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Article

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NudCL2 is essential for cytokinesis by stabilizing RCC2 with Hsp90 at the midbody

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Running Title: Regulation of cytokinesis by NudCL2
Abstract

Cytokinesis is required for faithful division of cytoplasmic components and duplicated nuclei into two daughter cells. However, its underlying mechanism remains elusive. Here, we show that an Hsp90 co-chaperone NudC-like protein 2 (NudCL2) regulates cytokinesis by stabilizing regulator of chromosome condensation 2 (RCC2) in mammalian cells. NudCL2 co-localizes with RCC2 at the midbody and is required for RCC2 stabilization. Either NudCL2 knockout (KO) or RCC2 depletion causes similar phenotypes, including cytokinesis failure, midbody disorganization, and multinucleation. Ectopic expression of RCC2 effectively reverses the cytokinesis defect induced by NudCL2 KO. Furthermore, Hsp90 is also found to co-localize with NudCL2 at the midbody and is involved in NudCL2-mediated RCC2 stability and cytokinesis. Interestingly, our results reveal that the mRNA and newly-synthesized peptides of RCC2 accumulate at the midbody. Loss of NudCL2 decreases the nascent RCC2, but not RCC2 mRNA, at the midbody. Taken together, our data provide a previously undescribed mechanism showing that NudCL2/Hsp90/RCC2 pathway is essential for cytokinesis at the midbody.

Keywords: cytokinesis; midbody; Hsp90; NudCL2; RCC2
Introduction

Cytokinesis is the final step of cell division driving the physical separation of daughter cells, which is controlled precisely during cell cycle. Failure of cytokinesis can result in chromosomal instability and polyploidy, and consequently contributes to the development of pathologies such as cancer. In animal cells, cytokinesis is initiated during anaphase with the assembly of an antiparallel interpolar microtubule bundle (the central spindle), which sequentially promotes the formation of an actomyosin-based contractile ring at the equatorial cortex that drives the ingestion of a cleavage furrow. During telophase, the contractile ring progressively compacts the central spindle to form a dense structure at the intercellular bridge called the midbody. Then, the ingressive cell membrane attaches to the midbody and then promotes the finally step of cytokinesis known as abscission on either side of the midbody. However, the underlying mechanism of cytokinesis still remains enigmatic.

Current studies indicate that cytokinesis is a multistage process that is regulated temporally and spatially. During anaphase, protein regulator of cytokinesis 1 (PRC1) firstly accumulates at the midzone during anaphase, promoting microtubule bundling and the central spindle formation. Then, polo-like kinase 1 (PLK1) is recruited into the central spindle and activates mitotic kinesin-like protein 2 (MKLP2) to help the localization of the key component of chromosomal passenger complex (CPC) Aurora B kinase into the central spindle. Aurora B phosphorylates centralspindlin subunits mitotic kinesin-like protein 1 (MKLP1) and Rac GTPase activating protein 1 (RACGAP1, also known as MgcRacGAP) in the midzone to promote further assembly of central spindle and the recruitment of Rho-GEF epithelial cell transforming 2 (ECT2) into the central spindle, respectively. In early telophase, ECT2 within the central spindle and the equatorial cortex further induce the recruitment and activation of the small GTPase Ras homolog family member A (RhoA) at the equatorial cortex; RhoA, in turn, promotes the formation and contraction of the cortical contractile ring by activating the Rho-associated kinases (ROCKs) and formin-homology protein until midbody formation. In late telophase,
CIT-K interacts with kinesin family member 14 (KIF14), MKLP1, and PRC1 at the midbody to stabilize the intercellular bridge\textsuperscript{22,23}. Finally, abscission is executed by the endosomal sorting complex required for transport (ESCRT) machinery\textsuperscript{24-26}. Collectively, accumulating studies about cytokinesis mostly focus on the regulation of central spindle and contractile ring in anaphase and early telophase, and abscission. However, little is known about the cytokinesis regulation at the midbody during late telophase.

In this study, we provide evidence that the NudCL2/Hsp90/RCC2 pathway is essential for cytokinesis at the midbody during late telophase. We find that an Hsp90 co-chaperone NudC-like protein 2 (NudCL2) co-localizes with and stabilizes regulator of chromosome condensation 2 (RCC2) at the midbody. Either loss of NudCL2 or depletion of RCC2 causes cytokinesis failure and multinucleation. The cytokinesis defect induced by \textit{NudCL2} KO is effectively reversed by ectopic expression of RCC2. Further data show that Hsp90 co-localizes with NudCL2 at the midbody and is involved in NudCL2-mediated RCC2 stability and cytokinesis.
Results

Loss of NudCL2 causes cytokinesis failure.

To explore the role of NudCL2 in cytokinesis, we employed CRISPR/Cas9-mediated genome editing to knock out NudCL2. A CRISPR/Cas9 plasmid with a short guide RNA (sgRNA) to target the first exon of NudCL2 was constructed and then transfected into HEK-293 cells (Fig. 1a). PCR amplification of genomic DNA followed by Sanger sequencing revealed indels that supposedly cause frameshift mutations at the NudCL2 DNA locus (Supplementary Fig. 1). Immunoblotting confirmed that NudCL2 protein was disappeared in two NudCL2 KO cell lines (KO-1 and KO-2) (Fig. 1b). Live cell imaging experiments showed that cells lacking NudCL2 failed to undergo cytokinesis, and about 50% of NudCL2 KO cells exhibited full-cleavage furrow ingression but eventually underwent cleavage furrow regression (Fig. 1c, d). Furthermore, deletion of NudCL2 led to a higher frequency of cells in telophase and cytokinesis compared to that of wild-type (WT) cells, which was efficiently reversed by ectopic expression of Myc-NudCL2 (Fig. 1e-g). In addition, fluorescence-activated cell sorting (FACS) analysis showed that loss of NudCL2 induced obvious cell cycle arrest in G2/M phase (Fig. 1h). Western blot analysis further revealed that knockout of NudCL2 suppressed telophase/cytokinesis progression (Fig. 1i). Together, our data suggest that NudCL2 plays an important role in cytokinesis.

Given that cytokinesis is a tightly regulated process required for proper chromosome segregation and cell division, whose failure results in multinucleation, we performed immunofluorescence analysis to further confirm the role of NudCL2 in cytokinesis. We found that deletion of NudCL2 caused a significant increase in multinucleation compared to the control, which was effectively reversed by ectopic expression of NudCL2 (Fig. 1j-l). A similar phenotype was also observed in NudCL2 KO HeLa cells (Supplementary Fig. 2). Taken together, our data strongly imply that knockout of NudCL2 causes cytokinesis failure.

