A vertebrate N-end rule degron reveals that Orc6 is required in mitosis for daughter cell abscission

Juan A. Bernal and Ashok R. Venkitaraman

Medical Research Council Cancer Cell Unit, Hutchison/Medical Research Council Research Centre, Cambridge CB2 0XZ, England, UK

Orc6, an evolutionarily conserved component of the origin recognition complex, is essential for deoxyribonucleic acid (DNA) replication initiation from yeast to humans. Whether vertebrate Orc6 has a mitotic function remains unresolved. In vertebrates, but not yeast, its depletion causes centrosome amplification and multinucleate division, but replication stress indirectly causes similar abnormalities. In this paper, we exploit Varshavsky’s N-end rule to create a temperature-sensitive degron form of avian Orc6. Orc6 depletion during the S phase triggers centrosome amplification suppressed by G2 checkpoint inhibition, reflecting an indirect consequence of aberrant DNA replication. However, Orc6 depletion during mitosis suffices to cause asymmetric division and failure in cytokinesis, with a delay in daughter cell abscission revealed by a fluorescence-bleaching assay. A mutant lacking the C-terminal 25 residues cannot rescue these defects. Thus, vertebrate Orc6 is necessary during mitosis for the abscission stage of cytokinesis. Our findings exemplify N-end rule degrons as tools to unravel functions of a single protein during different phases of the vertebrate cell cycle.

Introduction

Origin recognition complex proteins form a hexameric complex that helps to load replication initiation factors at origins of replication (Bell and Stillman, 1992; Rowles and Blow, 1997; Quintana and Dutta, 1999). Orthologues occur in Saccharomyces cerevisiae (Li and Herskowitz, 1993), Drosophila melanogaster (Gossen et al., 1995), Xenopus laevis (Gillespie et al., 2001), or Homo sapiens (Dhar and Dutta, 2000) and probably exist in all eukaryotes. Orc1–Orc6 are essential for the initiation of DNA replication in all systems so far tested (Duncker et al., 2009) but may also have additional biological functions (Bell et al., 1995; Pak et al., 1997; Bell, 2002; Prasanth et al., 2004; Shimada and Gasser, 2007). The most striking example is Orc6 (Prasanth et al., 2002; Chesnokov, 2007), which has no homology with the other origin recognition complex proteins and has rapidly diverged from budding yeast to humans (Dhar and Dutta, 2000).

Mammalian Orc6 has been implicated in mitosis because its depletion using RNAi causes centrosome amplification and multinuclear cell division (Prasanth et al., 2002). However, Orc6 depletion from asynchronously dividing cells triggered delayed and diverse mitotic phenotypes whose prominence depends on the duration of RNAi treatment. Genetic experiments in budding yeast fail to identify a corresponding role (Semple et al., 2006). Moreover, aberrant replication can itself induce G2 checkpoint activation (Dodson et al., 2004), which when persistent, provokes both centrosome amplification and abnormal cell division (Meraldi et al., 2002). Thus, whether the mitotic abnormalities triggered by mammalian Orc6 depletion reflect direct functions of the protein or are simply the indirect consequences of defective replication remains unresolved. To address this issue, we created a temperature-sensitive N-end rule degron form of vertebrate Orc6 in the avian DT40 cell line using a method we have recently established (Su et al., 2008).

Results and discussion

We made DT40 cells in which endogenous Orc6 was replaced by degron-Orc6 using the approach described in Fig. S1 (Su et al., 2008) to fuse endogenous Orc6 in frame at its 5′ end to a FLAG tag and a thermosensitive N-end rule mutant of Orc6, an evolutionarily conserved component of the origin recognition complex, is essential for deoxyribonucleic acid (DNA) replication initiation from yeast to humans. Whether vertebrate Orc6 has a mitotic function remains unresolved. In vertebrates, but not yeast, its depletion causes centrosome amplification and multinucleate division, but replication stress indirectly causes similar abnormalities. In this paper, we exploit Varshavsky’s N-end rule to create a temperature-sensitive degron form of avian Orc6. Orc6 depletion during the S phase triggers centrosome amplification suppressed by G2 checkpoint inhibition, reflecting an indirect consequence of aberrant DNA replication. However, Orc6 depletion during mitosis suffices to cause asymmetric division and failure in cytokinesis, with a delay in daughter cell abscission revealed by a fluorescence-bleaching assay. A mutant lacking the C-terminal 25 residues cannot rescue these defects. Thus, vertebrate Orc6 is necessary during mitosis for the abscission stage of cytokinesis. Our findings exemplify N-end rule degrons as tools to unravel functions of a single protein during different phases of the vertebrate cell cycle.

References

Bell, J.C., and Stillman, B. (1992). Nature 358, 343–346.

