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The Ino80 chromatin-remodeling enzyme regulates replisome function and stability

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Previous studies have demonstrated essential roles for ATP-dependent chromatin-remodeling and chromatin-modifying enzymes in gene transcription and DNA repair, but few studies have addressed how the replication machinery deals with chromatin. Here we show that the Ino80 remodeling enzyme is recruited to replication origins as cells enter S phase. Inducible degradation of Ino80 shows that it is required continuously for efficient progression of forks, especially when cells are confronted with low levels of replication stress. Furthermore, we show that stalling of replication forks in an *ino80* mutant is a lethal event, and that much of the replication machinery dissociates from the stalled fork. Our data indicate that the chromatin-remodeling activity of Ino80 regulates efficient progression of replication forks and that Ino80 has a crucial role in stabilizing a stalled replisome to ensure proper restart of DNA replication.

The template for eukaryotic DNA replication is a folded chromatin fiber that is estimated to be at least 30 nm thick, even in budding yeast[1]. These chromatin fibers are composed of long, linear arrays of nucleosomes that contain 147 bp of DNA wrapped around a histone octamer. Each histone octamer is organized as a central histone H3-H4 tetramer flanked by two heterodimers of histones H2A and H2B. As a replication fork proceeds along the chromosome, nucleosomes in front of the fork are disrupted, and parental H3-H4 tetramers are transferred to both sister chromatids closely behind the fork. Newly synthesized histones are then rapidly deposited by the fork-associated Caf1 and Asf1p histone chaperones to 'fill in the gaps'. Interestingly, the chaperone-dependent 'flow' of new and old histones seems to regulate fork progression[2].

Replication forks can stall when they encounter DNA damage or when nucleotide triphosphates becomes limiting. These stalled forks often 'back up' or regress, leading to the formation of unusual DNA structures and activation of the replication checkpoint[3,4]. The replication checkpoint machinery stabilizes the stalled replisome, ensuring that it remains competent for a subsequent restart event[5]. In the absence of the checkpoint, stalled forks collapse and the replication machinery is disassembled, causing increased genome instability and lethality[6]. Whether chromatin structure influences the efficiency of fork restart events is not clear, although previous studies have implicated the Asf1 histone chaperone and acetylation of histone H3K56 (refs. 7–11).

INO80 encodes a member of the Swi2/Snf2 family of DNA-stimulated ATPases, and Ino80 is the catalytic subunit of a multisubunit, ATP-dependent chromatin-remodeling complex that is conserved from yeast to mammals[12]. Previous genetic studies in yeast have shown that Ino80 has roles in transcriptional regulation, DNA double-strand break (DSB) repair and regulation of the DNA-damage cell-cycle checkpoint response[13–16]. Early studies also suggested that Ino80 might have a role in DNA replication, as *ino80* mutants are sensitive to hydroxyurea (HU), an inhibitor of ribonucleotide reductase that causes stalling of replication forks and activation of the DNA replication checkpoint[17]. Importantly, gene expression profiling has provided strong evidence that the DNA repair and replication functions of yeast Ino80 are not due to transcriptional defects[15]. For instance, inactivation of Ino80 has no significant effect on the expression of known DNA repair or DNA replication enzymes, cell-cycle checkpoint factors or enzymes involved in production of nucleotide triphosphates[14]. Likewise, Ino80 is not required for the transcriptional induction of DNA repair or DNA replication factors in response to DNA damage or replication stress[15]. And, finally, the Ino80 complex is actively recruited to the chromatin surrounding a DNA DSB, confirming that it has a direct role in DNA repair[14,15].

Here we investigate possible roles for *Saccharomyces cerevisiae* Ino80 in chromosomal DNA replication. We find that *ino80* mutants are sensitive to low concentrations of HU, and chronic HU treatment leads to lethality. Although the HU lethality is reminiscent of a defect in the replication checkpoint machinery[18,19], we show that Ino80 is not required for checkpoint activation in response to replication stress. In contrast, we find that Ino80 is recruited to replication forks and it is continuously required for replication fork progression when cells encounter low levels of replication stress. Notably, stalled replication forks rapidly collapse in the absence of Ino80, implicating ATP-dependent chromatin remodeling as a key regulator of fork stability and replication fork restart.

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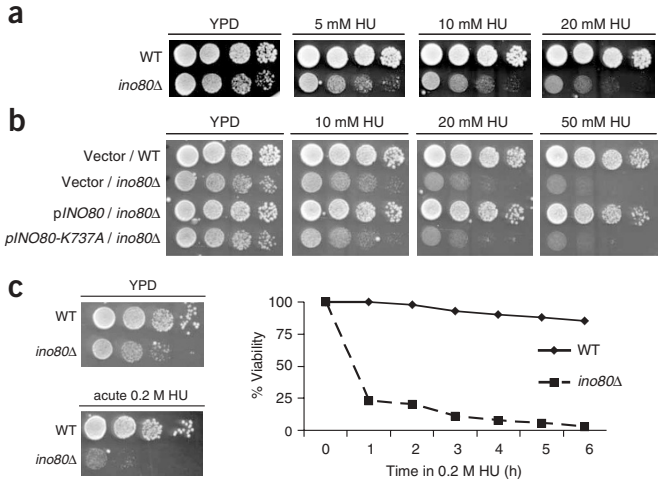

Figure 1 An *ino80*Δ mutant is hypersensitive to replication stress caused by dNTP depletion. (**a**) Wild-type (WT) and *ino80*Δ cells were plated in ten-fold serial dilutions on YPD plates containing the indicated concentrations of hydroxyurea (HU). (**b**) WT and *ino80*Δ cells harboring either the empty plasmid vector, a plasmid expressing wild-type *INO80* (p*INO80*) or an ATPase-defective allele of *INO80* (p*INO80-K737A*) were plated in ten-fold serial dilutions on YPD plates containing the indicated HU concentrations. (**c**) The *ino80*Δ mutant strain cannot survive acute replication stress. Left, log-phase WT and *ino80*Δ cells were grown for 6 h in YPD media containing 0.2M HU, before being plated in ten-fold serial dilutions onto YPD plates. Right, WT and *ino80*Δ mutant cells were initially arrested in G1 phase of the cell cycle by treatment with α-factor and subsequently released into YPD media containing 0.2M HU. Cell aliquots were then removed at the indicated times and plated onto YPD plates. Viability was assessed after 2–4 d as colony growth.