NudCL2 localizes at the midbody and loss of NudCL2 disrupts the midbody
The midbody is a transient structure forming at the intercellular bridge during cytokinesis and playing a key role in cell division\textsuperscript{10}. Accumulating studies have shown that a number of proteins involved in cytokinesis are midbody-associated proteins\textsuperscript{8,22}. To further address the function of NudCL2 in cytokinesis, we first detected the localization of NudCL2 during cytokinesis using anti-NudCL2 and anti-\(\alpha\)-tubulin antibodies and found that NudCL2 accumulated at the midbody in both HEK-293 and HeLa cells (Fig. 2a, b). The immunofluorescence staining using anti-NudCL2 together with anti-Aurora B (a marker of midbody arm) or anti-MKLP1 (a marker of midbody core) antibodies showed that NudCL2 appeared to almost co-localize with Aurora B rather than MKLP1, implying that it mainly dispersed at the midbody arm region (Fig. 2c-f). Moreover, we purified the midbodies from synchronized telophase HEK-293 cells and carried out western blot experiment. Our results revealed that NudCL2 was able to copurify with midbody proteins, including Aurora B, MKLP1, and MKLP2 (Fig. 2g). Together, our data strongly indicate that NudCL2 is a midbody-associated protein.

It is well known that the midbody serves as a key platform for recruiting and organizing crucial proteins that regulate cytokinesis, and the mislocalization of these proteins can lead to cytokinesis failure\textsuperscript{22}. Since NudCL2 localizes at the midbody and its deletion results in cytokinesis failure, we asked whether a lack of NudCL2 may disrupt midbody assembly. To answer this question, we performed immunofluorescence experiments to detect the localization of three characterized midbody components: Aurora B, MKLP1, and PLK1 (a marker of midbody core) in control and \textit{NudCL2} KO HEK-293 cells. Our results showed that deletion of NudCL2 resulted in obvious mislocalization of all three midbody proteins during telophase (Fig. 2h-l). The data showed that the Aurora B spread from the midbody arm into the midbody core (Fig. 2h), while MKLP1 and PLK1 failed to precisely gather at the center of the midbody core in \textit{NudCL2} KO cells (Fig. 2j). Similar results were also found in \textit{NudCL2}-deleted HeLa cells (Supplementary Fig. 3). Together, our data imply that deletion of NudCL2 may disrupt the midbody organization.
Knockout of NudCL2 decreases the stability of RCC2.

Since NudCL2 localizes at the midbody, and our previous data had shown that NudCL2 may acts as Hsp90 cochaperone to regulate protein stability\textsuperscript{30-34}. To explore the regulatory mechanism of NudCL2 in cytokinesis, we firstly measured the expression level of several well-known midbody proteins, such as PRC1, PLK1, Aurora B, MKLP1, RhoA, et al in NudCL2 KO cells. Western blot results showed that knockout of NudCL2 had no obvious effect on these proteins compared to the controls (Fig. 3a). To further investigate the potential regulator involved in NudCL2-mediated cytokinesis regulation, we performed isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomic analysis in WT and NudCL2 KO cells and found hundreds of differentially expressed proteins (KO/WT fold change >1.2 or <0.83, p < 0.05) in NudCL2 KO cells (Fig. 3b-e and Supplementary Table 1, 2). Then, we compared downregulated proteins in NudCL2 KO-1 and KO-2 cells with midbody proteins from MiCroKiTS 4.0 database (http://microkit.biocuckoo.org/)\textsuperscript{40} (Supplementary Table 3) and found two midbody proteins, regulator of chromosome condensation 2 (RCC2) and annexin A2 (ANXA2), that overlapped in three datasets (Fig. 3f, g).

To further verify the regulation of NudCL2 in protein levels of RCC2 and ANXA2, we performed western blot analysis in two NudCL2 KO cell lines and found that only the RCC2 protein level was substantially decreased, which was rescued by ectopic expression of NudCL2 (Fig. 3h, i). Similar results were also observed in NudCL2 KO HeLa cells (Supplementary Fig. 4). The quantitative real-time polymerase chain reaction (qRT-PCR) experiments further showed that the messenger RNA (mRNA) level of RCC2 had no obvious change in NudCL2 KO cells compared to the control (Fig. 3j). In addition, cycloheximide (CHX) chase analysis revealed that the rate of RCC2 degradation was faster in NudCL2 KO cells than in control cells (Fig. 3k and Supplementary Fig. 5), implying that NudCL2 KO reduced RCC2 protein stability. To further explore the degradation mechanism of RCC2 upon NudCL2 KO, we employed the proteasome inhibitor MG132 to treat NudCL2 KO cells and found that the
degradation of RCC2 was inhibited by MG132 treatment (Fig. 3l). Furthermore, co-immunoprecipitation (Co-IP) and GST-pull down results displayed that NudCL2 was able to interact with RCC2 in vivo and in vitro (Fig. 3m-o), and the N-terminal fragment (1-102 aa) of RCC2 may be required for its interaction with NudCL2 (Supplementary Fig. 6). Immunofluorescence data also revealed that NudCL2 co-localized almost entirely with RCC2 at the midbody arm (Fig. 3p). Notably, our data showed that signals of RCC2 at the midbody were substantially reduced in NudCL2 KO cells, which was further confirmed by western blot experiment (Fig. 3q, r), suggesting that RCC2 at the midbody is also regulated upon NudCL2 deletion. Collectively, these data strongly suggest that NudCL2 plays an important role in RCC2 stabilization.

RCC2 is involved in the regulation of cytokinesis in NudCL2 KO cells.
RCC2, also known as telophase disc protein of 60 kDa (TD-60), was reported to take part in spindle assembly and cell migration \(^{35-39}\). We and other groups found that RCC2 localized at the midbody during cytokinesis (Fig. 3p-r)\(^ {35}\). However, whether RCC2 plays a role in cytokinesis is largely unknown. To examine the function of RCC2 in cytokinesis, we employed two small interference RNAs (RNAi) targeting two RCC2 mRNA regions (si RCC2-1 and -2). Western blot results showed that RCC2 protein was obviously decreased in HEK-293 cells (Fig. 4a). Importantly, the live cell imaging experiments displayed that depletion of RCC2 lead to a defect in cytokinesis, and about 50% of RCC2-depleted cells exhibited full-cleavage furrow ingression but then underwent cleavage furrow regression (Fig. 4b, c). In addition, RCC2-depleted cells displayed higher frequency of multinucleation than the controls (Fig. 4d). Depletion of RCC2 also induced the mislocalization of Aurora B, PLK1 and MKLP1 at the midbody (Fig. 4e-i). Together, our data suggest that RCC2 plays an important role in cytokinesis.

Since NudCL2 interacts with and stabilizes RCC2, and either NudCL2 KO or RCC2 depletion may induce failure of cytokinesis (Fig. 1-3), we predicted that RCC2 may be involved in NudCL2-mediated cytokinesis regulation. To test this hypothesis,
the rescue experiments were designed and carried out. Indeed, our data suggest that ectopic expression of RCC2 effectively rescued multinucleation caused by NudCL2 deletion, while ectopic expression of NudCL2 failed to rescue this phenotype induced by RCC2 depletion (Fig. 4 j-m). Collectively, our data suggest that RCC2 may function as downstream of NudCL2 to participate in the regulation of cytokinesis.

Hsp90 is involved in the regulation of cytokinesis by stabilizing RCC2 in NudCL2 KO cells.