Bell, J.C., and Stillman, B. (1995). Genes Dev. 9, 987–999.

Bell, J.C. (2002). Trends Genet. 18, 301–307.

Bohdanowicz, M., Karpova, I., and Dutta, A. (2005). Mol. Cell 18, 447–458.

Bournazou, L., and Dutta, A. (2008). Nature 451, 834–837.

Bournazou, L., and Dutta, A. (2009). Cell 139, 931–939.

Cesca, T., and Dutta, A. (2008). Cell 135, 882–893.

Cesca, T., and Dutta, A. (2009). Cell 137, 766–778.

Chesnokov, A. (2007). Cell 129, 779–791.

Chesnokov, A., and Dutta, A. (2007). Mol. Cell 27, 61–68.

De La Calle, M., and Dutta, A. (2007). Mol. Cell 28, 619–627.

Dodson, D., Lees, J., and Dutta, A. (2004). Mol. Cell 16, 79–90.

Duncker, R., Li, W., Pak, C., and Dutta, A. (2009). Mol. Cell 33, 428–439.

Dhar, A.K., and Dutta, A. (2000). Genes Dev. 14, 2675–2685.

Gillespie, K.W., Chen, C., and Dutta, A. (2001). Cell 105, 601–610.

Gossen, M., Bujard, H., and Sonenberg, N. (1995). Mol. Cell 6, 987–999.

Gruters, A., and Ploegh, H.L. (2000). Trends Biochem. Sci. 25, 304–310.

Herskowitz, I., and Hieter, P. (2000). Nat. Genet. 24, 225–226.

JCB: Report

Correspondence to Ashok R. Venkitaraman: arv22@cam.ac.uk

J.A. Bernal’s present address is Dept. de Desarrollo Cardiovascular y Reparación, Centro Nacional de Investigaciones Cardiovasculares, Madrid CP28029, Spain.

Abbreviations used in this paper: ANOVA, analysis of variance; KIF, knockin forward; KIR, knockin reverse; KOF, knockout forward; KOR, knockout reverse; MFI, mean fluorescence intensity; Nz, nocodazole.

© 2011 Bernal and Venkitaraman. This article is distributed under the terms of an Attribution–Noncommercial–Share Alike–No Mirror Sites license for the first six months after the publication date (see http://www.rupress.org/terms). After six months it is available under a Creative Commons License (Attribution–Noncommercial–Share Alike 3.0 Unported license, as described at http://creativecommons.org/licenses/by-nc-sa/3.0/).

Supplemental material can be found at: http://doi.org/10.1083/jcb.201008125
dihydrofolate reductase (Dohmen et al., 1994; Labib et al., 2000; Dohmen and Varshavsky, 2005). In cells exclusively expressing degron-Orc6 (Orc6\(^{-\text{deg}}\)), the fusion protein is rapidly degraded to <10% of its initial levels within 90 min when cells are shifted from a permissive temperature of 35°C to a nonpermissive temperature of 42°C (Fig. 1 A). Several lines of evidence confirm that degron-Orc6 functionally replaces endogenous Orc6. The viability and proliferation of Orc6\(^{-\text{deg}}\) cells is similar to that of controls (Fig. 1 B) at 35°C. However, when shifted to 42°C, Orc6\(^{-\text{deg}}\) cells fail to survive. Complementation with an EGFP-Orc6 fusion protein (which is similar in size to the degron-Orc6 cells) is stable at 35°C but undergoes degradation to <10% of its initial levels within 90 min when cells are shifted to a nonpermissive temperature of 42°C; Fig. 1 A). Depletion of Orc6 with caffeine, an inhibitor of G2 checkpoint kinases known to bypass G2 arrest, decreased the percentage of 4N cells from 25 to 66% of cell cycle prolongation. Indeed, the fraction of degron-Orc6–depleted cells with 4N DNA content increased from 25 to 66% over 24 h (Fig. 2 C), but there was no increase in M-phase cells stained with anti-MPM2 (1.3% at 24 h). Thus, these observations suggest that degron-Orc6 depletion during DNA replication causes arrest in the G2 phase, allowing the accumulation of supernumerary centrosomes and multipolar spindles.

Supporting this notion, phosphorylated Chk1, a G2 checkpoint effector that is induced by defective replication (Helfman et al., 2002; Xiao et al., 2003), accumulated in degron-Orc6–depleted cells (Fig. 2 D). Treatment of degron-Orc6–depleted cells with caffine, an inhibitor of G2 checkpoint kinases known to bypass G2 arrest, decreased the percentage of 4N cells from 66 to 42% after 24 h; it correspondingly reduced supernumerary centrosomes and multipolar spindle formation between 16 and 20 h after degron-Orc6 depletion (Fig. 2 E). Finally, cells synchronized with the microtubule poison nocodazole (Nz; Fig. 2 F) before release into mitosis without degron-Orc6 did not exhibit supernumerary centrosome formation (Fig. 2 G). Collectively, these findings argue against a direct role of Orc6 in centrosome duplication or mitotic spindle formation and suggest instead that the occurrence of centrosome and spindle anomalies in degron-Orc6–depleted cells is the result of persistent G2 arrest after defective DNA replication.