RESULTS

A previous study showed that strains harboring an *ino80* deletion allele were sensitive to moderate levels of HU (100 mM)[17]. We confirmed and extended this earlier study and found that growth of an *ino80*Δ strain is sensitive to very low concentrations of HU (**Fig. 1a**,**b**). We also tested how *ino80*Δ mutants respond to acute replication stress. In this case, wild-type and *ino80*Δ strains were transiently exposed to 0.2 M HU, and then cells were plated onto media lacking HU to score for viability. Whereas the wild-type strain fully recovered from the acute replication stress, the *ino80*Δ mutant showed extreme lethality rates (**Fig. 1c**). HU lethality is not observed for mutants that disrupt the DNA repair machinery (for example, *rad52*Δ)[19], suggesting that Ino80 is not required for recombinational repair of DNA DSBs that arise as a consequence of HU treatment. Likewise, an *ino80*Δ mutant is not sensitive to the topoisomerase I inhibitor camptothecin (CPT), which causes DSBs during S phase (**Supplementary Fig. 1** online). To further rule out the possibility that the inviability of *ino80*Δ cells is due to the presence of persistent DNA DSBs, we monitored the phosphorylation status of the DNA damage checkpoint mediator protein Rad9 to verify that the intra–S phase DNA-damage checkpoint was not activated. Indeed, Rad9p remained unmodified in HU-treated *ino80*Δ cells, indicative of an absence of DNA damage under these conditions (**Supplementary Fig. 2** online). These results show that the absence of Ino80 renders yeast cells hypersensitive to replication stress and suggest a role for Ino80 in the regulation of S-phase progression.

Ino80 is required for efficient S-phase progression

To further investigate the role of Ino80 during replication, we monitored S-phase progression of wild-type and *ino80*Δ cells by fluorescence-activated cell sorting (FACS) analysis. Cells were arrested in G1 phase by treatment with α-factor and then released from the G1 block in the presence or absence of a low amount of HU (40 mM) (**Fig. 2a**,**b**). This medium also contained nocodazole to block cells in the subsequent G2-M phase. In the absence of HU, wild-type cells proceeded rapidly through S phase, and cells reached the G2-M block by 40 min. However, the *ino80*Δ cells replicated their DNA much more slowly than wild-type cells, taking 100–120 min for completion (**Fig. 2a**). The slow progression to G2 observed in the absence of *INO80* is not a result of aberrant DNA structures or DNA damage, because the intra–S phase checkpoint remains quiescent, as shown by the lack of Rad53p kinase activity (**Fig. 2c**).

When cells are released into medium containing 40 mM HU, wild-type cells initially stall in S phase and subsequently proceed to slowly replicate their genome (**Fig. 2b**). In this case, the intra–S phase checkpoint is activated and maintained throughout S phase, as indicated by the transient kinase activity of Rad53 (**Fig. 2d**, above, and **Supplementary Fig. 3** online). In contrast, *ino80*Δ cells activate the replication checkpoint under HU treatment but are unable to complete DNA synthesis and permanently arrest, with high Rad53 kinase activity and as large-budded cells with DNA content close to 1 C (haploid; data not shown, **Fig. 2b** and **Fig. 2d**, below). Given that S-phase entry is a prerequisite for activation of the replication checkpoint[20,21], these results suggest a role for Ino80 after initiation of replication.

We considered the possibility that the slow progression of *ino80*Δ mutants from G1 to G2 in the absence of replication stress may be due to a delay in S-phase entry rather than a defect in replication *per se*. To address this possibility, we first monitored entry into S phase by assaying the emergence of small daughter buds, a morphological event that correlates with the G1-S transition. Whereas 50% of wild-type cells formed small buds ~40 min after release from a G1 block, bud emergence in the *ino80*Δ mutant was delayed by ~15 min (**Fig. 2e**). We note that this delay is substantial but not sufficient to completely explain the ~80-min delay in progression to G2 observed in the absence of Ino80. To further investigate the kinetics of replication initiation, we monitored activation of the Cdc7 kinase, a key regulator of replication initiation[22]. Cdc7 was immunoprecipitated from extracts of synchronized cultures of wild-type or *ino80*Δ cells, and *in vitro* kinase assays were used to detect phosphorylation of the associated Dbf4 substrate. When cells were released from the G1 block into media that contained 200 mM HU, maximal levels of Cdc7 kinase activity were detected by 60 min in both wild-type and *ino80*Δ cells (**Fig. 2f**). Likewise, release of cells into media that lacked HU further showed that the Cdc7 kinase was activated with similar kinetics in wild-type and *ino80*Δ cells (**Fig. 2g**). Thus, although Ino80 may be involved in the initiation of DNA replication, these results indicate that Ino80 has a significant role in DNA replication subsequent to initiation and is essential for completion of S phase when cells are exposed to low levels of replication stress. Further evidence that Ino80 functions subsequently to initiation is provided below.