Given that NudCL2 functions as an Hsp90 cochaperone and enhances RCC2 stability, we attempted to ask whether Hsp90 is involved in RCC2 stability and cytokinesis in mammalian cells. To test this hypothesis, HEK-293 cells were treated with an Hsp90 inhibitor, geldanamycin (GA) or radicicol (RA), respectively. Inhibition of Hsp90 ATPase activity obviously reduced the protein level of RCC2 compared to the controls (Fig. 5a, b and Supplementary Fig. 7). In addition, our data showed that signals of RCC2 at the midbody were reduced after Hsp90 inhibition (Fig. 5c and Supplementary Fig. 8). Moreover, Hsp90 inhibition also induced in multinucleation and midbody disorganization (Fig. 5d-g). Together, our data suggest that Hsp90 may play a role in cytokinesis.

Since either depletion of NudCL2 or inhibition of Hsp90 ATPase activity destabilizes RCC2 and causes cytokinesis failure (Fig. 3 and 5a-g), we speculated that Hsp90 might be involved in RCC2 stabilization through NudCL2. To test this hypothesis, we carried out rescue experiments and found that exogenous expression of Hsp90 efficiently reversed the instability of RCC2 and its functional defect induced by NudCL2 depletion (Fig. 5h, i). By contrast, ectopic expression of NudCL2 failed to reverse the degradation of RCC2 and the cytokinesis defect caused by Hsp90 inhibition (Fig. 5j, k). Furthermore, Co-IP and GST-pull down experiments showed that NudCL2, RCC2 and Hsp90 were able to interact with each other both in vivo and in vitro (Fig. 5l-o). Interestingly, the immunofluorescence data showed that endogenous Hsp90 was able to localize at the midbody (Fig. 5p). The similar localization was also observed by ectopic expressing myc-Hsp90 (Fig. 5q).
addition, Hsp90 was found to co-localize with Aurora B and NudCL2 at the midbody arm (Fig. 5r, s and Supplementary Fig. 9). Furthermore, western blot results revealed that Hsp90 was able to copurify with midbodies from synchronized telophase HEK-293 cells (Fig. 5t). Collectively, these data suggest that Hsp90 may act a downstream regulator of NudCL2 to participate in RCC2-mediated cytokinesis.

Knockout of NudCL2 decreases nascent peptides of RCC2 at the midbody.

Accumulating studies have indicated that the local mRNA translation is a key mechanism for spatial and temporal regulation of proteins and plays essential roles in subcellular regions\textsuperscript{40,41}. Previous studies have shown that a number of mRNA processing and translation-related proteins are enriched in the proteomes of midbodies\textsuperscript{22,42}. Our data also show that Hsp90, a molecular chaperone involved in the correct folding of nascent polypeptide chains and the degradation of misfolded proteins, co-localizes with NudCL2 and RCC2 at the midbody and is involved NudCL2-mediated RCC2 stability regulation. Thus, we are interested to know whether there exists the local translation of RCC2 at the midbody. To answer this question, we first performed single-molecule fluorescent in situ hybridization (smFISH) to detect the presence of \textit{RCC2} mRNA at the midbody\textsuperscript{43,44}. Our results showed that \textit{RCC2} mRNAs accumulated at the midbody and exhibited a fine colocalization with RCC2 protein in both HEK-293 and HeLa cells (Fig. 6a, b).

To further detect whether \textit{RCC2} mRNA is locally translated at the midbody, we first employed the metabolic labeling of puromycin to label sites of newly synthesized peptides in HEK-293 cells\textsuperscript{45,46}. Our results revealed that strong positive signals of puromycin were presented at the midbody in both HEK-293 and HeLa cells, and these signals co-localized with \textit{RCC2} mRNA and its protein (Fig. 6c, d). In addition, immunofluorescence data also showed that ribosomal protein S3 (Rps3), a component of the 40S ribosomal subunit, was enriched at the midbody in these two cell lines (Fig. 6e, f). More importantly, immunoprecipitation with RCC2 antibody followed by western blot showed that abundant nascent polypeptides of RCC2 were enriched in extracts of midbodies, and these signals dramatically decreased after CHX treatment.
(Fig. 6g, line 1, 2). These data strongly imply that local translation of RCC2 may occur at the midbody region. Interestingly, our data also showed that deletion of NudCL2 led to a substantial decrease in nascent peptides but not RCC2 mRNA at the midbody (Fig. 6g, line 3-6, and Fig. 6h, i). In addition, polysome profile analysis showed that NudCL2 KO had no obvious effect on the RCC2 translation (Supplementary Fig. 10). Taken together, these data suggest that NudCL2 may regulate the stabilization of newly-synthesized peptides of RCC2 at the midbody during cytokinesis.
**Discussion**

In this study, we provide evidence that the NudCL2/Hsp90/RCC2 pathway is essential for cytokinesis at the midbody in mammalian cells. Hsp90 co-localizes with NudCL2 at the midbody and participates in NudCL2-mediated RCC2 stability and cytokinesis (Fig. 6j).

NudCL2 was originally cloned and characterized as a homolog of mammalian nuclear distribution gene C (NudC, a key upregulator of the LIS1/dynein pathway) by our research group. Our previous studies show that NudCL2 functions as an Hsp90 cochaperone to stabilize both lissencephaly protein 1 (LIS1) and myosin-9 to regulate cell migration. NudCL2 also plays important roles for centriole duplication and sister chromatid cohesion by stabilizing the HECT and RLD domain containing E3 ubiquitin protein ligase 2 (HERC2) and the cohesin subunits during S-phase and prometaphase, respectively. Additionally, NudCL2 acts as an autophagy receptor to promote ciliogenesis by mediating the selective degradation of centrosomal protein of 110 kDa (CP110) at mother centrioles. However, whether NudCL2 is required for cytokinesis is still unknown. Here, we observed that NudCL2 is required for cytokinesis in mammalian cells. NudCL2 localizes at the midbody during cytokinesis. Deletion of NudCL2 causes cytokinesis failure, multinucleation, and midbody disorganization, which is able to be reversed by ectopic expression of NudCL2. Further iTRAQ-based quantitative proteomic analysis and western blot experiments displayed that RCC2 is an important downstream target of NudCL2 to regulate cytokinesis. Depletion of RCC2 leads to a similar phenotype as NudCL2 KO. Ectopic expression of RCC2 is able to reverse the cytokinesis defect induced by NudCL2 KO (Fig. 1 and 4). Thus, our data provide a previously undescribed function of NudCL2 in cytokinesis.

RCC2, a member of regulator of chromatin condensation 1 (RCC1) superfamily, is initially identified as a telophase disk-binding protein during mitosis and acts as a specific guanine exchange factor (GEF) for small G proteins, Rac1 and RalA. Previous study has reported that RCC2 bind to the nucleus and microtubules during interphase and plays an important role in interphase progression. RCC2 also is...
considered as a member of the chromosomal passenger complex (CPC) and dynamically ties to the inner centromeres during prophase. It binds to the midzone of the mitotic spindle during anaphase, and then accumulates at the midbody during cytokinesis\textsuperscript{35,36}. RCC2 is required for prometaphase to metaphase progression by regulating proper kinetochore-microtubule attachments\textsuperscript{37,38}. However, little is known on the role of RCC2 in cytokinesis. Here, our data show that RCC2 plays an important role in cytokinesis. RCC2 is found at the midbody, which is consistent with the findings of previous studies\textsuperscript{35,36}. Depletion of RCC2 causes failure of cytokinesis, multinucleation, and midbody disorganization. In addition, we found that NudCL2/Hsp90 pathway is involved in the regulation of stability of RCC2 to enable cytokinesis. However, the detailed mechanism how RCC2 regulates cytokinesis still needs to be better explored.