Does Orc6 have a direct role in cytokinesis independent of its role in DNA replication? To answer this question, we depleted degron-Orc6 from Nz-arrested cells by incubation at 42°C before release into mitosis (as in Fig. 2 F). Representative frames occur corresponds to ~1.5–2 times the typical 10-h doubling time of DT40 cells (Li and Dodgson, 1995), which is indicative of cell cycle prolongation. Indeed, the fraction of degron-Orc6–depleted cells with 4N DNA content increased from 25 to 66% over 24 h (Fig. 2 C), but there was no increase in M-phase cells stained with anti-MPM2 (1.3% at 24 h). Thus, these observations suggest that degron-Orc6 depletion during DNA replication causes arrest in the G2 phase, allowing the accumulation of supernumerary centrosomes and multipolar spindles.
Figure 2. Degron-Orc6 depletion during the S phase triggers supernumerary centrosome formation suppressed by G2 checkpoint inhibition. [A] Enumeration of cells with multiple anti-γ-tubulin–stained centrosomes. Control cells expressing degron-Orc6 are compared with Orc6−/−/deg cells lacking Orc6 at different time points after shifting to 42°C. The same cells maintained at 35°C represent controls. At least 200 cells were analyzed per time point. The percentage of cells...
from serial time-lapse images show that \emph{Orc6}\textsuperscript{Δdeg} cells lacking degron-Orc6 displayed asymmetric cell cleavage and failure in cytokinesis in contrast to the symmetric division of controls (Fig. 3, A–E). Moreover, during cytokinetic furrow ingression, degron-Orc6–depleted cells exhibited cortical blebbing, furrow regression, and failure to divide, forming multinucleated cells (Fig. 3, B and C). Defective cell division was strikingly more frequent in cells lacking full-length degron-Orc6 than in controls (Fig. 3 F, compare \emph{Orc6}\textsuperscript{Δdeg} and \emph{Orc6}\textsuperscript{Δdeg/EGFP-Orc6Δ25} with \emph{Orc6}\textsuperscript{Δdeg} and \emph{Orc6}\textsuperscript{Δdeg/EGFP-Orc6Δ25} cells). Full-length EGFP-Orc6 could complement these defects in \emph{Orc6}\textsuperscript{Δdeg} cells, whereas EGFP alone or the EGFP-Orc6Δ25C mutant could not (Fig. 3, E and F).

Thus collectively, our findings show that the C-terminal region of Orc6 is essential during mitosis for the late stages of cell division and, in particular, that it promotes correct abscission and cell separation. However, EGFP-Orc6Δ25C (like EGFP-Orc6) decorates chromosomes from anaphase to telophase independent of cell division fate and also accumulates in interphase nuclei (Fig. 3, G and H), suggesting that mislocalization does not explain its defective function.

Abscission defects late in cytokinesis are difficult to detect by conventional light microscopy. To determine whether degron-Orc6 depletion during early mitosis induces delayed abscission, we used a fluorescence-bleaching approach to test whether there was a cytoplasmic connection between daughter cells transfected with free monomorphic EGFP (Fig. 4 A). We first depleted degron-Orc6 during mitosis (as in Fig. 2 F) and, during division, photobleached EGFP from one daughter cell and measured over time the mean fluorescence intensity (MFI) of EGFP in its partner (Fig. 4 B). As long as a cytoplasmic connection exists between the two cells, the MFI of the unbleached cell will equilibrate with that of its partner (see example in Fig. 4 C). When abscission is complete and no cytoplasmic connection exists, the MFI of the bleached and unbleached cells will not equilibrate (Fig. 4 C). 2 h after release into mitosis, 92% of \emph{Orc6}\textsuperscript{−/−/EGFP} cells (n = 25) have completed abscission, whereas only 24% of \emph{Orc6}\textsuperscript{−/−/EGFP\textsuperscript{Δdeg}} cells (n = 25) lacking degron-Orc6 did so in the same time frame (Fig. 4 D). To exclude that this difference was not simply the result of delays in mitosis before the abscission step, we measured the precise timing of abscission and the completion of cell separation by modifying a recently developed fluorescence-bleaching assay (Steigemann and Gerlich, 2009; Steigemann et al., 2009). Repeated bleaching of EGFP in one daughter cell decreases the MFI in the unbleached daughter cell as long as they maintain their cytoplasmic connection. The decrease in MFI terminates after abscission, when the daughter cells separate (Fig. 4 E). When comparing \emph{Orc6}\textsuperscript{+/−/EGFP} and \emph{Orc6}\textsuperscript{−/−/EGFP} cells, we find a significant delay in the timing of abscission after degron-Orc6 depletion (78 ± 3.8 min, mean ± SEM; n = 15) compared with control cells (44 ± 1.1 min, mean ± SEM; n = 15; Fig. 4, F–H). This delay could not be explained by the persistence of DNA bridges between the two daughter cells that might impede abscission (Fig. S3, A and B).