Rapid stalling of replication forks in the absence of Ino80

The results from the FACS analyses indicate that *ino80*Δ mutants arrest early in S phase when challenged with low levels of replication stress. To directly monitor the proficiency of DNA synthesis *in vivo*, we first examined the structure of yeast chromosomes by pulse field gel electrophoresis (PFGE) as cells are released

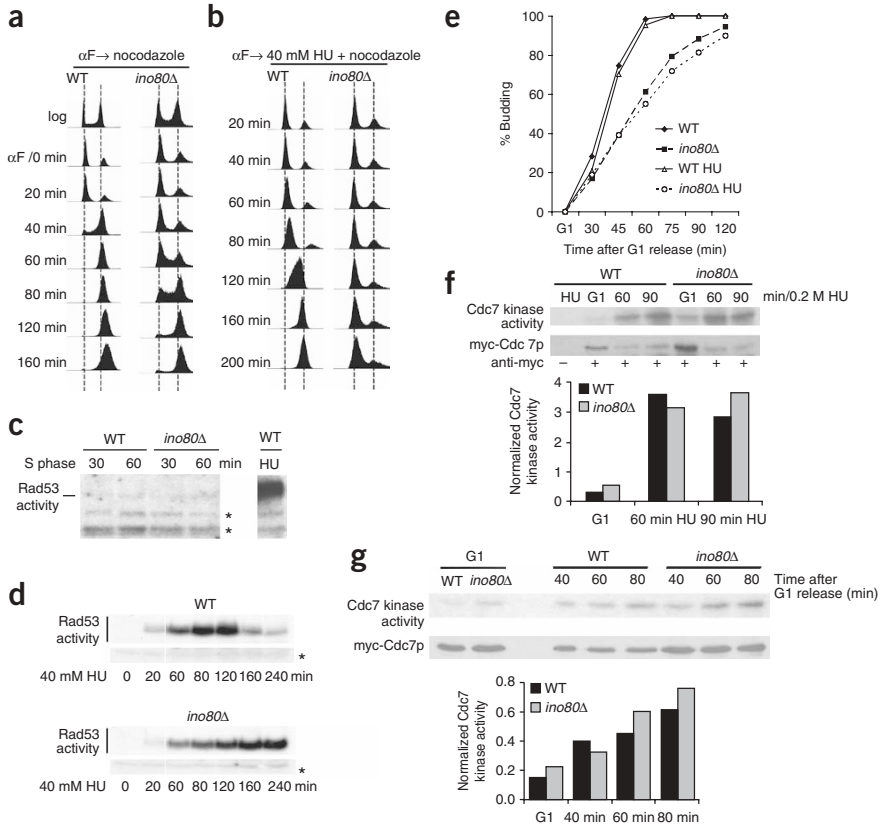

Figure 2 Ino80 is essential for progression of S phase during replication stress. (**a**–**d**) Wild-type (WT) and *ino80*Δ cells were synchronized in G1 phase with α-factor (αF) and subsequently released into nocodazole-containing YPD media without (**a**,**c**) or with (**b**,**d**) 40 mM hydroxyurea (HU). (**a**,**b**) Cell samples were collected at the indicated times and analyzed for DNA content by flow cytometry analysis. (**c**,**d**) Protein samples were acid-extracted from the same cultures as in **a** and **b** at the indicated times and analyzed by an *in situ* autophosphorylation (ISA) assay for Rad53 activity. The HU lane in **c** indicates the relative position of the Rad53 activity in the gel. An asterisk (*) at **c** and **d** indicates kinase activity independent of Rad53 and serves as loading control. (**e**) Cells from the indicated strains were arrested in G1 with αF and released in YPD media with or without HU. Cell samples were collected at the indicated times and assessed for bud emergence by microscopy. (**f**,**g**) Cdc7p kinase activity is intact in the *ino80*Δ strain. (**f**) G1-arrested wild-type and *ino80*Δ cells expressing a myc epitope–tagged *CDC7* allele were released into media containing 0.2 M HU, and protein extracts were isolated at the indicated times and subjected to immunoprecipitation with polyclonal anti-myc antibody. Above, the immunoblot at the top shows a kinase assay on the immunoprecipitation from WT and *ino80*Δ strains. The phosphorylated protein appearing at ~80 kDa corresponds to Dbf4, which is both a subunit of the DDK complex and a substrate for the Cdc7p kinase[44]. The immunoblot of the immunoprecipitated fraction with monoclonal anti-myc antibody. Below, quantification of the myc-Cdc7p kinase activity normalized to the immunoprecipitated levels of the myc-Cdc7 protein. (**g**) G1-arrested WT and *ino80*Δ cells expressing a myc epitope–tagged *CDC7* allele were released into YPD media, and protein extracts were isolated at the indicated times and analyzed as in **f**.

from a G1 block into either 50 mM or 200 mM HU (**Fig. 3a**). In G1-arrested cells, PFGE analysis yields the full complement of chromosomes in both wild-type and *ino80*Δ cells (**Fig. 3a**). As the wild-type cells enter S phase, chromosome bands smear and do not escape the gel well during PFGE analysis, both characteristics typical of replicating chromosomes[23]. In contrast, the chromosome banding pattern remains intact throughout the time course in samples from the *ino80*Δ mutant, indicating that little replication occurs on any chromosome in the presence of either 50 mM or 200 mM HU (**Fig. 3a**).

To monitor the extent of replication adjacent to an individual origin of replication, we monitored accumulation of single-stranded DNA (ssDNA) replication intermediates (RIs) originating from the early origin, ARS305 (**Fig. 3b**). G1-arrested wild-type and *ino80*Δ cells were released into media containing 0.2 M HU, and genomic DNA was isolated from several time points. Samples were electrophoresed on alkaline gels, and RIs from the ARS305 locus were detected by Southern blot[24]. As expected, robust accumulation of RIs was detected in the wild-type strain immediately after release into HU media (**Fig. 3b**, left). In contrast, the abundance of RIs in the *ino80*Δ mutant remained extremely low throughout the time course, suggesting that forks may stall rapidly in the absence of Ino80. Furthermore, replication intermediates were not detected from the late origin, ARS501, from either wild-type or *ino80*Δ strains, consistent with the presence of a functional intra–S phase checkpoint[24] (**Fig. 3b**, right).