It is well established that proteins are translated on ribosomes in the cytoplasm and then targeted to the functional locations by the diffusion or trafficking pathways\textsuperscript{41}. Recent studies also indicate that mRNAs can be transported and localized to the special organelles, including dendrites, axons, cilia and centrosomes, to control in situ protein synthesis and local cell physiology\textsuperscript{40,41}. For example, the mRNAs encoding β-actin and several proteins are found in dendrites in neurons and translated locally to respond to the structural and functional remodeling of spines\textsuperscript{50}. Axons of neurons also are enriched in groups of mRNAs and nascent proteins that play important roles for neuronal development, function and survival\textsuperscript{51}. Recently, the study reports that multicilia of mouse ependymal cells contain the protein translation machinery and locally synthesize proteins such as tubulin to sustain their ultrastructure and functions\textsuperscript{52}. It is also reported that several mRNAs and their corresponding proteins localize to the centrosome to participate in the functional regulation of centrosome during mitosis\textsuperscript{53}. Midbody is a transient organelle-like structure, consisting of a compact, dense matrix of proteins that are indispensable for cytokinesis\textsuperscript{8,10}. The previous studies of midbody proteomes indicated that a number of proteins involved in mRNA processing and translation are enriched at the midbody\textsuperscript{22,42}. However, whether the local translation occurs at the midbody is still unknown. Here, our data
show that abundant puromycylated nascent peptides and a ribosomal component Rps3 were enriched at the midbody (Fig. 6c-f). More importantly, we found that RCC2 mRNA accumulated at the midbody, where it co-localized with its protein (Fig. 6a, b). Furthermore, our data show that abundant nascent polypeptides of RCC2 were presented in the midbody extract, and dramatically decreased with CHX treatment (Fig. 6g). Thus, our data suggest that the local translation of RCC2 might occur at the midbody. However, further experimental work is required to elucidate the regulatory mechanism of the local translation of RCC2, which will contribute to a comprehensive understanding on the role of midbody local translation.

Hsp90, a constituting molecular chaperone, is reported to be involved in folding, stabilization, activation, and assembly of a wide range of client proteins, thereby participating in many cellular processes, including transcriptional regulation, chromatin remodeling, DNA repair, and the immune response.\(^{54-56}\) Furthermore, previous studies also showed that Hsp90 plays important roles in cell cycle progression by regulating a number of cell cycle-related proteins including cyclin-dependent kinases (such as CDK1, 2, 4 and 6) and cell cycle protein (such as cyclin B, D and E).\(^{57,58}\) Inhibition of Hsp90 function results in the cell cycle arrest in G2/M.\(^{59}\) Recently, Hsp90 was found to play a role in cytokinesis in fission yeast and African trypanosomes.\(^{60,61}\) However, whether Hsp90 is required for cytokinesis in mammalian cells is largely unknown. Here, our data suggest that Hsp90 localized to the midbody during cytokinesis, and inhibition of its ATPase activity caused failure of cytokinesis (Fig. 5). Furthermore, our data show that Hsp90 interacts and co-localizes with NudCL2 and RCC2, and its inhibition results in RCC2 instability at the midbody. Overexpression of Hsp90 rescued RCC2 instability and multinucleation in NudCL2-depleted cells (Fig. 5). Together, our data suggest that Hsp90 plays an important role in cytokinesis by participating in NudCL2-mediated RCC2 stabilization at the midbody in mammalian cells. However, the detailed role of Hsp90 in the local translation of RCC2 at the midbody is still needed to be explored.
Methods

Plasmids and small interfering RNAs (siRNAs). The human Myc-NudCL2, GST-NudCL2, Myc-Hsp90, and His-Hsp90 vectors were previously constructed by our group\textsuperscript{30-32,47}. Full-length human RCC2 cloned by PCR from pSA-RCC2\textsuperscript{39} (a kind gift from Y Yin, Peking University Institute of Systems Biomedicine, Beijing, China) was inserted into pcDNA 3.1/Myc-His C (Myc/His-tag vector, Invitrogen, USA) and pET-28a (His-tag vector, Novagen, China). His-RCC2-ΔN, -ΔR1, -ΔR2, -ΔR3, -ΔR4, -ΔR5, -ΔR6, -ΔR7, and -ΔC were constructed with an N-terminal flag tag by the mutagenesis of His-RCC2 plasmids using the Mutagenesis Kit (Vazyme, China), respectively. All of these constructs were confirmed by DNA sequencing.

All siRNAs were synthesized by GenePharma (Shanghai, China). A control siRNA (Qiagen, USA) that shows no homology to any known mammalian genes were used as a negative control. The sequences of the sense strands of the siRNA duplexes were as follows:

\begin{itemize}
  \item si RCC2-1: 5′-AAGAGATGAAAGTGAGACTGA-3′;
  \item si RCC2-2: 5′-AAGGGGCAGCTGGGACATGGT-3.
\end{itemize}

Generation of NudCL2 KO cell lines by CRISPR/Cas9-mediated genome editing. The two sgRNAs, 5′-GAAGTTCAGGTGCCGCCAGG-3′ and 5′-TGGGATTCCGCGCGTGCGCTCGG-3′, targeting the first exon of the NudCL2 gene were designed and synthesized by YSY Biotech Ltd (Nanjing, China). Then, the CRISPR/Cas9 plasmid was constructed by cloning the sgRNA into its backbone. HEK-293 and HeLa cells were transfected with this plasmid for 48 h followed by treatment with 1 μg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA) for 48 h. After selection, cells were counted and diluted to a density of 1 cell per 200 μl of medium and seeded into 96-well (Bio-Rad, Laboratories, Hercules, CA, USA) plates to obtain single colonies. NudCL2 KO colonies were identified by western blot and genomic DNA sequencing analyses. Two pairs of primers used to amplify the target regions are as follows:

\begin{itemize}
  \item NudCL2 sgRNA-F1: 5′-AGGCGTAGCTAAGCGTGAGGATTC-3′;
\end{itemize}
**NudCL2 sgRNA-R1**: 5′-ACCCAACAGTCGTTCAGGGAAACG-3′;