Moreover, the Aurora B kinase, which delays abscission when chromosome bridges are present (Steigemann et al., 2009), localizes normally to the midbody in degron-Orc6–depleted cells and did not colocalize with DAPI-stained DNA (Fig. S3 B).

How vertebrate Orc6 may regulate cytokinesis is unclear, although we show that a C-terminal region of 25 residues is necessary for abscission. Silencing of the \emph{D. melanogaster} Orc6 orthologue also causes multinuclear cell division; its conserved C-terminal region binds to the septin “peanut” (Chesnokov et al., 2003; Huijbregts et al., 2009). However, we could not detect an interaction between vertebrate Orc6 and the peanut homologue septin 7 (Fig. S3). Thus, vertebrate Orc6 might exert its functions during abscission through a different mechanism.

In summary, we have exploited an N-end rule degron to show that vertebrate Orc6 is necessary during mitosis for symmetric cell division and cytokinetic abscission; this is distinct from its established function in DNA replication. In contrast, our results suggest that centrosome amplification induced by Orc6 depletion reflects an indirect consequence of aberrant replication, rather than the direct role suggested by previous studies (Prasanth et al., 2002; Chesnokov et al., 2003). Thus, our work illustrates the utility of vertebrate N-end rule degrons to unravel complex phenotypes associated with different functions of a single protein during different phases in the cell cycle.
Figure 3. Degron-Orc6 depletion during mitosis induces asymmetric cell division and failed cytokinesis. (A–D) Representative mitotic phenotypes in Orc6−/− cells, Orc6−/deg/EGFP-Orc6−/deg or controls at 42°C. Orc6−/+ controls uniformly exhibit symmetric division (A), whereas Orc6−/deg cells exhibit a variety of abnormal phenotypes, including asymmetric division (B) and failed division (C). (D) Symmetric division after reconstitution with Orc6−/deg/EGFP-Orc6.
Our work highlights an evolutionary difference between vertebrate and yeast Orc6 (Fig. 5). Conditional genetic experiments in yeast indicate that yeast Orc6 exclusively functions during DNA replication. In contrast, our findings indicate that Orc6 has acquired an additional and distinct role in the completion of cell division during the transition from unicellular to multicellular organisms, providing fresh insight into the evolution of the mechanisms that coordinate DNA replication and mitosis in eukaryotes.

Materials and methods

Construction of plasmids and targeting vectors
The Gallus gallus Orc6 (ENSGal00000006929, 1261 bp; ENSGal00000006918, 267 aa) was cloned from DT40 first-strand cDNA by PCR using PF1 and PR1 primers. The PCR product was digested with NolI and KpnI and ligated into pEGFP-N1 digested with the same enzymes to create pN1-GgOrc6. To generate pEGFP-Orc6 and pEGFP-Deg-Orc6Δ25, the vector pEGFP-C1 was digested with EcoRI and KpnI and ligated to the PCR products generated with the primers EGFP and EGF1 or EGF2, respectively, cut with the same enzymes.

We created ploxDegron-GgOrc6 by PCR amplifying the degron cassette from pK1187 using the primers forward and gallus reverse before digestion with NheI and KpnI and ligation into pN1-GgOrc6. A targeting vector, ploxNeo/KO-Orc6, for knocking out the entire Orc6 gene, including sequences 2 kb 5′ from the ATG (~6.5 kb in all), was constructed as follows. A left arm from position –4 kb to –2 kb from the ATG (homology A) was amplified by PCR using the primers PF2 and PR2, digested with KpnI and SalI, and ligated into ploxNeo digested with the same enzymes. This construct was then digested with Sp and NotI and ligated to a right arm from the 3′ end of the gene to 2 kb downstream (homology B) prepared by PCR using primers PF3 and PR3.