To further investigate the replication defect of the *ino80*Δ mutant, two-dimensional gel analyses were performed. Wild-type and *ino80*Δ strains were synchronized in G1 and then released into media containing 0.2 M HU. DNA from several time points was analyzed by two-dimensional gel analysis, and replication of the ARS305 region was detected by Southern blot. In wild-type cells, a typical bubble arc was detected within 1 h of release from G1, reflecting origin firing and early elongation (**Fig. 3c**). Within 2 h, characteristic X structures could be detected, reflecting the stalling of forks. In contrast, neither bubble arcs nor X structures were detected in the *ino80*Δ strain, even by 4 h after release from G1 (**Fig. 3c**). Similar results are also seen for two-dimensional analysis of ARS607 (**Supplementary Fig. 4** online).

The collective results of PFGE, RI analysis and two-dimensional gel analysis indicate that replication forks stall rapidly during replication stress in the absence of Ino80. To confirm that some DNA synthesis has indeed occurred in the absence of Ino80, we used real-time PCR to monitor replication of DNA directly adjacent to ARS305. In G1-arrested wild-type or *ino80*Δ cells, origin-proximal DNA was present at levels equivalent to the *ASI1* amplicon that is located 30 kb from the nearest origin (**Fig. 3d**). However, when wild-type cells were released into media that contains 200 mM HU, nearly twice as much origin-proximal DNA was detected, reflecting replication of this region. Similarly, this origin-proximal region was replicated in the absence of Ino80, as the ratio of origin-proximal to origin-distal PCR product was 1.7 ± 0.3 by 80 min after release into HU. Thus, although

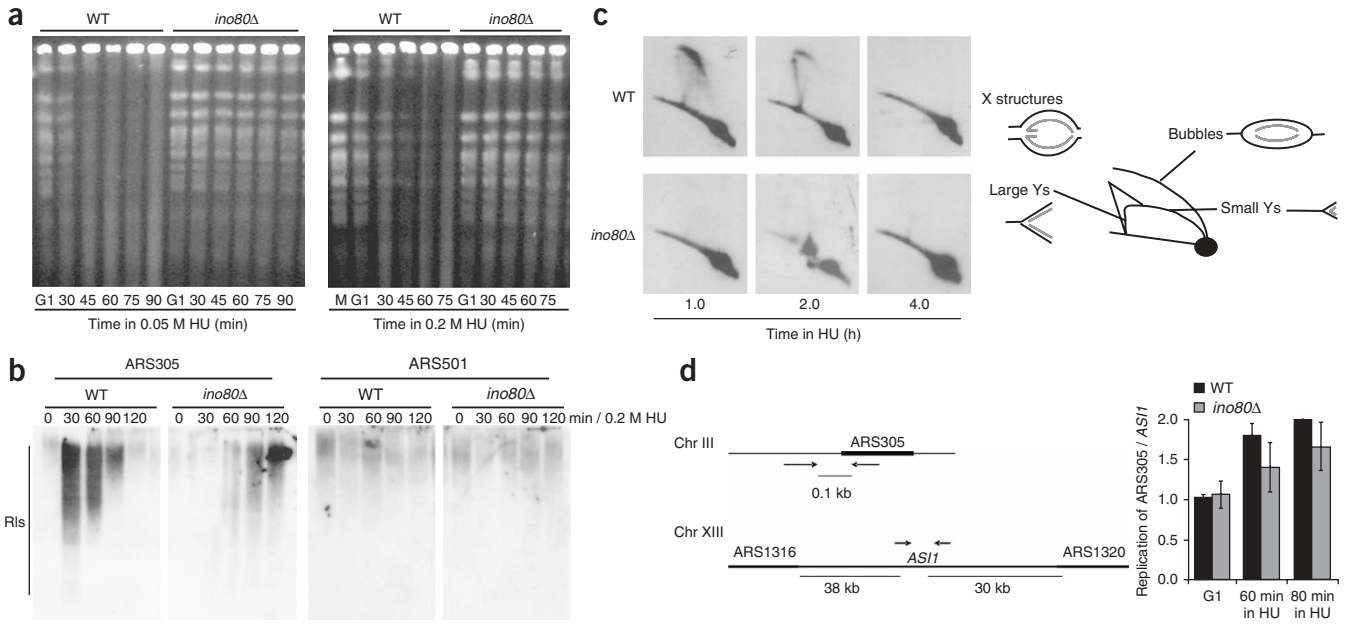

Figure 3 Rapid stalling of replication forks in the absence of Ino80. (**a**) Pulsed field gel electrophoresis (PFGE) analysis of yeast chromosomes. G1-arrested wild-type (WT) and *ino80*Δ cells were released into YPD media containing 50 mM (left) or 200 mM (right) hydroxyurea (HU), and chromosomal DNA was collected at the indicated time points and subjected to PFGE. Yeast chromosomes were visualized by ethidium bromide staining. (**b**) Low abundance of replication intermediates (RIs) from the early-firing origin ARS305 in the absence of *INO80*. G1-arrested wild-type and *ino80*Δ cells were released into media containing 0.2 M HU, and genomic DNA was isolated from the indicated time points. Samples were electrophoresed on alkaline gels, and RIs from the ARS305 and ARS501 loci were detected by Southern blot. (**c**) Left, replication fork structures do not accumulate in the absence of Ino80. G1-arrested WT and *ino80*Δ cells were released into media containing 0.2 M HU. DNA samples were collected at the indicated times, digested with NcoI and subjected to two-dimensional gel analysis, using a probe for ARS305. Right, schematic representation of replication intermediates as detected by two-dimensional gel analysis, adapted from ref. 42. (**d**) Replication of DNA proximal to ARS305 takes place in the absence of Ino80. Left, schematic representation of chromosomes III and XIII and the location of the primers used in the assay. Right, G1-arrested WT and *ino80*Δ cells were released into media containing 0.2 M HU, and genomic DNA was isolated from the indicated time points and subjected to real-time PCR analysis. Values reflect the amount of the ARS305 DNA normalized over a region of the *ASI1* ORF. The *ASI1* ORF is located between ARS1320 and ARS1316, which are mapped 30 kb and 38 kb, respectively, from the *ASI1* region. Error bars represent s.d. from three separate experiments.

replication does initiate in the absence of Ino80, it seems that Ino80 is essential for substantial elongation of replication forks when cells are exposed to low levels of replication stress.