**NudCL2 sgRNA-F2**: 5′-ACTTAGGGGACGGTGTAGTGA-3′;

**NudCL2 sgRNA-R2**: 5′-GGCGGCACCTGAACTTCAAT-3′.

**Cell culture, transfection, and drug treatment.** HEK-293 and HeLa cells were purchased from the Cell bank of Chinese Academy of Sciences (Shanghai, China) and cultured in Dulbecco’s modified Eagle’s Medium (Corning, USA) supplemented with 10% FBS (fetal bovine serum, ExCell Bio, China) at 37°C in 5% CO₂. Plasmids were transfected with PolyJet (SignaGen Laboratories, Rockville, MD, USA) and the siRNA duplexes were transfected with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA). The transfection processes were performed according to the manufacturer’s instructions. To synchronize cells, HEK-293 cells were incubated with 2 mM thymidine (Selleck, USA) for 18 h. After washing twice with phosphate-buffered saline (PBS), cells were released in fresh culture medium for 6 h. After release, cells were incubated with 2 mM thymidine for another 18 h. Cells were released in fresh culture medium, harvested at the indicated time points by centrifugation at 1000 × g for 3 min and frozen immediately in dry ice and stored at -80°C. To synchronize cells in telophase, cells were synchronized with a double-thymidine block and released. Next, cells were cultured for additional 13 h in fresh complete medium containing 20 ng/ml nocodazole (Sigma-Aldrich, USA) and then harvested by mitotic shake-off. Mitotic cells were washed three times with PBS, and released in fresh medium for 90 min to be collected in telophase. Cells were then harvested and frozen immediately in dry ice and stored at -80°C. For CHX chase analysis, 100 µg/ml CHX (Sigma-Aldrich, USA) was used as described in the text.

HEK-293 cells were treated with 1 µM MG132 (Millipore, Billerica, MA, USA) for 12 h to block the proteasome dependent degradation pathway. Geldanamycin (GA, Tocris, UK) and radicicol (RA, Tocris, UK) were stored at -20°C as a stock solution of 5 mM in DMSO and ethanol respectively. Cells were treated with geldanamycin for the indicated concentrations and times as described in the text.
Antibodies. The following antibodies for western blot (WB) and immunofluorescence (IF) were used in this study: Two types of anti-NudCL2 antibodies were prepared, including a rabbit polyclonal anti-NudCL2 antibody that generated as described previously and a mouse monoclonal antibody against NudCL2 peptide (GAEISGNYTKGGPDSNLEK, 138-157 aa) as antigens. A rabbit polyclonal anti-RCC2 antibody was prepared commercially (HuaBio, China) against the mixed peptides (RAGPRKRGPAGRKRE, 22-37aa and RVAIFIEKTDGQILP, 311-326 aa) as antigens using conventional protocols. The following antibodies and dilutions were commercially acquired for WB and IF analyses: mouse monoclonal anti-α-tubulin (Sigma-Aldrich, T6199 dilutions for WB 1:5000, for IF 1:400), mouse monoclonal anti-β-actin (Sigma-Aldrich, T1978 dilution for WB 1:5000), mouse monoclonal anti-c-Myc (Cell Signaling Technology, 9B11 dilutions for WB 1:2000, for IF 1:200), mouse monoclonal anti-His (Proteintech, 6005-1-Ig dilution for WB 1:1000), mouse monoclonal anti-GST (Santa Cruz Biotechnology, sc-138 dilution for WB 1:1000), mouse monoclonal anti-cyclin B1 (Santa Cruz, sc-70898 dilution for WB 1:2000), rabbit polyclonal anti-phospho histone H3 pS10 (Merck, 06-570 dilution for WB 1:5000), rabbit polyclonal anti-Plk1 (Sigma-Aldrich, SAB4502211 dilutions for WB 1:2000, for IF 1:200), rabbit polyclonal anti-Aurora B (diagbio, db2045 dilutions for WB 1:2000, for IF 1:200), rabbit polyclonal anti-MKLP1 (diagbio, db5469 dilutions for WB 1:1000, for IF 1:100), rabbit polyclonal anti-MKLP2 (Proteintech, 15911-1-AP dilution for WB 1:1000), rabbit polyclonal anti-Anillin (Bethyl laboratories, a301-406a dilutions for WB 1:2000, for IF 1:200), mouse monoclonal anti-GAPDH (ABclonal, AC002 dilution for WB 1:5000), rabbit polyclonal anti-Hsp90 (diagbio, db621 dilutions for WB 1:1000, for IF 1:100), mouse monoclonal anti-Puro mycin (Sigma-Aldrich, MABE343 dilutions for WB 1:10,000, for IF 1:500) and rabbit polyclonal anti-Rps3 (Cell Signaling Technology, 2579S dilution for IF 1:25). The secondary antibodies used for immunofluorescence analysis were Alexa Fluor 488-and 568-conjugated anti-rabbit or anti-mouse IgG (Invitrogen). Goat anti-mouse or anti-rabbit secondary antibody (LI-COR, Lincoln, NE, USA) c
onjugated to either Alexa Fluor 680 or IRDye 800 was used for western blot analysis.

**GST pull down assay.** GST pull-down assays were performed as described previously\(^6^2\). In brief, GST, GST-NudCL2, His-RCC2, different types of His-tagged RCC2 truncates, including His-RCC2-\(\Delta R1\), -\(\Delta R2\), -\(\Delta R3\), -\(\Delta R4\), -\(\Delta R5\), -\(\Delta R6\), -\(\Delta R7\) and-\(\Delta C\), and His-Hsp90 proteins were purified from *Escherichia coli BL21*. The purified proteins were incubated in PBS at 4\(^\circ\)C for 4 h, and then glutathione-agarose beads were added and incubated for 2 h. The beads were washed and then subjected to western blot with the respective antibodies as indicated in the text.

**Immunoprecipitation and western blot.** Immunoprecipitation was performed as previously described\(^3^2\). Briefly, cells were lysed in TBSN buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 0.5% Nonidet P-40, 5 mM EGTA, 1.5 mM EDTA, 0.5 mM Na\(_3\)VO\(_4\), 20 mM pinitrophenyl phosphate) containing a cocktail of protease inhibitors (Roche, Basel, Switzerland) and then subjected to immunoprecipitation with the indicated antibodies. The proteins were separated in a sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% BSA at room temperature for 1 h, incubated with the indicated primary antibodies followed by the secondary antibodies and then detected by ChemiDoc Touch Imaging System (Bio-Rad, USA) or LI-COR Odyssey imaging systems (LI-COR, USA).

**Immunofluorescence staining.** Cells grown on microscope glass coverslips were fixed with 3.7% [v/v] paraformaldehyde at room temperature for 20 min or ice-cold methanol for 15 min at -20\(^\circ\)C. After washing three times with PBST (0.1% Triton X-100 in PBS) for 15 min, cells were incubated in blocking buffer (3% [w/v] BSA in PBS) for 30 min at room temperature. The coverslips were incubated with the primary antibodies (diluted in PBS and 3% [w/v] BSA) indicated in the text for 2 h at room
temperature. After washing with PBST for 15 min, cells were incubated with secondary antibodies (Alexa Fluor 488-, 568- or 647-conjugated anti-rabbit or mouse IgG, Invitrogen, USA)) for 1 h at room temperature. DNA was stained with 4’,6-diamidino-2-phenylindole (DAPI) (Beyotime Technology, Shanghai, China). Finally, the mounted coverslips were analyzed by confocal fluorescence microscopy (Zeiss, LSM 800, Germany).