A targeting vector, ploxBS/Ki-deg-Orc6, for knocking in the degron sequence into the endogenous DT40 Orc6 gene was constructed as follows. A left arm from intron 1 to intron 4 (homology D) was amplified by PCR using the primers PF4 and PR4, digested with SalI and ligated into ploxBS right-arm. A left arm ploxBS/Ki-deg-Orc6 was assembled using ZeroBlunt as a backbone in two steps. In the first step, a region from the 3′ end of exon 1 to –2 kb from the ATG (homology C) was amplified using the primers PF5 and PR5 and cloned into ZeroBlunt (Invitrogen) to create ZeroBlunt-leftarm. In the second step, the degron in the first exon of Orc6 was amplified using the primer pair PF6 and PR6 and cloned into ZeroBlunt-leftarm using NotI–NdeI. The cassette was digested with NotI and KpnI and ligated into the ploxBS-rightarm construct as described in this paragraph. All constructs were verified by nucleotide sequencing. Site-directed mutagenesis was used to restore a Kozak sequence 5′ to the ATG.

The following primers were used in this paper (sequences run 5′ to 3′): PF1, ATATCGGATTCTTATGACACCTGGCCTCCTCCTGCGGAGG; PR1, TTAATACGACTCACTATAGGGGACGTGTTCGAGCTCA; PF2, TGAATACGACTCACTATAGGGGACGTGTTCGAGCTCA; PR2, TGAATACGACTCACTATAGGGGACGTGTTCGAGCTCA; PF3, ATATCGGATTCTTATGACACCTGGCCTCCTCCTGCGGAGG; PR3, TTAATACGACTCACTATAGGGGACGTGTTCGAGCTCA; PF4, GTGCAGGTCGTCGGACTTCGGGACAGAAGGTTG; PR4, GTGCAGGTCGTCGGACTTCGGGACAGAAGGTTG; PF5, CGGCGCGGCGCTGCAGGATGGAAGCTGAGAAGG; PR5, CGGCGCGGCGCTGCAGGATGGAAGCTGAGAAGG; PF6, CATATGAGCTCTTGCTGAGTTCTGATTTTGGCTGAG; PR6, CATATGAGCTCTTGCTGAGTTCTGATTTTGGCTGAG; PF7, GGGCTGAGTTCTGATTTTGGCTGAG; PR7, GGGCTGAGTTCTGATTTTGGCTGAG; PF8, GGGCTGAGTTCTGATTTTGGCTGAG; PR8, GGGCTGAGTTCTGATTTTGGCTGAG; PF9, GGGCTGAGTTCTGATTTTGGCTGAG; PR9, GGGCTGAGTTCTGATTTTGGCTGAG; PF10, GGGCTGAGTTCTGATTTTGGCTGAG; PR10, GGGCTGAGTTCTGATTTTGGCTGAG.

Generation of DT40 cell lines
All targeting constructs were transfected into DT40 as described previously (Su et al., 2008). In brief, 105 cells were washed with PBS and resuspended in a final volume of 400 µl PBS. 20 µg of targeting construct was linearized with ApaI and dissolved in 400 µl PBS. Cells and DNA were mixed in an electroporation cuvette (Bio-Rad Laboratories) and kept on ice for 10 min. Electroporation was performed with an electroporation system (Gene Pulser II; Bio-Rad Laboratories) at 950 µF and 250 V followed by a 10-min incubation on ice. Cells were seeded into five 96-well plates, and the relevant antibiotics were added 24 h later at final concentrations of 1.5 mg/ml G418 (Invitrogen) and 30 µg/ml blasticidin (InvivoGen). Orc6+/+ and Orc6+/deg cells were generated by sequential transfection of ploxNeo/KO-Orc6 and ploxBS/Ki-deg-Orc6 targeting constructs into wild-type DT40 cells. Genetic DNA was extracted from the candidate clones by DNAzol (Invitrogen) according to the manufacturer’s instructions, and the identification of successful targeting events was performed by PCR product analysis generated by primers 5′ knockout forward (KOF) and 5′ knockout reverse (KOR; 5′ in the knockout) and 3′ KOR and 3′ KIR (3′ in the knockout) for the heterozygous allele. Degron-positive clones were reselected 2 wk later by their ability to grow at 35°C and lack of viability at 42°C under neomycin and blasticidin selection. These knockin clones were identified using the primers 3′ knockin forward (KIF) and 3′ knockin reverse (KIR; 5′ in the knockin) and 3′ KIF and 3′ KIR (3′ in the knockin).

The resistance cassettes were then floxed out by transient expression of Cre recombinase. In brief, cells were transfected with the pPGK-Cre plasmid (gift from K.J. Patel, Laboratory of Molecular Biology, Cambridge, England, UK) using solution from Nucleofector Kit T (Lonza) according to the manufacturer’s guidelines. Clones with restored sensitivity to Blasticidin were identified using the primers 3′ knockin forward (KIF) and 5′ knockin reverse (KIR; 5′ in the knockin) and 3′ KIF and 3′ KIR (3′ in the knockin).