Ino80 is continuously required for DNA replication

It is possible that Ino80 might act specifically at the beginning of S phase, or alternatively that Ino80 function might be required throughout S phase. To distinguish between these two possibilities and to directly demonstrate a role for Ino80 during S phase, we used a yeast strain (*ino80-td*) that allows for the rapid, inducible degradation of Ino80 (**Fig. 4a** and ref. 13). Growth of this strain in galactose media at 37 °C leads to rapid degradation of Ino80 (**Fig. 4b**; see also ref. 13). As shown in **Figure 4c**,**d**, wild-type and *ino80-td* cells were arrested in G1 at 24 °C and subsequently released at 24 °C into galactose medium for 1 h to induce *UBR1* expression. At this time, >50% of the wild-type and *ino80-td* cells had budded, indicating that S phase had initiated (data not shown). Moreover, if cells were released from the G1 block at the permissive temperature into 50 mM HU, the Rad53 checkpoint kinase was activated by 45 min, which is also consistent with the onset of S phase (**Fig. 4c**). After the 1 h release at 24 °C, cultures were shifted to 37 °C in galactose medium to induce degradation of Ino80 (**Fig. 4d**, above). As shown in **Fig. 4d** (left), depletion of Ino80 within S phase dramatically slowed replication, even in media that lacked HU. Moreover, loss of Ino80 during 50 mM HU treatment blocked the progression of replication forks, leading to permanent arrest of cells in S phase (**Fig. 4d**, right). Collectively, these data suggest a previously unknown and continuous role for Ino80 in DNA replication and raise the possibility that the Ino80 remodeling enzyme acts directly at replication forks.

Ino80 is recruited to a replication fork

To investigate whether Ino80 acts directly at replication forks, we monitored recruitment of Ino80 to replication origins in HU-treated cells by chromatin immunoprecipitation (ChIP). A strain harboring an epitope-tagged allele of *INO80* (*INO80-13Myc*) was released from G1 arrest into media containing 0.2 M HU. Samples collected at various time points were analyzed for the association of Ino80 with the replication fork of the early-firing origin, ARS305. To monitor the progression of the fork during the time course, the essential replication factor PCNA was immunoprecipitated from the same chromatin samples. During G1, the level of Ino80 at ARS305 was comparable to the low levels found at the control *ASI1* open reading frame (**Fig. 5a**). However, upon release from G1, Ino80 was enriched at ARS305. Furthermore, as cells progressed into S phase, less Ino80 was detected at the origin, and increased levels were associated with the adjacent DNA that is 1.5–2.5 kb distal (**Fig. 5b–d**). Similar results were obtained at another early origin, ARS607 (**Supplementary Fig. 5** online). Notably, the temporal changes in Ino80 association are similar to the pattern of PCNA recruitment (**Fig. 5a–d**), and Ino80 was not recruited to the silenced, late-firing origin ARS501

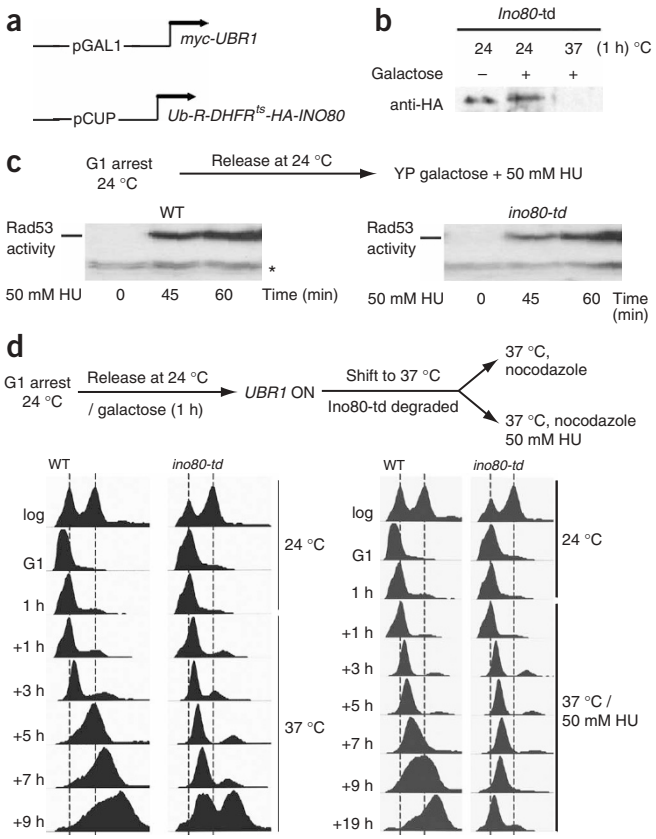

Figure 4 Ino80 has a continuous role in DNA replication. (**a**) Schematic representation of the *ino80-td* degron strain (YZ106)[3] bearing a galactose-inducible *UBR1* gene and a temperature-sensitive, degron-tagged *INO80* allele (*INO80*-td). (**b**) The Ino80-td protein is rapidly degraded in yeast cells upon shifting to 37 °C. Immunoblot analysis of the *ino80-td* strain. Whole-cell protein extracts were isolated under the indicated conditions, separated by SDS page and analyzed for the presence of hemagglutinin (HA)–Ino80-td with antibody directed against the HA epitope. (**c**) Replication has initiated in the *ino80-td* degron strain before Ino80 degradation. Above, schematic representation of the experimental protocol used to assess replication initiation in the *ino80-td* degron. Below, wild-type (WT) and *ino80-td* cells were synchronized at 24 °C in G1 phase with α-factor and released in galactose-containing media with 50 mM hydroxyurea (HU), according to the protocol. HU induces the Rad53 kinase activity in a post-replication initiation step[20,21,24]. Protein samples were acid-extracted at the indicated times and analyzed by an *in situ* autophosphorylation ISA assay for Rad53 activity as in **Figure 2c**,**d**. (**d**) Above, schematic representation of the protocol used for degradation of the Ino80-td protein within S phase. WT and *ino80-td* cells were synchronized at 24 °C in G1 phase with α-factor and released in galactose-containing media with nocodazole according to the experimental protocol, without (below left) or with (below right) 50 mM HU. Samples were collected at the indicated time points and analyzed for DNA content by FACS. + indicates the time after shifting cultures to 37 °C.