**Live cell image.** Cells were plated on the μ-Slides (4 well; Ibidi) and analyzed with differential interference contrast (DIC) images. Imaging was performed on the OLYMPUS Spin10 (CSU-W1) inverted digital microscope by the UPLXAPO 40× oil NA 1.40 objective at 37°C in 5% CO₂.

**Fluorescence activated cell sorting (FACS).** For analysis of cell cycle, 1 × 10⁶ cells/ml were washed twice with ice-cold PBS and fixed in 70% ethanol overnight at 4°C. The fixed cells were stained with 1 ml solution containing 50 μg/ml RNase (Sigma-Aldrich, USA) and 20 μg/ml propidium iodide (Sigma-Aldrich, USA). Cells were incubated for at least 30 min at 37°C and analyzed using the Cytomic FC 500 MCL Flow Cytometer (Beckman Coulter, USA).

**Proteomic analysis (iTRAQ) and quantification.** Isobaric tags for the relative and absolute quantitation (iTRAQ) based proteomic analysis was performed as described previously. Briefly, samples including three independent repetitions of whole-cell lysates from WT, NudCL2 KO-1 and NudCL2 KO-2 cells were lysed in a SDT buffer (4%(w/v) SDS, 100mM Tris/HCl pH7.6, 0.1M DTT). After centrifugation, the protein concentration of the supernatant was determined using BCA assay (Thermo Fisher Scientific, USA). An appropriate amount of protein from each sample was subjected to trypsin hydrolysis using the filter aided protein preparation (FASP) method, and then used C18 solid-phase extraction to desalt the peptides. Finally, the desalted peptide samples were dried in a vacuum concentrator for peptide iTRAQ labeling. The proteomic analysis of the samples was performed as iTRAQ 4-plex experiments.
according to the manufacturer’s instructions (AB-SCIEX, USA). The labeled peptides of each group were mixed and graded by Akta purifier 100. Then each graded sample was separated by HPLC liquid phase system. After chromatographic separation, the samples were analyzed by Q Exactive mass spectrometer (Thermo Scientific, USA). The original mass spectrometry file (raw) generated by Q Exactive mass spectrometer was analyzed with MASCOT2.2 (Matrix Science, London, UK; version 2.2) and Proteome Discoverer 1.4 (Thermo Fisher Scientific, USA) software for identification and quantitation analysis.

**RNA extraction and quantitative real-time PCR (qRT-PCR).** Total RNA was extracted with TRIzol (Invitrogen, USA) and reverse transcribed to obtain complementary DNA (cDNA) with HiScript II Q RT SuperMix (Vazyme, China). The LightCycler 480 II system (Roche) or CFX-96 (Bio-Rad) system was used to perform qRT-PCR using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). All of the reactions were performed in triplicate. Primers used to amplify the target region of \textit{RCC2} mRNA are as follows:

- **Forward:** 5′-GTGGGAAGAGCAGCATCATT-3′;
- **Reverse:** 5′-GAAGACTTGGGCTTGTGGTC-3′.

**Midbody purification.** Midbodies were purified according to the method previously reported\textsuperscript{22}. HEK-293 cells (at least 3 \times 10\textsuperscript{7}) were synchronized using the thymidine-nocodazole block and release procedure previously described. 5 μg/ml taxol (Selleck, USA) was added to the medium for 2-3 min to stabilize microtubules \textit{in vivo} before collection. Then cells were collected by centrifugation at 250 × g for 3 min. After wash once with pre-warmed H\textsubscript{2}O\textsubscript{2}, cells were gently resuspended in 25 ml of swelling solution (1 mM PIPES pH 7.0, 1 mM MgCl\textsubscript{2}, 5 μg/ml taxol and Roche Complete Protease Inhibitors) and immediately centrifuged at 250 × g for 3 min. The cell pellet was then resuspended in 40 ml of lysis buffer (1 mM PIPES pH 7, 1% [v/v] NP-40, 1 mM EGTA, 3 U/ml DNase I, 10 μg/ml RNAse A, 1 U/ml micrococcal nuclease, 5 μg/ml taxol, and Roche Complete Protease Inhibitors) and vortexed
vigorously for 1 min. After the addition of 0.3 volumes of cold 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 6.3, the sample was incubated on ice for 20 min and then centrifuged at 200 × g for 10 min at 4°C, and the supernatant was transferred to a new tube and centrifuged at 650 × g for 20 min at 4°C to pellet midbodies. The midbody pellet was then resuspended in 4 ml of 50 mM MES pH 6.3 and centrifuged through a 25 ml glycerol cushion (40% [w/v] glycerol diluted in 50 mM MES pH 6.3) at 2800 × g for 45 min at 4°C. After removal of the glycerol cushion, the midbody pellet was washed with 2 ml of 50 mM MES pH 6.3, transferred to a 15 ml conical tube and centrifuged at 2800 × g for 20 min at 4°C. The sample was then centrifuged at 3500 × g for 10 min at 4°C, the supernatant was carefully discarded and the pellet was left to dry for 5-10 min at room temperature. Precipitated proteins were stored at -80°C until further processing.

Single-molecule fluorescent in situ hybridization (smFISH). FISH with the single-molecule DNA probe sets was performed as described previously. Briefly, mixed DNA probes for the detection of the transcripts of RCC2 were designed and their sequences are listed in Supplementary Table 4. Cells grown on coverslips were fixed with 4% PFA in PBS for 20 min and washed in PBS for three times (5 min each) and permeabilized in 75% ethanol overnight at 4°C. After rinsing once with PBS, the cells were washed with 10% formamide in 2× SSC for 10 min, followed by pre-hybridization in pre-hybridization buffer (10% formamide, 10% dextran sulfate in 2 × SSC) at 37°C for 1 h. Subsequently, the cells were incubated with 488-labeled smFISH probes in hybridization buffer (10% dextran sulfate, 50% formamide, 1 mg/ml yeast tRNA, 5 mM Ribonucleoside Vanadyl Complex in 1×SSC) for 18 h at 37°C (100 nM probes). After three washes with 10% formamide in 2× SSC (5 min each) at 37°C, the follow-up immunofluorescence was carried out as described in the immunofluorescence staining section.

Puromycin labeling assay. Puromycin labeling assay for the detection of nascent peptides was performed as described previously. Briefly, cells were treated with
10 μg/ml puromycin (Sigma-Aldrich, P8833) for 30 min and fixed in 4% PFA in PBS for 20 min, then subjected to smFISH experiment to detect RCC2 mRNA, followed by immunofluorescence with mouse anti-puromycin and rabbit anti-RCC2 antibodies. After three times washes with 0.1% Triton X-100 in PBS (5 min each), the cells were incubated with donkey anti-rabbit IgG conjugated with Alexa Fluor 555 and donkey anti-mouse IgG conjugated with Alexa Fluor 647 for 1 h. For puromycin labeling of purified midbodies, cells were synchronized with a thymidine-nocodazole block/release, then treated with or without 10 μg/ml CHX for 30 min, and then incubated with 10 μg/ml puromycin for 30 min. Then the midbodies were purified as described in the midbody purification section and subjected to immunoprecipitation analysis using anti-RCC2 antibody.