Cell culture and viability assay
DT40 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10−5 M β-mercaptoethanol, 10% fetal calf serum, and 5% chicken serum (Invitrogen) between 35 and 42°C. DT40 cells were synchronized at early mitosis by the addition of 500 ng/ml –1 Nz (Sigma-Aldrich) for 5 h or at the G1/S boundary by exposure to 400 µM mimosine (Sigma-Aldrich) for 5 h (Su et al., 2008). Cells were released by washing...
Figure 4. *Degron-Orc6 depletion during mitosis delays in cell abscission.* (A) Experimental design for the diffusion-based fluorescence-bleaching assay to detect the completion of cytokinetic abscission. (B) Selected frames from time-lapse series of Orc6^{+/deg/EGFP} cells (expressing degron-Orc6 and EGFP) or Orc6^{+/+/EGFP} controls (expressing wild-type Orc6 and EGFP) after synchronization and release from Nz. Free EGFP was bleached from one of the two daughter
Distinct contributions of Orc6 to normal mitosis in vertebrate cells versus budding yeast. In budding yeast, Orc6 functions exclusively in DNA replication, whereas in vertebrate cells, it has acquired a distinct role in promoting symmetric cell division and the abscission step of cytokinesis independent of DNA replication. Other cell cycle abnormalities (e.g., supernumerary centrosome formation) triggered by Orc6 inactivation during the S phase stem indirectly from the cellular response to aberrant replication.

**Figure 5.** Distinct contributions of Orc6 to normal mitosis in vertebrate cells versus budding yeast. In budding yeast, Orc6 functions exclusively in DNA replication, whereas in vertebrate cells, it has acquired a distinct role in promoting symmetric cell division and the abscission step of cytokinesis independent of DNA replication. Other cell cycle abnormalities (e.g., supernumerary centrosome formation) triggered by Orc6 inactivation during the S phase stem indirectly from the cellular response to aberrant replication.
for 4 min. The soluble nuclear fraction was collected and mixed with the cytoplasmic soluble protein to constitute the soluble fraction. The insoluble chromatin pellet was then resuspended in buffer A with 1 mM CaCl2 and 0.2 U micrococcal nuclease incubated at 37°C for 10 min. The chromatin fraction was collected by high speed centrifugation. Protein concentration was quantified using the bicinchoninic acid assay (Sigma-Aldrich). Cell extracts were resolved by 4–12% SDS-PAGE (Invitrogen) and transferred to nitrocellulose membranes (Millipore) before blocking with the appropriate antibodies (anti-EGFP [JL-18; BD], anti-Mek2 [BD], anti–histone H3 [Cell Signaling Technology], anti–FLAG [M2; Sigma-Aldrich], anti–CHK1 [G-4], septin 7 [H-120; Santa Cruz Biotechnology, Inc.], anti-Mcm2 [ab4461; Abcam], anti–phospho-CHK1 [S345; Cell Signaling Technology], or anti–β-actin [Sigma-Aldrich]). HRP-conjugated secondary antibodies against rabbit and mouse were used at 1:20,000 or 1:40,000 dilutions, respectively. Signal intensities were quantified by integrated density measurements on ImageJ (National Institutes of Health).

Live-cell microscopy

Cells were plated in glass-bottomed Mat-Tek dishes. Before imaging, growth medium was replaced with phenol red-free L15 imaging medium. Confocal live imaging was performed on a customized microscope (LSM 510 Axiosvert; Carl Zeiss, Inc.) using a 100× 1.4 NA oil Plan Apochromat objective (Carl Zeiss, Inc.) or a microscope [IX81; Olympus] using a 40× 1.4 NA objective. Both microscopes were equipped with piezo focus drives and incubation chambers, providing a humidified atmosphere at 35 or 42°C. Long-term videos for Fig. 3 were acquired on an IX81 microscope [Cell R software]. Sample illumination was kept to a minimum and had no adverse effect on cell division and proliferation. Image analysis was preformed by LSM 510 and ImageJ software. No contrast adjustments were applied.

FRAP and fluorescence loss in photobleaching

For experiments shown in Fig. 4, EGFP was bleached by radiating a defined region with a 488-nm laser at 100% transmission. Bleaching of EGFP on a microscope [LSM 510] was preformed by epifluorescence illumination. FRAP experiments used 50 iterations of photobleaching at 100% transmission of a 488-nm Ar ion laser at a 15-mW output at regions similar to the one indicated in Fig. 4 A. The recovery kinetics of MFI was measured in a region of constant size. Fluorescence loss in photobleaching experiments used 20 iterations of photobleaching at 100% transmission of a 488-nm laser at regions similar to the one indicated in Fig. 4 D. Bleaching was repeated every 5 cycles of 120 s. Mean fluorescence was measured in regions of constant size as indicated in Fig. 4 D.