(**Fig. 5e**). These results are consistent with the view that Ino80 is recruited to and moves with the replication fork.

Ino80 stabilizes stalled replication forks

Considering that *ino80*Δ cells cannot survive transient replication stress (**Fig. 1c**), we proposed that Ino80 may be essential for maintaining replisome integrity under stress conditions. To test this hypothesis, we monitored the recruitment of the replication factors Mcm4, Orc2, PCNA, RPA and DNA polymerase-α (DNA Polα) to ARS305 in G1 and during release of cells into media containing 0.2 M HU. Mcm4 and Orc2 are components of the pre-replication complex that is assembled before onset of S phase[25,26]. Consistent with a role for Ino80 after replication initiation, ChIP analyses of Orc2 and Mcm4 showed that both proteins are efficiently recruited to ARS305 in the absence of Ino80, suggesting that the pre-replication complex is formed normally (**Fig. 6a**,**b**).

When wild-type cells are released from the G1 block, DNA Polα, RPA and PCNA are recruited to ARS305, and they are redistributed downstream of the origin with increasing time, which reflects the stalling of the fork[27–29] (**Fig. 6c–e**). We also detected a large increase in Mcm4 association with ARS305 as cells enter S phase, which is believed to reflect increased recruitment of Mcm4 (refs. 27,28; **Fig. 6f**). H2AX phosphorylation is also detected adjacent to ARS305 in both wild-type and *ino80*Δ cells, consistent with stalling of replication forks and localized recruitment of checkpoint kinases (**Supplementary Fig. 6** online). Notably, DNA Polα was nearly undetectable in HU-treated *ino80*Δ cells, suggesting rapid dissociation from stalled forks (**Fig. 6c**). Likewise, low levels of RPA were detected at ARS305 in the absence of Ino80, which may be due to limited production of ssDNA (**Fig. 6d**). The low level of RPA that is detected at forks in the absence of Ino80 may also explain why we reproducibly observed slower activation of the intra–S phase checkpoint in an *ino80*Δ mutant[30] (**Fig. 2d**). On the other hand, PCNA was recruited in the absence of Ino80, albeit with slower kinetics (**Fig. 6e**). Recruitment of PCNA, but not DNA Polα, is consistent with origin firing and subsequent collapse[31–33]. Similar

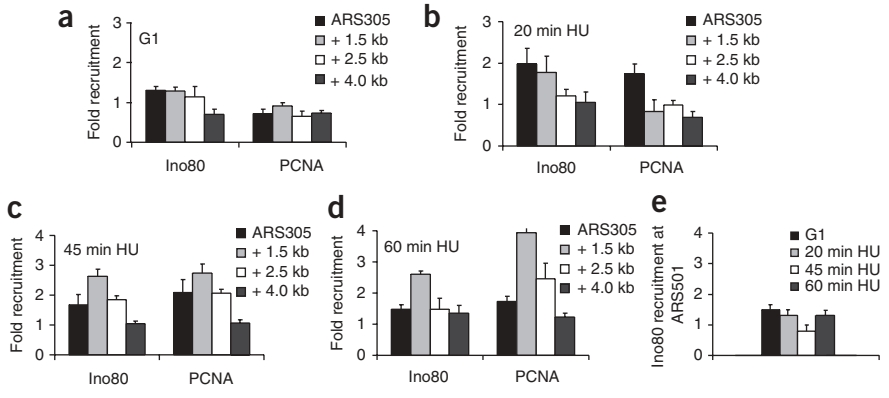

Figure 5 The Ino80 complex associates with replication forks. (**a**–**d**) Ino80 is recruited to the ARS305 origin and localizes at stalled replication forks. ChIP analysis of Ino80 was conducted in a wild-type strain bearing a myc epitope–tagged *INO80* allele using polyclonal anti-myc antiserum. ChIP analysis of PCNA was conducted from the same chromatin using polyclonal anti-PCNA antiserum. G1-arrested cells were released into 0.2 M hydroxyurea (HU) and samples were collected at the indicated time points. Occupancy at ARS305 and at sites 1.5 kb, 2.5 kb and 4.0 kb proximal to the origin was measured by quantitative PCR. Values reflect the fold enrichment of the tested DNA relative to a region of the *ASI1* ORF after correction for the ratios of amplification achieved using input DNA. Error bars represent s.d. between two ChIP experiments. (**e**) Ino80 is not recruited to a silent origin of replication. ChIP analysis of Ino80 at the late firing origin ARS501 was conducted as in **a**.