**Polysome profiling.** Control or NudCL2 KO cells were treated with 100 μg/ml CHX for 7 min at 37°C in fresh culture medium. Then the cells were washed and collected with ice-cold PBS (DEPC-treated water) containing 100 μg/ml CHX. The cell suspension was centrifuged at 250 × g for 5 min and the cell pellet was washed by cold PBS. Lysis buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 150 mM KCl, 5 mM DTT, 0.5% Triton X-100, 100 μg/ml CHX, freshly added protease inhibitor and 40 U/ml RNase inhibitor) was added to suspend the cells and incubated on ice for 15 min. After centrifugation at 15,000 × g for 15 min, the supernatant was collected and layered onto a 10-50% w/v sucrose gradient prepared in the polysome buffer (20 mM Tris-HCl pH 7.4, 100 mM NaCl, 5 mM MgCl2, and 100 μg/ml CHX). The gradients were centrifuged at 4°C for 2.5 h at 36,000 rpm (Beckman, rotor SW41Ti). The sample was then fractioned by Gradient Station (BioCamp) equipped with an ECONO UV monitor (Bio-Rad) and collected with a fraction collector (FC203B, Gilson). Total RNA from the indicated fractions was isolated by TRIzol reagent for qRT-PCR analysis.

**Imaging analysis.** All fluorescence imaging data were analyzed and quantified by Image J. For all images, the background was subtracted. To quantify the fluorescence
intensity at the midbody in Fig. 5 and Fig. 6, the identically sized areas at the
midbody were selected in each image using Photoshop and normalized by the mean
fluorescence intensity of all images in the control group using ImageJ. The GraphPad
Prism 8.4.0 was used for statistical analysis and graphic representation. Two-tailed
unpaired Student’s $t$-test was performed for comparison of two groups. Statistical
significance was specified as $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

**Statistics and reproducibility.** Data are representative of at least three independent
experiments. Means and standard deviations (SD) were calculated for all quantitative
experiments. Two-tailed unpaired Student’s $t$-test was performed to determine
statistically significant differences between two groups (GraphPad Prism 8.4.0).
Statistical significance was specified as $^*P < 0.05$, $^{**}P < 0.01$, or $^{***}P < 0.001$.

**Data availability**

The quantitative proteomics source data generated in this study is provided in
Supplementary Information. Source data are provided with this paper.
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Author contributions

X.X. and Y.H. designed and performed the major experiments. F.Y. provided assistance for quantitative proteomic analysis. X.S., R.L. and J.S. performed the experiments. X.X. and Y.H. wrote the original draft, and then Y.Y., J.F and M.Y further refined this manuscript. Y.Y. and T.Z. edited the manuscript and supervised the overall project. All authors discussed the results and commented on the manuscript.
Competing interests