Quantitative immunofluorescence microscopy

Cells cytospun onto glass slides were stained with rabbit polyclonal anti–phospho-CHK1 [S345; Cell Signaling Technology], anti-FLAG [M2; Sigma-Aldrich], anti-CHK1 [G-4], septin 7 [S345; Cell Signaling Technology], or anti–β-actin [Sigma-Aldrich]. HRP-conjugated secondary antibodies against rabbit and mouse were used at 1:20,000 or 1:40,000 dilutions, respectively. Signal intensities were quantified by integrated density measurements on ImageJ (National Institutes of Health).

Online supplemental material

Fig. S1 shows the generation and characterization of a cell line expressing functional degron-Orc6. Fig. S2 shows that degron-RADS1 depletion during the 5 phase triggers supernumerary centrosome formation. Fig. S3 shows that obscission defects after Orc6 depletion correlate neither with abnormal DNA bridges during division nor an interaction with septin 7.

Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201008125/DC1.

We thank the members of the Venkitaraman laboratory for helpful discussion and technical advice and Dr. K.J. Patel for the gift of reagents. This work was supported by grants to A.R. Venkitaraman from the Wellcome Trust and the Medical Research Council. J.A. Bernal was also supported by the Spanish Ramón y Cajal program (RYC-2009-04341) from the Ministry of Science and Innovation.

Submitted: 23 August 2010
Accepted: 18 February 2011

References

Balasov, M., R.P. Huijbrsregt, and I. Chesnokov. 2009. Functional analysis of an Orc6 mutant in Drosophila. Proc. Natl. Acad. Sci. USA. 106:10672–10677. doi:10.1073/pnas.0902670106
Bell, S.P. 2002. The origin recognition complex: from simple origins to complex functions. Genes Dev. 16:659–672. doi:10.1101/gad.696902
Bell, S.P., and B. Stillman. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature. 357:128–134. doi:10.1038/357128a0
Bell, S.P., J. Mitchell, J. Leber, R. Kobayashi, and B. Stillman. 1995. The multi-domain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. Cell. 83:563–568. doi:10.1016/0092-8674(95)90069-9
Chesnokov, I.N. 2007. Multiple functions of the origin recognition complex. Int. Rev. Cytol. 256:69–109. doi:10.1016/S0076-6879(07)5003-1
Chesnokov, I.N., O.N. Chesnokova, and M. Botchan. 2003. A cytokinetic function of Drosophila Orc6 protein resides in a domain distinct from its replication activity. Proc. Natl. Acad. Sci. USA. 100:9150–9155. doi:10.1073/pnas.1633580100
Dhar, S.K., and A. Dutta. 2000. Identification and characterization of the human ORC6 homolog. J. Biol. Chem. 275:54983–54988. doi:10.1074/jbc.M006069200
Dodson, H., E. Bourke, L.J. Jeffers, E. Vagnarelli, L.J. Jeffers, P. Vagnarelli, E. Sonoda, S. Takeda, W.C. Earnshaw, A. Merdes, and C. Morrison. 2004. Centrosome amplification induced by DNA damage occurs during a prolonged G2 phase and involves ATM. EMBO J. 23:3864–3873. doi:10.1038/sj.emboj.7600393
Dohmen, R.J., and A. Varshavsky. 2005. Heat-inducible degron and the making of conditional mutants. Methods Enzymol. 399:799–822. doi:10.1016/S0076-6879(05)90052-6
Dohmen, R.J., P. Wu, and A. Varshavsky. 1994. Heat-inducible degron: a method for constructing temperature-sensitive mutants. Science. 263:1273–1276. doi:10.1126/science.263.5181.1273
Duncker, B.P., I.N. Chesnokov, and B.J. McConkey. 2009. The origin recognition complex protein family, Genome Biol. 10.214. doi:10.1186/gb-2009-10-3-214
Gillespie, P.J., A. Li, and J.J. Blow. 2001. Reconstitution of licensed replication origins on Xenopus sperm nuclei using purified proteins. BMC Biochem. 2:15. doi:10.1186/1471-2091-2-15
Gossen, M., D.T. Pak, S.K. Hansen, J.K. Acharya, and M.R. Botchan. 1995. A heat-shock promoter directs expression of transgenes at DNA break ends. Mol. Cell. Biol. 20:270–281. doi:10.1128/MCB.25.16.6948-6955.2005
Heffernan, T.P., D.A. Simpson, A.R. Frank, A.N. Heinloth, R.S. Paules, M. Cordeiro-Stone, and W.K. Kaufmann. 2002. An ATR- and Chk1-dependent S checkpoint inhibits replicon initiation following UV-induced DNA damage. Mol. Cell. Biol. 22:8552–8561. doi:10.1128/MCB.22.24.8552-8561.2002
Huijbrsregt, R.P., A. Svitin, M.W. Stinnett, M.B. Renfrow, and I. Chesnokov. 2009. Drosophila Orc6 facilitates G$\nu$Pase activity and filament formation of the septin complex. Mol. Biol. Cell. 20:270–281. doi:10.1091/mbc.E08-07-0754
Kikuchi, K., Y. Taniguchi, A. Hatanaka, E. Sonoda, H. Hochegger, N. Adachi, Y. Matsuzaki, H. Koyama, D.C. van Gent, M. Jasim, and S. Takeda. 2005. Fen-1 facilitates homologous recombination by removing divergent sequences at DNA break ends. Mol. Cell. Biol. 25:6948–6955. doi:10.1128/MCB.25.16.6948-6955.2005
Labib, K., J.A. Tercero, and J.F. Diffley. 2000. Uninterrupted MCM2-7 function required for DNA replication fork progression. Science. 288:1643–1647. doi:10.1126/science.288.5471.1643
Li, J.J., and L. Herskovitz. 1993. Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science. 262:1870–1874. doi:10.1126/science.8266975
Li, Y., and J.B. Dodgson. 1995. The chicken HMG-17 gene is dispensable for cell growth in vitro. *Mol. Cell. Biol.* 15:5516–5523.