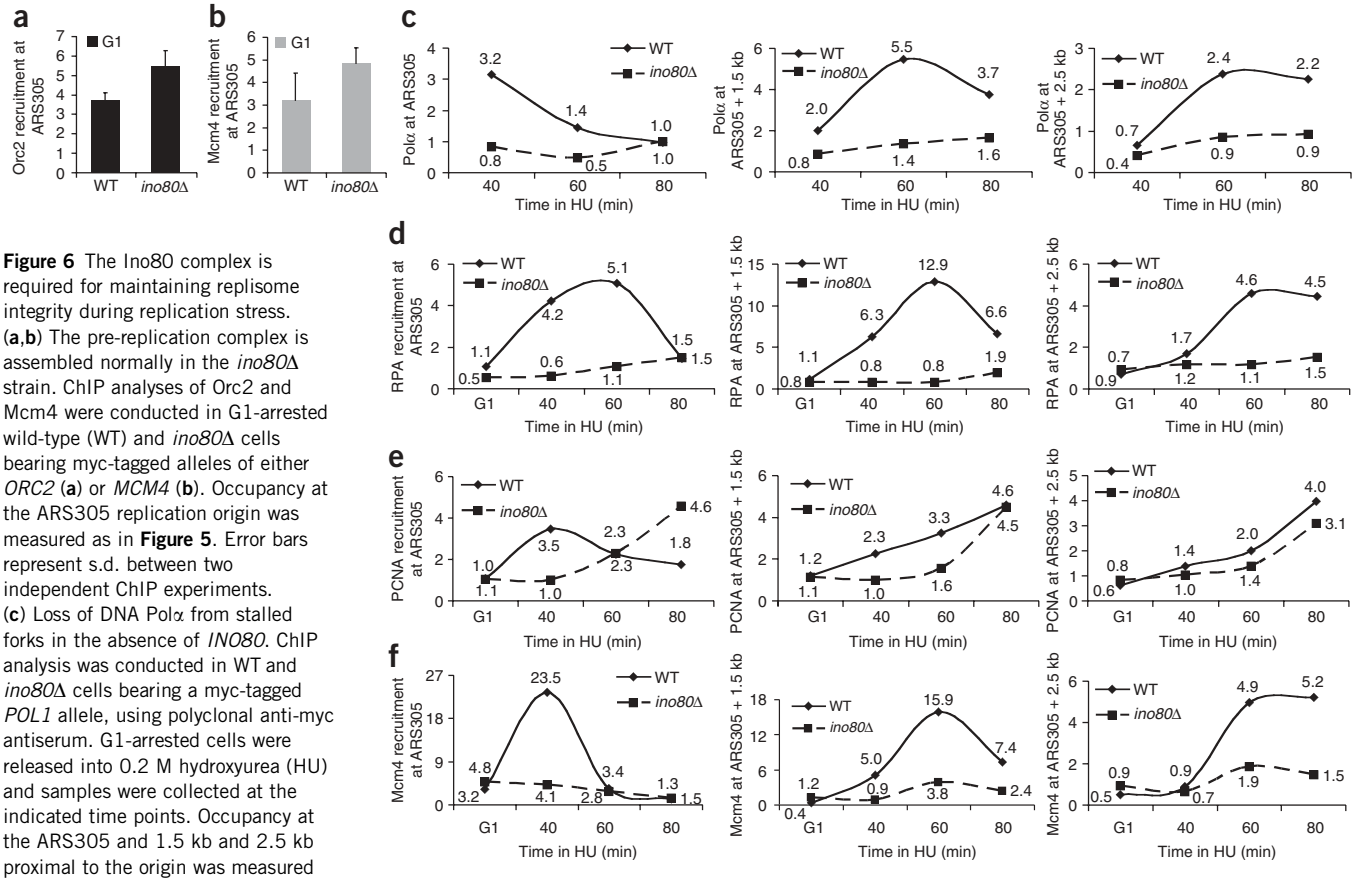

Figure 6 The Ino80 complex is required for maintaining replisome integrity during replication stress. (**a**,**b**) The pre-replication complex is assembled normally in the *ino80*Δ strain. ChIP analyses of Orc2 and Mcm4 were conducted in G1-arrested wild-type (WT) and *ino80*Δ cells bearing myc-tagged alleles of either *ORC2* (**a**) or *MCM4* (**b**). Occupancy at the ARS305 replication origin was measured as in **Figure 5**. Error bars represent s.d. between two independent ChIP experiments. (**c**) Loss of DNA Polα from stalled forks in the absence of *INO80*. ChIP analysis was conducted in WT and *ino80*Δ cells bearing a myc-tagged *POL1* allele, using polyclonal anti-myc antiserum. G1-arrested cells were released into 0.2 M hydroxyurea (HU) and samples were collected at the indicated time points. Occupancy at the ARS305 and 1.5 kb and 2.5 kb proximal to the origin was measured as in **a**. (**d**,**e**) Loss of RPA but not of PCNA from stalled forks in the absence of *INO80*. ChIP analysis of RPA was conducted in WT and *ino80*Δ cells bearing a myc-tagged *RFA1* allele, using polyclonal anti-myc antiserum. ChIP analysis of PCNA was conducted from the same chromatin using polyclonal anti-PCNA antiserum. G1-arrested cells were released into 0.2 M HU, and samples were collected at the indicated time points. Occupancy was measured as in **c**. (**f**) Reduced recruitment of the MCM helicase in the absence of *INO80*. ChIP analysis was conducted in WT and *ino80*Δ cells bearing a myc-tagged *MCM4* allele, using polyclonal anti-myc antiserum. G1-arrested cells were released into 0.2 M HU, and samples were collected at the indicated time points. Occupancy was measured as in **c**. Data shown are representative of experiments performed at least twice. Equal expression of all tagged proteins in wild-type and *ino80*Δ strains was confirmed by western blot analysis (data not shown).

results for PCNA, RPA and DNA Polα were also found at ARS607 (**Supplementary Fig. 7** online). Notably, the large increase in Mcm4 association during S phase onset was also not observed in the *ino80*Δ mutant (**Fig. 6f**). Collectively, these data suggest that the function of the Ino80 complex is essential for the progression and integrity of stalled replication forks.