The authors declare no competing interests.
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Fig. 1 Knockout of NudCL2 causes cytokinesis failure. (a) Schematic representation of NudCL2 gene targeting strategy. (b) Western blot analysis of NudCL2 protein in WT and NudCL2 KO HEK-293 cells. (c) DIC stills of the live cell imaging experiment of the control or NudCL2 KO cells. Time point 00:00 (hours:minutes) refers to the first frame where the separating sisters are observed. (d) Percentage of cells showing cytokinesis failure was calculated. More than 100 cells were counted in each experiment. (e) Cells were stained with DAPI and anti-α-tubulin antibody for immunofluorescence analysis. The percentage of cells in each mitotic
phase and cytokinesis was calculated. More than 800 cells were counted in each experiment. (f) Cells transfected with Myc or Myc-NudCL2 vector were subjected to western blot with the antibodies as shown. (g) Cells transfected with Myc or Myc-NudCL2 vector were stained with DAPI and anti-α-tubulin antibody for immunofluorescence analysis. The percentage of cells in telophase and cytokinesis was calculated. More than 700 cells were counted in each experiment. (h) Cells were synchronized by a double-thymidine block/release, and harvested at the indicated time points, then analyzed using FACS. (i) Cells were synchronized by a double-thymidine block/release, and harvested at the different stage of the cell cycle, then subjected to western blot analysis with the antibodies as shown. (j) Cells were stained with DAPI and anti-α-tubulin antibody for immunofluorescence. Scale bars, 10 μm. (k) The percentages of the multinucleated cells were calculated. More than 900 cells were counted in each experiment. (l) Cells transfected with the indicated vectors were stained to detect DNA and α-tubulin. The percentages of the multinucleated cells were calculated. More than 700 cells were counted in each experiment. β-actin, a loading control. Quantitative data are expressed as the mean ± SD (at least three independent experiments). *p < 0.05, **p < 0.01, ***p < 0.001; ns, no significance; Student’s t test.
Fig. 2 NudCL2 localizes at the midbody and loss of NudCL2 disrupts the midbody architecture. (a-f) Cells were fixed and subjected to immunofluorescence analyses with the antibodies as shown. Higher magnifications of the boxed regions are displayed. (g) HEK-293 cells were synchronized with a thymidine-nocodazole block, released and harvested. The proteins from the total cells and midbodies were extracted and subjected to western blot analysis with the indicated antibodies. (h, i) Control and NudCL2 KO cells were fixed and subjected to immunofluorescence analysis with anti-Aurora B and anti-α-tubulin antibodies. Higher magnifications of the boxed regions are displayed. The frequencies of cells with mislocalization of Aurora B at the midbody were calculated. (j-l) Control and NudCL2 KO cells were fixed and...
subjected to immunofluorescence analysis with anti-MKLP1 and anti-PLK1 antibodies (j). Higher magnifications of the boxed regions are displayed. The frequencies of cells with mislocalization of MKLP1 (k) or PLK1 (l) at the midbody were calculated. DNA was visualized with DAPI. Scale bars, 5 μm. Quantitative data are expressed as the mean ± SD (at least three independent experiments). More than 150 cells were counted in each experiment. **p < 0.01, ***p < 0.001; ns, no significance; Student’s t test.
Fig. 3. Deletion of NudCL2 decreases RCC2 protein stability. (a) Western blot analysis of proteins from control and NudCL2 KO cells using the indicated antibodies. (b) Schematic representation of the isobaric tags for relative and absolute quantitation (iTRAQ)-based quantitative proteomic analysis. (c, d) Volcano plot showing p values (−log 10) versus the protein ratio of KO-1/WT cells (log 2) or KO-2/WT cells (log 2). Proteins exhibiting a fold change >1.2 or <0.83, p < 0.05 were defined as
“differentially expressed proteins” (red dots). Others were defined as “no change” (blue dots). (e) Scatter plot showing the protein ratio of KO-1/WT cells (log2) versus KO-2/WT cells (log2). Each point represents a single protein identified. (f) Venn diagram showing the overlap among the proteins downregulated in KO-1 and KO-2 of the quantitative proteomic analysis and the midbody proteins from the MiCroKiTS database. (g) Two midbody proteins that overlap in Fig. 3f are shown. (h) Western blot analysis of proteins from control and NudCL2 KO cells using the indicated antibodies. (i) Western blot analysis of proteins from control and NudCL2 KO cells transfected with Myc or Myc-NudCL2 vector with the indicated antibodies. (j) The qRT-PCR analysis of RCC2 mRNA in control and NudCL2 KO cells. Quantitative data are expressed as the mean ± SD (at least three independent experiments). ns, no significance, Student’s t test. (k) Western blot analysis of proteins from control and NudCL2 KO cells treated with 100 μg/ml CHX using the indicated antibodies. (l) Western blot analysis of protein from cells treated with 20 μM MG132 for 2 h. (m, n) Immunoprecipitation analysis was performed using the indicated antibodies. 5% of total input is shown. (o) In vitro GST pull-down assays using purified GST, GST-NudCL2 and His-RCC2 protein. (p, q) Cells were subjected to immunofluorescence analyses with the indicated antibodies. (r) Western blot analysis of total protein extracts and midbodies purified from control and NudCL2 KO cells with the indicated antibodies. DNA was visualized with DAPI (blue in the merged panels). Higher magnifications of the boxed regions are displayed below. Scale bars, 5 μm. β-actin, a loading control.
Fig. 4. RCC2 is involved in the regulation of cytokinesis in NudCL2 KO cells. (a) HEK-293 cells transfected with control or RCC2 siRNAs for 72 h were subjected to western blot analysis using anti-RCC2 antibody. (b) DIC stills of the live cell imaging experiment of cells transfected with RCC2 siRNAs. (c) Percentage of cells showing cytokinesis failure was calculated. More than 100 cells were counted in each experiment. (d) HEK-293 cells transfected with the siRNAs as shown were fixed and stained to detect DNA and α-tubulin. The percentages of the multinucleated cells were calculated. More than 800 cells were counted in each experiment. (e-i) HEK-293 cells
transfected with the indicated siRNAs were fixed and subjected to immunofluorescence analyses with anti-α-tubulin and anti-Aurora B (e), anti-MKLP1 and anti-PLK1 (g) antibodies. The frequencies of cells with mislocalization of Aurora B (f), MKLP1 (h) or PLK1 (i) were calculated. More than 150 cells were counted in each experiment. (j) Control and NudCL2 KO cells were transfected with Myc or Myc-RCC2 vector and subjected to western blot. (k) Control and NudCL2 KO cells transfected with Myc or Myc-RCC2 vector were fixed and stained with DAPI and anti-α-tubulin antibody for immunofluorescence. The percentages of the multinucleated cells were calculated. More than 800 cells were counted in each experiment. (l) Control or RCC2 siRNA cells transfected with Myc or Myc-NudCL2 vector were subjected to western blot. (m) Control or RCC2 siRNA cells transfected with Myc or Myc-NudCL2 vector were stained with DAPI and anti-α-tubulin antibody for immunofluorescence. The percentages of the multinucleated cells were calculated. More than 800 cells were counted in each experiment. β-actin, a loading control of Western blot analysis. DNA was visualized with DAPI (blue). Scale bars, 5 μm. Higher magnifications of the boxed regions are displayed. Quantitative data are expressed as the mean ± SD (at least three independent experiments). **p < 0.01, ***p < 0.001; ns, no significance; Student’s t test.
Fig. 5. Hsp90 localizes at the midbody and is involved in the RCC2-mediated regulation of cytokinesis in NudCL2 KO HEK-293 cells. (a, b) Cells treated with different concentrations of GA for 48 h (a) or 0.5 μM GA at the indicated time points (b) were subjected to western blot analyses with the indicated antibodies. (c) Cells were subjected to immunofluorescence analysis with the indicated antibodies. (d) Cells were stained with DAPI and anti-α-tubulin antibody. The percentages of the multinucleation were calculated. More than 800 cells were counted in each group. (e-g) Cells were subjected to immunofluorescence experiment as described in Fig. 2h-l. The frequencies of cells with Aurora B (e), MKLP1 (f) or PLK1 (g) mislocalization were calculated. More than 150 cells were counted in each group. (h, i) Cells transfected with Myc or Myc-Hsp90 were subjected to western blot and immunofluorescence analyses. The percentages of the multinucleation were calculated. More than 800 cells were counted in each group. (j, k) Cells treated with or without GA transfected with the indicated vectors were subjected to western blot and immunofluorescence analyses. The percentages of the multinucleation were calculated. More than 800 cells were counted in each group. (l-n) Immunoprecipitation analysis was performed using the indicated antibodies. 5% of total input is shown. (o) In vitro GST pull-down assays using purified GST-NudCL2 with His-RCC2 and His-Hsp90 proteins. (p) Cells were subjected to immunofluorescence analysis with the indicated antibodies. (q) Cells transfected with Myc-Hsp90 vector were subjected to immunofluorescence analysis with the indicated antibodies. (r, s) Cells were subjected to immunofluorescence analyses with the indicated antibodies. (t) Western blot analysis of total protein extracts and midbodies purified from telophase cells with the indicated antibodies. DNA was visualized with DAPI. Scale bars, 5 μm. Higher magnifications of the boxed regions are shown. β-actin, a loading control. Quantitative data are expressed as the mean ± SD (at least three independent experiments). ***p < 0.001; ns, no significance; Student’s t test.
Fig. 6. **RCC2 mRNA accumulates at the midbody and deletion of NudCL2 decreases nascent peptides of RCC2 at the midbody.** (a, b) Cells were fixed and subjected to single molecule RNA fluorescence in situ hybridization (smFISH) to detect RCC2 mRNA, then followed by immunofluorescence analysis with anti-RCC2 antibody. (c, d) Cells labeled with puromycin for 30 min were fixed and subjected to smFISH, then followed by immunofluorescence analysis with the anti-RCC2 antibody. (e, f) Cells were fixed and subjected to immunofluorescence analyses with the antibodies as shown. (g) Cells were synchronized with a thymidine-nocodazole block/release and labeled with puromycin for 30 min with or without CHX. Then, the midbodies were purified and subjected to immunoprecipitation analysis with
anti-RCC2 antibody. (h) Control and *NudCL2* KO cells were fixed and subjected to
smFISH to detect *RCC2* mRNA, followed by immunofluorescence analysis with the
anti-RCC2 antibody. (i) The qRT-PCR analysis of *RCC2* mRNA in total extracts and
midbodies purified from synchronized telophase control and *NudCL2* KO HEK-293
cells. (j) Working model for the role of NudCL2 in cytokinesis. NudCL2, Hsp90, and
the mRNA and protein of RCC2 all localize at the midbody arm. NudCL2 regulates
cytokinesis by stabilizing RCC2 protein with Hsp90 at the midbody. Loss of NudCL2
decreases RCC2 at the midbody, resulting in cytokinesis failure and multinucleation.
DNA was visualized with DAPI. Higher magnifications of the boxed regions are
displayed. Scale bars, 5 μm. β-actin, a loading control of Western blot analysis.
Quantitative data are expressed as the mean ± SD (at least three independent
experiments). ns, no significance, Student’s *t* test.
Supplementary Files

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