Meraldi, P., R. Honda, and E.A. Nigg. 2002. Aurora-A overexpression reveals tetraploidyization as a major route to centrosome amplification in p53â¬/â¬ cells. *EMBO J.* 21:483–492. doi:10.1093/emboj/21.4.483

Pak, D.T., M. Pflumm, I. Chesnokov, D.W. Huang, R. Kellum, J. Marr, P. Romanowski, and M.R. Botchan. 1997. Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell.* 91:311–323. doi:10.1016/S0092-8674(00)80415-8

Prasanth, S.G., K.V. Prasanth, and B. Stillman. 2002. Orc6 involved in DNA replication, chromosome segregation, and cytokinesis. *Science.* 297:1026–1031. doi:10.1126/science.1072802

Prasanth, S.G., K.V. Prasanth, K. Siddiqui, D.L. Spector, and B. Stillman. 2004. Human Orc2 localizes to centrosomes, centromeres and heterochromatin during chromosome inheritance. *EMBO J.* 23:2651–2663. doi:10.1038/sj.emboj.7600255

Quintana, D.G., and A. Dutta. 1999. The metazoan origin recognition complex. *Front. Biosci.* 4:D805–D815. doi:10.2741/Quintana

Rowles, A., and J.I. Blow. 1997. Chromatin proteins involved in the initiation of DNA replication. *Curr. Opin. Genet. Dev.* 7:152–157. doi:10.1016/S0959-437X(97)80123-2

Semple, J.W., L.F. Da-Silva, E.J. Jervis, J. Ah-Kee, H. Al-Attar, L. Kummer, J.J. Heikkila, P. Pasero, and B.P. Duncker. 2006. An essential role for Orc6 in DNA replication through maintenance of pre-replicative complexes. *EMBO J.* 25:5150–5158. doi:10.1038/sj.emboj.7601391

Shimada, K., and S.M. Gasser. 2007. The origin recognition complex functions in sister-chromatid cohesion in *Saccharomyces cerevisiae.* *Cell.* 128:85–99. doi:10.1016/j.cell.2006.11.045

Steigemann, P., and D.W. Gerlich. 2009. Cytokinin abscission: cellular dynamics at the midbody. *Trends Cell Biol.* 19:606–616. doi:10.1016/j.tcb.2009.07.008

Steigemann, P., C. Wurzenberger, M.H. Schmitz, M. Held, J. Guizetti, S. Maar, and D.W. Gerlich. 2009. Aurora B-mediated abscission checkpoint protects against tetraploidization. *Cell.* 136:473–484. doi:10.1016/j.cell.2008.12.020

Su, X., J.A. Bernal, and A.R. Venkitaraman. 2008. Cell-cycle coordination between DNA replication and recombination revealed by a vertebrate N-end rule degron-Rad51. *Nat. Struct. Mol. Biol.* 15:1049–1058. doi:10.1038/nsmb.1490

Xiao, Z., Z. Chen, A.H. Gunasekera, T.J. Sowin, S.H. Rosenberg, S. Fesik, and H. Zhang. 2003. Chk1 mediates S and G2 arrests through Cdc25A degradation in response to DNA-damaging agents. *J. Biol. Chem.* 278:21767–21773. doi:10.1074/jbc.M300229200