DISCUSSION

The Ino80 complex may be the most versatile member of the ATP-dependent chromatin-remodeling family, with roles in gene transcription, DNA repair and DNA replication. Here we have investigated the role of Ino80 in DNA replication and find that Ino80 is essential for progression of replication forks during low levels of replication stress. Indeed, we were unable to detect substantial levels of replication in the absence of Ino80 when cells are released from a G1 block into a low concentration of HU. Numerous lines of evidence indicate that replication forks do initiate in the absence of Ino80 but subsequently stall: (i) the replication checkpoint is activated; (ii) H2AX is phosphorylated in chromatin surrounding the presumptive stalled fork; (iii) the Cdc7 kinase is activated; (iv) small buds are formed; (v) PCNA is recruited, albeit slowly, to early origins; and (vi) conditional degradation of Ino80 subsequent to replication initiation leads to rapid arrest within S phase. We were also able to use a PCR-based assay to demonstrate duplication of DNA directly adjacent to the ARS305 origin in the absence of Ino80 during exposure to 200 mM HU. Thus, our results indicate that when cells encounter replication stress, Ino80 is essential to prevent forks from stalling throughout S phase and to stabilize a stalled fork so that the replisome can restart.

How is Ino80 recruited to replication forks? The Ino80 complex is recruited to sites of DNA damage by interacting with a large chromatin domain of phosphorylated histone H2AX[14,15]. Histone H2AX is also phosphorylated in chromatin surrounding a stalled replication fork, but the domain of phosphorylation is smaller than that arising from DNA damage[34,35]. We found that Ino80 is recruited to replication forks when cells are released from G1 into 200 mM HU, conditions that stall forks and induce H2AX phosphorylation. Similar results have been reported previously[36]. Thus, a simple model posits that Ino80 is recruited to a stalled replication fork, where its remodeling activity is used to facilitate a fork restart event. However, strains that harbor alterations in H2AX that remove the phosphorylation site (H2A S129A) are not sensitive to HU, suggesting that other factors may be more important in recruiting Ino80 to a stalled fork.

Does Ino80 function during S phase in the absence of replication stress? We found that cells traverse a normal S phase much slower in

the absence of Ino80, suggestive of a role for Ino80 in an unperturbed S phase. Some of this delay, however, may be due to a lag in replication initiation, as suggested by delayed kinetics of bud emergence. Conditional degradation of Ino80 within S phase also slows progression, providing further evidence that Ino80 has a key role in a normal S phase. Whether the slower S phase progression reflects enhanced stalling of forks in the absence of Ino80 remains to be investigated.

How does Ino80 stabilize stalled forks and facilitate replication fork progression? Ino80 is the catalytic subunit of a multisubunit ATP-dependent chromatin-remodeling complex that can mobilize nucleosomes and enhance the accessibility of nucleosomal DNA[17]. One possibility is that the Ino80 complex travels with the replication fork, mobilizing or disrupting nucleosomes ahead of the fork, thereby facilitating fork elongation. A similar model has been proposed for the role of the ISWI remodeling enzyme during replication of heterochromatin in mammals[37]. In addition, Ino80 may function to mobilize nucleosomes at a stalled replication fork. In this case, nucleosomes behind the fork may need to be removed by Ino80 in order for the fork to 'back up' or regress, and inefficient regression may lead to replisome collapse. On the basis of our previous study[16], Ino80 may also function to control incorporation of histone variants (for example, Htz1) that may form unusual chromatin structures[38] that inhibit fork progression or fork restart events.

METHODS

Yeast strains and plasmids. Strains used in this study are derivatives of the LS20 strain[39]. All strains were grown at 30 °C unless otherwise indicated. We deleted the *INO80* gene by replacing the *INO80* coding region (nucleotides 1–4440) with the *KAN-MX6* gene, which confers resistance to the drug G418 (ref. 40). The *hta1-S129A hta2-S129A* strain is a derivative of JKM179 and has been described previously[16]. The *RAD53* gene disruption was generated in the *sml1* mutant background as described[40]. All of the disruptions were confirmed by PCR analysis and the constructed mutant strains showed the expected sensitivity on methylmethanesulfonate (MMS), camptothecin (CPT) or HU plates. *INO80, RAD9, ORC2, MCM4, POL1, CDC7* and *RFA1* were tagged at the C terminus with 13-Myc at their original chromosomal loci according to ref. 40. The *ino80-td* (YZ106) and its parental strain (*YUBR1*) have been described previously[13]. The plasmid-borne copy of *INO80* and the plasmid *INO80-K737A* have been described previously[16].

Chromatin immunoprecipitation. ChIPs were performed as described[41] using commercially available polyclonal antibody raised against the Myc epitope or the phosphorylated form of Ser129 of histone H2A (AbCam), or polyclonal antibody raised against yeast PCNA[7]. The recovered DNA was subjected to quantitative real-time PCR. All ChIPs and PCR reactions were performed at least twice, and the variation between experiments was 10–30%. The primers covering the region next to the origins ARS305 and ARS607 are proximal to the centrosome. Primers used in the PCR reactions are available upon request.

Protein analysis and *in situ* autophosphorylation assay. Protein analysis from yeast whole-cell extracts and the *in situ* autophosphorylation assay were conducted as described previously[16,21].

Replication intermediates analysis. Analysis for the replication intermediates was conducted as described[24]. Probes specific for the origins ARS305 and ARS501 were generated as described[24].

Two-dimensional gel assay. DNA for two-dimensional gel analysis was purified using the cetyltrimethylammoniumbromide (CTAB) method, and two-dimensional gel analysis was conducted as described[42].

Pulsed field gel electrophoresis. PFGE analysis was conducted as described[23].

Cdc7 protein kinase assay. The Cdc7 protein kinase assay was performed using protein extracts from yeast strains carrying a Myc epitope–tagged *CDC7* allele and was conducted as described[43]. Cdc7 kinase activity and Cdc7 protein levels were quantified with the ImageJ software (US National Institutes of Health).

Flow cytometry analysis. Cell-cycle arrest and FACS were performed as described previously[7].

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

All experiments were designed and executed by M.P.-C., and C.L.P. helped with data interpretation; C.L.P. and M.P.-C. wrote the manuscript together.

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