 0.01 ***p < 0.001.

DATA AND SOFTWARE AVAILABILITY
Raw data have been deposited to Mendeley Data and are available at http://doi:10.17632/x8tvm6r6fb.1
